THE POTENTIAL OF LEAF EXTRACT FROM *Clinacanthus nutans* AS ANTIBACTERIAL AGENTS

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The Potential of Leaf Extract from *Clinacanthus nutans* as Antibacterial Agents

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research has not been submitted for a higher degree to any universities or institutions.

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I certify that the report of this final year project entitled "The Potential of Leaf Extract from *Clinacanthus nutans* as Antibacterial Agents" by Nurin Qistina Binti Mohd Zamri, matric number F18A0283 has been examined and all the correction recommend by examiners have been done for the degree of Bachelor of Applied Science (Food Security), Faculty of Agro-Based Indusrty, University Malaysia Kelantan.

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EMA	European Medicine Agency	7
XDR	Extensively drug resistant	7
PDR	Pan-drug-resistant	7
UV	Ultra violet	14
DMSO	Dimethyl sulfoxide	17
NA	Nutrient agar	19
CFU	Colony forming unit	19
MHA	Mueller Hinton Agar	19
TLC	Thin Layer Chromatography	22
FeCl3	Ferric chloride	23
dH_2O	Distilled water	23
NaOH	Sodium hydroxide	24
HCL	Hydrochloride acid	24
KOH	Potassium hydroxide	25
SD	Standard deviation	28

LIST OF SYMBOLS

		Page
%	Percentage	1
cm	Centimeter	11
mm	Millimeter	11
<	Less than	14
°C	Degree celcius	16
h	Hour	17
g	Gram	17
mL	Milliliter	17
mg	milligram	17
µm	Micrometer	17
°	Degree angle	19
µL	Microliter	19
min	Minute	19
µg	Microgram	20
≤	Less than or equal to	21
-	No inhibition zone	21
+	Small inhibition zone	21
++	Medium inhibition zone	21
+++	Large inhibition zone	21
M	Molar	24
±	Plus minus	31

The Potential of Leaf Extract from *Clinacanthus nutans* as Antibacterial Agents

ABSTRACT

Antibacterial resistance has becoming one of the major problems facing by medical and pharmaceutical industry nowadays. There are more than 3,00 species of plants in Malaysia that possesses medicinal value due to presence of bioactive compound which can act as antibacterial agents. Therefore, aims of this study were to investigate the antibacterial potential of different extracts of *C. nutans* leaf on the selected Gram-positive and Gram-negative bacteria using disc diffusion assay as well as preliminary screen the group of organic compound present in the leaf extract. Preparation of the sample has been done by collecting leaves sample and extracted with five different solvents (hexane, chloroform, acetone, ethyl acetate and methanol). Disc diffusion susceptibility test was used for antibacterial activity determination using Gram-positive bacteria (*B. cereus*, MRSA, *S. aureus* and *B. subtilis*) and Gram-negative bacteria (*Y. Enterocolitica*, *K. pheumoniae* and *E. coli*)**.** The preliminary screening of the presence of organic compound in the plant extracts was conducted using thin layer chromatography. The result from disc diffusion susceptibility test showed ethyl acetate extract demonstrated the most prominent antibacterial activity where it can inhibit–five out of seven tests. *B. cereus* and *K. pneumoniae* were the most susceptible to the *C. nutans* extracts as it exhibited 80% of antibacterial activities from five different extract. Gram-positive bacteria were more susceptible to the extracts compared to Gram-negative bacteria. There were three bioactive compounds detected in each of the extracts where hexane showed the least diverse of compound as compared to the other extracts. *C. nutans* extracts were shown to have potential antibacterial activity against a variety of bacteria in this study.

Keywords: Antibacterial agent, medicinal, bacteria, inhibition zone, bioactive compound

Potensi ekstrak daun daripada pokok belalai gajah sebagai agen antibakteria

(*Clinacanthus nutans*)

ABSTRAK

Rintangan antibakteria telah menjadi salah satu masalah utama yang dihadapi oleh industri perubatan dan farmaseutikal pada masa kini. Terdapat lebih daripada 3,00 spesies tumbuhan di Malaysia yang mempunyai nilai perubatan kerana kehadiran sebatian bioaktif yang boleh bertindak sebagai agen antibakteria. Oleh itu, tujuan kajian ini adalah untuk menyiasat potensi antibakteria bagi ekstrak berbeza daun C. nutans pada bakteria Gram-positif dan Gram-negatif terpilih menggunakan ujian resapan cakera serta skrin awal kumpulan sebatian organik yang terdapat dalam ekstrak daun. . Penyediaan sampel telah dilakukan dengan mengumpul sampel daun dan diekstrak dengan lima pelarut berbeza (heksana, kloroform, aseton, etil asetat dan metanol). Ujian kepekaan resapan cakera digunakan untuk penentuan aktiviti antibakteria menggunakan bakteria Gram-positif (B. cereus, MRSA, S. aureus dan B. subtilis) dan bakteria Gram-negatif (Y. Enterocolitica, K. pheumoniae dan E. coli). Saringan awal kehadiran sebatian organik dalam ekstrak tumbuhan telah dijalankan menggunakan kromatografi lapisan nipis. Hasil daripada ujian kepekaan resapan cakera menunjukkan ekstrak etil asetat menunjukkan aktiviti antibakteria yang paling menonjol di mana ia boleh menghalang lima daripada tujuh ujian. B. cereus dan K. pneumoniae adalah yang paling mudah terdedah kepada ekstrak C. nutans kerana ia mempamerkan 80% aktiviti antibakteria daripada lima ekstrak berbeza. Bakteria Gram-positif lebih mudah terdedah kepada ekstrak berbanding bakteria Gram-negatif. Terdapat tiga sebatian bioaktif yang dikesan dalam setiap ekstrak di mana heksana menunjukkan kepelbagaian sebatian paling sedikit berbanding dengan ekstrak lain. Ekstrak C. nutans telah ditunjukkan mempunyai potensi aktiviti antibakteria terhadap pelbagai bakteria dalam kajian ini

Kata kunci: Ejen antibakteria, perubatan, bakteria, zon perencatan, sebatian bioaktif

CHAPTER 1

INRODUCTION

1.1 Research background

Natural resource has become one of the most important resources that can perform various function either in biological activities or even in pharmaceutical industry. Medicinal plants are one of the examples that are significantly related with the source of traditional medicine as well as act as antibacterial agents. Medicinal plant refers to plants that contain medicinal properties which are rich in bioactive compound to be extracted and used in pharmaceutical products. In Malaysia, more than 3,000 species of plants have been identified to possess medicinal value which have been consumed in traditional healthcare system (Jantan, 1998). Besides, medicinal plants increasingly gaining not only public attention but also researchers as they are affordable, more efficient as well as the belief that natural drugs are more authentic (Elvin-Lewis, 2001). In fact, there is an increase in percentage of demand of medicinal herbs by 8%- 15% per year in Asia, Europe and North America (Sheetal Verma, 2008).

Clinacanthus nutans or also known as 'belalai gajah' or snake grass mainly can be found in tropical countries such as Malaysia, Thailand, Vietnam and Indonesia. It

belongs to Acanthaceae family where it is one of the largest sources among medicinal plants that are being used for anti-inflammatory, antifungal and antibacterial agents (Alam *et al.*, 2016). In addition, the effect from fungi *P. oryzae* and antibacterial activity on Gram-positive and Gram-negative can be inhibit by *C. nutans* leaves which was tested on three different bacteria which are *Bacillus cereus, Salmonella enterica* and *Escherichia coli* (Arullappan *et al.*, 2014).

Furthermore, according to phytochemical investigation, what makes *C. nutans* leaf getting higher demand is because it contains wide range of bioactive compounds or phytochemical properties. The chemical constituents consist of flavonoids, stigmasterol, lupeol, belutin and myricyl alcohol (Yang *et al.,* 2013). These bioactive compounds which synthesized in *C. nutans* can activate antibacterial response causes by the presence of carbonyl group (Rathee *et al.*, 2009). Therefore, a lot of way can be done by the researchers to elucidate the pharmacologically active compounds from this potential plant and at the same time new generation of antibiotics may be developed.

1.2 Problem statement

Nowadays, production of affordable and effective medicines has been one of the major challenges facing by global health care especially in developing countries like South Africa, Philippines and even in our country. Not only that, the arising of antibacterial resistance also significant with healthcare issue (Talbot *et al.*, 2006). The emergence of antibacterial resistance could inhibit the effectiveness of available antimicrobial agents like penicillin, gentamicin, tobramycin, amikacin etc. Common

bacteria that involve in global prevalence of infectious disease consist of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis*, and *Proteus vulgaris* (Pitout, 2008). This resistance was developed due to the unrestrained and inappropriate usage of antibiotic as well as increased transboundary passage of antibiotic-resistant bacteria (Torgersen *et al.,* 2000).

Hence, the introduction towards extracting medicinal plants as antibacterial agents in pharmaceutical is urgently needed. *C. nutans* which particularly produced secondary metabolites with remarkable antimicrobial potential is depicted as one of the best sources as natural antibacterial agent. Flavonoids which is one of the bioactive compound contain in *C. nutans* considered to have bacteriostatic or killing effect against bacteria (Awad *et al.,* 2012). According to research done by Arullappan *et al*. (2014), this plant showed more influential towards Gram-positive bacteria compared to Gram-negative bacteria. In fact, most natural products include medicinal plants are more valuable and efficient in terms of bacterial infection's treatment. Possibility for this plant to be used as a therapeutic remedy is very promising as it represents less toxicity towards human health.

1.3 Hypothesis

Extract of leaf *C. nutans* is believed to possess antibacterial activity against a few selected Gram-negative and Gram-positive bacteria. This extract is hypothesised to have a lot of antibacterial potential and could be used as a natural medicine or antimicrobial agent in the future.

1.4 Scope of study

The current study was focusing on the antibacterial activity of the leaf of local medicinal plant, *C. nutans.* The preparation of *C. nutans* leaf extract was performed using sequential extraction method using the increasing polarity of solvent from hexane, chloroform, acetone, ethyl acetate till methanol. The antibacterial potential of this leaf extract was evaluated by using a few selected pathogenic Gram-negative and Gram-positive bacteria to determine the potential of this medicinal plant as natural antibiotics using disc diffusion susceptibility test. The qualitative analysis of bioactive compounds in the extracts was performed using TLC plates and sprayed with respective reagents to detect the presence of group of organic compounds.

1.5 Significant of study

Medicinal plants are highly getting demand and their acceptance also continuously increasing particularly in pharmaceutical industry. This plant is believed to be the source of bioactive compound which can possess significant antibacterial effect against Gram- Positive and Gram-negative bacteria. These bacteria were becoming phenomenon as their resistance towards multiple antibiotics getting attention by public health and pharmaceutical industry. Therefore, current study is carried out to determine the antibacterial potential of *C. nutans* leaves. Hence, the findings of this study may

provide new insight to pharmaceutical companies in the development of natural antibacterial agents derived from medicinal plants.

1.6 Research objective

1. To prepare the leaves extract of *C. nutans* using sequential solvent extraction method

2. To investigate the antibacterial potential of different extracts of *C. nutans* leaf on the selected Gram-positive and Gram-negative bacteria using disc diffusion assay.

3. To preliminary screen the group of compound present in the leaf extract of *C. nutans*

CHAPTER 2

LITERATURE REVIEW

2.1 The history of antibiotic resistant

Since 1952, it has been reported about antibiotic resistant where it involves staphylococcal infections that resistance to penicillin (Finland, 1965). It started when the discovery of penicillin by Sir Alexander Fleming in 1928 became a phenomenon where it saved millions of live and successfully controlled infectious disease during World War II (Ventola, 2015). Shortly thereafter, there were penicillin resistance evolved which also brought threat to another antibiotic as well. Few decades ago, up until now there were antibiotic resistance reported become a threat to pharmaceutical industry which are Methicillin-Resistant Staphylococcus Aureus (MRSA), Vancomycin-Resistant Enterococci (VRE) and many more. MRSA is among the earliest antibiotic resistance discovered in 1962 (Johnson, 2011). It had caused many deaths where it involved 11,285 lives per year in the U.S (Gross, 2013). While, VRE caused major illness in bloodstream and urinary tract infections (Sengupta *et al.*, 2013).

The resistance exist commonly due to irresponsible and immoderate amount of antibiotic uses. According to Ventola (2015), crisis happened in public health is because

of antibiotic resistance that spread quicker than introduction of new antimicrobial agent into clinical practice. Generally, bacteria can undergo process of changing in a way of eliminate or reduce the productiveness of antibiotic. This can be done due to bacterial evolution or mutation (Read *et al.*,2014). Even they have different type of mechanisms to escape effectiveness of antibiotic which are pumping them outside of the cell, neutralize antibiotics and modified their outer structure so that the attachment of drugs cannot be done (Breijyeh *et al.,* 2020). Therefore, there must be an effort to educate people and give awareness over misuse of the antibiotic that could contribute to spread antibiotic resistance.

2.2 Development of antibacterial agent

Antimicrobial agent is predominantly imitative of antibiotic which developed to combat infection that become major concern in human health nowadays. Over many years, antibacterial agent has provided strong pressure on bacteria leading to preferential survival and spread of those harboring antibiotic resistance mechanisms (Jackson *et al.,* 2018). The development of antibacterial agent had given impactful towards surgery and medicine's development particularly in countering with pathogenic bacteria which are Gram-positive and Gram-negative. Since 2017, there are many antibacterial agents that have been approved by Food and Drug Administration (FDA) and European Medicine Agency (EMA) which they focusing on antibacterial agents that can combat bacteria that are extensively drug resistant (XDR) and pan-drug-resistant (PDR) (Theuretzbacher,

2017). As bacteriological information in this case is very important in analyzing the suitable antibacterial agents.

Some of the examples of antibacterial agents are Vaborbactam, Eravacycline and Omadacycline. Each of antibacterial agents have their own antibiotic class where each of them acts differently towards different bacteria. As for Eravacycline, it has the same classes like Omadacycline which is Tetracycline while for Vaborbactam it belongs to Boronate BLI and carbapenem. Even though Eravacycline and Omadacycline are in the same classes but both expected to acts with different bacteria where Eravacycline will against Carbapenem-resistant Enterobacterales (CRE) while Omadacycline will against mostly other pathogens mostly Gram-positive.

In addition, plant products can also act as antibacterial agents. The extraction of bioactive compound in plants can helps in dealing with bacterial activity. The development of antibacterial plant extract is intended to help people in rural areas who are mostly relying on natural resources (Tura *et al.,* 2017). Therefore, this plant extract is developed for another alternative to cure infectious disease causes by bacteria

2.3 Medicinal plant extract as source of antibacterial agent

More than 35,000 plant species are used for medicinal purposes in different human cultures around the world (Philip *et al.,* 2009). Medicinal plant had become significant for public health due to the acceptance of traditional medical system as well as discovery of medicinal plants which has been shown to have tremendous healing effect stated in indigenous pharmacopeias. In fact, it believes to give less toxic than other

synthetic pharmaceutical agents. Plants certainly have important role in our ecosystem as they essentially providing benefits not only for human way of life but giving an assurance for other living organisms for example food chain (Singh, 2002).The dependency on medicinal plants as traditional drugs increasingly across the world. Among traditional medicine, traditional Chinese medicine is the most well-known followed by Indian traditional medicine. According to research done by Salmerón-Manzano *et al*., (2020), the results obtained from 159 countries on medicinal plants publication shown that China had the most publication with more than 10,000 publication followed by India. Hence, it is undeniable that China has a great influence in medicinal plants.

Whereas, in Peninsular Malaysia there are more than 1,000 species of plants with medicinal properties (Jantan, 1998). Most of the plants are aromatic and used to cure human illness as a good antioxidant and antibacterial. Phenolic compound and carotenoids which give antibacterial and antioxidant effect naturally contain in different parts of plant such as flowers, leaves, stems, seeds etc. (Sultana *et al.,* 2014). Some examples of medicinal plants are *Amaranthus spinosus* L., *Callicarpa arborea* Roxb, *Coleus amboinicus* Lour, *Clinachantus nutans* and many more. These plants not only being used in Malaysia but also other countries. For example, in India they had used *Callicarpa arborea* Roxb to treat skin disease which related to microbial activity (Sultana *et al.,* 2014). In Brazil whereas use *Coleus amboinicus* Lour to treat skin ailments and act as antimicrobial agent (Gurgel *et al.*, 2009).

2.4 Clinacanthus nutans

One of the biggest dicotyledonous flowering plants families is Acanthaceae family. Family of Acanthaceae consisting around 346 genera and 4300 species *(Khan et al.,* 2017). The family can be found mainly in South East Asia for example Indonesia, Malaysia and Thailand even in subtropical regions like Brazil and Africa (Khan *et al.,* 2017). Most of them are shrubs, tropical herbs and some are from epiphytes. Plants come from this family can be found in varieties of habitats including bushes, damp field, open forest and many more (Meyer *et al* ., 2004).

Clinacanthus nutans is one of the species from this family. In Malaysia, it is known as Belalai Gajah or Sabah Snake Grass, in Indonesia they called as Kijatan while among the Thais *C. nutans* is popular with the name of Phaya Yor (Ronald Watson, 2008). Taxonomy classification of *C. nutans* as below:

Table 2.1**:** Scientific classification of *Clinacanthus nutans*:

Kingdom	**Plantae**
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Lamiales
Family	Acanthaceae
Genus	*Clinacanthus*
Species	*nutans*
Scientific name	*Clinacanthus nutans*

C. nutans is an annual shrub which can grow up to 1-3 meters tall. The leaves are simple with lanceolate-ovate 2.5 – 13 cm long and 0.5-1.5cm wide and the arrangement of leaves are opposite. The leaf can grow up to 1-4 cm width and 7- 12 cm length. Both surfaces of leaves are pubescent when young then glabrescent. The leaf base usually oblique but can be obtuse rounded or cuncate. The stem is cylindrical, striate and glabrescent that will turn into yellow once it dry. The flowers are greenish yellow in color and dense cymes at the top of branches, covered with 5-alpha cymules. The ovary is divided into two cells, each of which contains two ovules. Capsule is oblong and has a short, strong stalk with four seeds. Size of the seed usually has diameter of 2mm.

Figure 2.1: The leaves of *C. nutans*

2.5 Techniques of extraction and isolation of natural products

In order to get the desired natural product from the plant sample, extraction is the first step before proceed to another stages. Extraction is the process of extracting desired active portions from inactive components using specific solvents and following standard procedures (Handa *et al.,* 2008) . There are few stages to go through before extract solute is produce. First, penetration between solvent and solid matrix. Next, solute will dissolve into the solvents. Third, solute will be diffused out from solid matrix and finally the desired extract is produced (Zhang *et al.,* 2018). The selection of solvents is important for extraction method. This is because, when the polarity value between solvents and solutes are closer, they are likely to perform better. Next, the particle size of the sample also plays an important role as smaller the particle size is, the larger surface area provided which enhanced the diffusion of solute into the solvents.

Generally, there are several common techniques of extraction of medicinal plants. Maceration is a technique that takes up to 3 days long. The plant powder will be placed in stoppered container that contain solvent and left at room temperature for 3 days. After that, the mixture will be strained by filtration. Another technique is infusion where it applies same principle like maceration but the difference is it has short period of time and the sample is soaked in cold or boiling water. The results gain from decoction technique whereas contains large number of water-soluble impurities. This technique requires less time and the sample will be boiled into specific volume of water. Percolation technique is the most frequently used to extract active components where it uses percolator as main component. Plant powder is moistened in a closed container and let stand for about 4 hours with sufficient amount of specified solvent. More solvent is added and the mixture

is left for another 24 hours. After 24 hours, the lid of percolator is opened and the mixture that contained liquid is allowed to drip. Then, the mixed liquid will undergoes filtration and decanting (Handa *et al.*, 2008).

2.6 Organic Solvent

Generally, organic solvent can be categorized as carbon-based substance which are used to dissolve certain substances and materials for any given application. Organic solvents are valuable because of their unrivalled ability to remain chemically stable while dissolving a wide range of useful compounds (Firestone & Gospe, 2009). They also called as volatile organic compound due to their physical properties which tend to vaporize under room temperature and frequently release pungent odour into surrounding (Firestone & Gospe, 2009). Since the Industrial Revolution, a variety of organic solvents have been widely used. It is worth notices that, until now the uses of organic solvents relatively prevalent today with their use of crude isolate in pharmaceutical industry along with products consisting of enhanced mixes of many chemically related chemicals.

In line with the herbal industry, organic solvents widely in used as the classic way of extraction which called solvent extraction. The extraction of the product or compound from the aqueous phase can be used by the high solubility products in organic solvents (Schügerl, 1994). Indeed, the extraction conditions can have an impact on chemical isolation and characterization. Therefore, type of solvent employed to extract bioactive

chemicals will determine the quantity of varieties compound present in solvent extraction (Ben Yakoub *et al.,* 2018).

There are few parameters need to be considered for the solvents used in extracting bioactive compound, which are boiling point, solubility and pka value. However, the polarity of the solvents is the main indicator in choosing the right solvents due to polarity of the solute of interest. Chemical constituents will be effectively extracted and properly dissolved through the same polarity as the solvents (Altemimi *et al.,* 2017). In order to minimise the number of similar compounds in the desired yield, multiple solvents might be utilised successively. The arrangement of polarity must be from least polarity until most polarity where few common solvents are being used in this study as follows: hexane < chloroform < acetone < ethyl acetate < methanol.

2.7 Identification of bioactive compound in medicinal plants

Plant secondary metabolites or also known as phytochemicals originally produced by plants which responsible for protection against UV, pigmentation and pathogens as well as improve chances of pollination which lead to survivability of the plant from being directly involved in critical functions such as growth and reproduction (Bagniewska-Zadworna *et al.,* 2008). There has been a significant increase in scientific interest in these compounds and their benefits to human health over the last few decades, as many exhibit significant antioxidant and antibacterial activity (Bansal *et al.,* 2013).

Acknowledging the presence of phytochemicals in medicinal plants is favourable, and the discovery of new drug compounds or lead molecules from plants is currently based primarily on the systematic examination of various plant extracts or plant-based products. Furthermore, this preliminary knowledge can be used to comprehend a new source of economically valuable chemical compounds. Numerous secondary metabolites have been shown to have antibacterial activity such as flavonoid, alkaloid, phenol, glycosides, terpenes and tannins. They have been proven to have synergistic effects with currently antimicrobial drugs.

Phenols are the most common antioxidants found in nature. Depending on the number of phenol groups, this large group of over 164,800 different compounds can be divided into two main categories. The first category includes simple phenols with a single phenol group (a hydroxyl group attached to a phenyl ring), while the second includes polyphenols with multiple phenol groups. Polyphenolic compounds have piqued the interest of researchers due to their antibacterial, antiviral, anti-allergic, anti-inflammatory, anticancer, and immunostimulant properties (Tan & Lim, 2015). These phenolic compounds in plants provide defence against various pathogens, regulate cell division and growth, and aid in pigmentation and a variety of other metabolic pathways (Lattanzio *et al.*, 2006).

Different bioactivity in each plants primarily affected by accumulation and synthesis of secondary metabolism which is due to the environmental changes or variation in geographical (Mediani et al., 2012). Flavonoids is one of the phenolic compounds that were found in all leaves extract of medicinal plant. Flavonoids are well-known for their ability to scavenge free radicals, which highlights their antibacterial properties *(Siew et al.*, 2014).

CHAPTER 3

METHODOLOGY

3.1 Preparation of plant material

3.1.1 Collection of Plant Sample

C. nutans leaves was collected from the Herbs Garden of University Malaysia Kelantan Jeli Campus. The fresh leaves samples of *C. nutans* were washed under running tap water to removes any dust and debris from the top and bottom surfaces of the leaves. Next, the cleaned leaves were put into the basket or container and let them to dry in a room temperature of 37 °C for a week. The dried leaves become shrinks, brittle and the green colour of leaves changed to dark brownish. Then, the dried leaves were cut into small pieces followed by grinding into a fine

powder form. The powdered leaves were kept in a zip lock plastic bag in a desiccator to prevent moisture loss and contamination.

3.1.2 Preparation of plant extract

The powdered material was dried again in an oven at 40°C for 4 h and used for extraction. A total of 47g of powdered leaf sample was extracted with 940 ml of hexane according to ratio 1:20 proposed by (Chirinos et al., 2007). This process was repeated with another four solvents which are chloroform, acetone, ethyl acetate and methanol until the residual marc got exhaustively extracted and finally extracts was concentrated to dryness in rotary evaporator.

3.1.3 Preparation of extract solution

A total of five extracts was added into 0.5 mL dimethyl sulfoxide (DMSO). After the extract was completely dissolved, a total of 0.5 mL of sterile distilled water was added into the extract to yield a stock with 20 mg/ mL concentration (the concentration of

DMSO in the extract stock solution was 50%). Then the extract solution was filtered using 0.2 µm pore size of sterile nylon membrane

3.2 Susceptibility test

3.2.1 Test microorganisms

In this study, antibacterial activities of the leaf extract of *C. nutans* had been tested on two different types of bacteria, Gram-positive and Gram-negative bacteria:

Gram- positive bacteria

i) *Staphylococcus aureus*

ii) *Methilin-resistant Staphylococcus aureus*

iii) *Bacillus cereus*

iv) *Bacillus subtilis*

Gram-negative bacteria

i) *Yersina Enterocolitica*

ii) *Klebsiella pheumoniae*

iii) *E. coli*

3.2.2 Preparation of test inoculum and seeded agar plate

A loopful of a pure bacterial colony was picked from a 24 hours old bacterial culture on nutrient agar (NA) (Oxoid, England) followed by suspended in a 5 ml of sterile physiological saline (0.85% sodium chloride) solution. The resulting suspension had been vortex uniformly and the suspension turbidity was adjusted visually to match 0.5 McFarland standards (approximately 1.5 x 108 CFU/mL). After that, a sterile cotton swab was dipped into the bacterial suspension and pressed hardly on the inside wall of universal bottle to ensure the removal of any excessive inoculum from the cotton swab. The cotton swab was streaked over the whole Mueller Hinton Agar (MHA) surface thrice with the rotation angle of 60^0 to ensure the uniform distribution of bacterial inoculum (CLSI, 2006)

3.2.3 Preparation of susceptibility test

Whatman no. 1 filter papers (0.14mm of thickness) that had punched to become a 6 mm of diameter disc was autoclaved at 1210 C for 15 min for the sterilization purpose. After that, 10 µL of extract solution (20 mg/mL) was pipetted onto sterile disc and left it to air dry for a moment prior to impregnated the disc with another 10 µL of extract to

produce the disc with 0.4 mg of extract and 1% DMSO. This disc then had left air dried prior to placing onto the agar plate that will be seeded with test microorganisms

3.2.4 Disc diffusion susceptibility test (Antibacterial activity)

Sterile Whatman antibiotic disc was placed on the surface of inoculated medium. The negative control of 1% DMSO was included for solvent effect detection whilst 30 µg per disc (20 µL of 1.5 mg/ mL) chloramphenicol was served as positive control for bacteria. After that, the plates were incubated at 37^0 C for 16 to 18 hours. The diameter of inhibition zones formed around the discs was measured and the experiment was conducted in triplicates in separate occasion starting from the initial step until the diameter measurement of clear inhibition zone.

3.3 Statistical analysis

All the experiments were independently repeated three times, and average zone of inhibition of test extracts relative to negative control was calculated using Microsoft Excel 2019 software.

3.4 Interpretation of results

The formation zone of inhibition around the agar plugs by Gram negative and Gram-positive bacteria was observed and measured after 24 hours of incubation time and the measurements were done thrice. For the positive control, chloramphenicol (30 μg/mL) was used for bacteria. The result was recorded as zone inhibition -, +, ++ and +++.

- = No inhibition zones

\+ = Small inhibition zone (≤10 mm)

++ = Medium inhibition zone (11 to ≤ 20 mm)

+++ = Large inhibition zone (≥ 21 mm)

3.5 Preliminary chemical profiling of extract

3.5.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was used to separate compounds of five different extracts. The type of chromatography used was normal phase chromatography. The TLC plate was cut into strips of 2cm x 12cm and lines were draws across the TLC plate 1.5cm from bottom and 0.5cm from top using pencil. The TLC plates were activated in an oven at 80℃ for about 30 minutes. After complete cooling, they were ready to be used. A volume of 1.0 µl of the extract solution was spotted on the baseline of the plate to form tiny round spot. The TLC plate was swing for speed drying purpose.

C. nutans extract contain various of compounds with different polarity. In order to attain the best separation, solvent system was prepared using three solvent which are petroleum ether, ethyl acetate and methanol. Petroleum ether, ethyl acetate and methanol were adjusted to the ratio of (v/v/v) 1:1:1 respectively. Solvent system was used in order to act as mobile phase while silica gel acted as stationary phase. Prepared TLC plates were placed in closed chamber containing solvent system covered with glass lid in order to prevent from evaporation occur (Figure 3.1). Mobile phase of spotted extract was then left to run up the TLC plate. The TLC plates were removed immediately from the chamber as the mobile phase reached top line (0.5cm from top) of TLC plates. Then, developed TLC plates were air dried prior to further detection. TLC plates developed in solvent system were used in detection of bioactive compounds include flavonoid, alkaloid, phenol, lactone and anthraquinone.

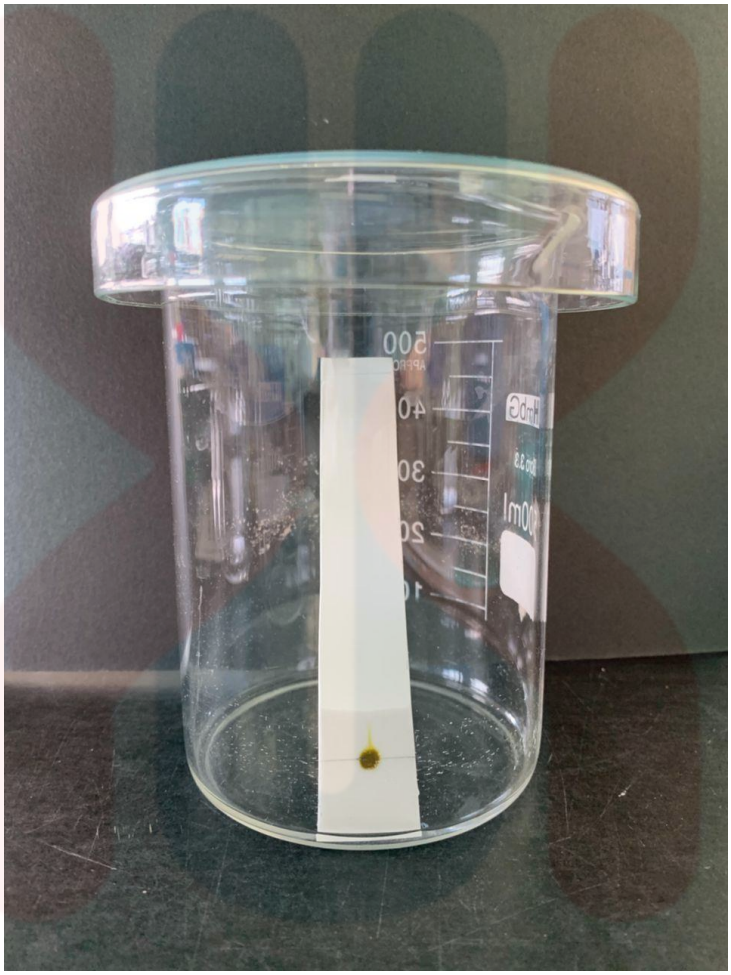


Figure 3.1 Chromatogram developed in closed chamber

3.5.2 Detection of phenol

Phenolic compounds can be detected using freshly prepared 1% aqueous ferric chloride ($FeCl_3$). The reagent was prepared by dissolving 1g of $FeCl_3$ in distilled water (dH_2O). The developed TLC plate was sprayed with reagent The presence of phenol was

indicated by appearance of green, purple, blue or black colours (Richardson & Harborne, 1985).

3.5.3 Detection of alkaloid

Alkaloids were detected using Wagner's reagent (Raaman, 2006). The reagent was prepared by dissolving 1.27g of iodine and 2g of potassium iodide in 5ml distileed water, afterwards top up with distilled water until 100ml. The developed TLC plate was sprayed with Wagner' reagent. The presence of alkaloid was indicated by appearance of reddish brown colour.

3.5.4 Detection of flavonoid

The presence of flavonoid was tested by spraying with increasing amount of 1M sodium hydroxide (NaOH). The presence of flavonoid was indicated by appearance of yellow colour and discolours after addition of 1M of Hydrochloride acid (HCl) (Ganatra *et al.*, 2012).

3.5.5 Detection of anthraquinone

TLC plate was sprayed with 10% methanolic potassium hydroxide (KOH), a solution of 100ml methanol and 10g of KOH. The presence of anthraquinone was indicated by appearance of red, violet, green or purple colours (Richardson & Harborne, 1985).

3.5.6 Detection of lactone

Lactone were detected by placing the developed plates in chamber containing iodine crystals. The presence of lactone was indicated by appearance of brown spot

CHAPTER 4

RESULT & DISCUSSION

This chapter discusses the results of inhibition zone diameter against test microorganisms on the different types of extracts using disc diffusion susceptibility test and the screening of bioactive compound groups present.in the extracts using spray reagents on developed TLC plates

4.1 Extraction of *C. nutans* leaf on different types of solvents

In the current study, *C. nutans* leaf was extracted by using five different types of solvents which are hexane, chloroform, acetone, ethyl acetate and methanol. The extraction of *C. nutans* leaf was done sequentially based on polarity starting from non-polar solvent until polar solvents. The extracts were then concentrated to dryness in rotary evaporator. The result of extract yield of *C nutans* leaf was tabulated in Table 4.1.

Table 4.1 Results of colour and yield extracts of *C. nutans* leaf

Extracts	Yield (%)	Colour
Hexane	0.07	Yellowish green
Chloroform	0.08	Black
Acetone	0.08	Green
Ethyl Acetate	0.01	Light Green
Methanol	0.96	Dark green

Based on Table 4.1, it showed the percentage yield and appearance of different extracts of *C. nutans* leaf. Methanol extract had the highest percentage of yield with 0.96% while ethyl acetate had the lowest percentage with only 0.01%. As for chloroform and acetone they had the same amount of yield percentage which is 0.08% while Hexane produced 0.07%. Meanwhile, the colour of each extracts produced were different where Chloroform had the highest colour intensity which is black followed by methanol (dark green), acetone (green), hexane (yellowish green) and lastly ethyl acetate for the lowest colour intensity which is light green.

Application of different solvents were used in order to extract and separate as much as possible bioactive compound that presents in the plant sample. Different solvents showed a significant influence on the total yield of the extracts. Based on the results, methanol extract had the highest percentage of yield as compared to others due to its polarity which is polar solvents. It is clearly indicated that there is higher presence of polar compound in plant. Similar finding was also shown by Swamy (2015) which total yield of methanol extracts of *L. camara* leaves was the highest as compared to ethyl acetate and acetone. Similarly, Gaikwad (2015) also showed yield percentage of methanolic extracts from *Cissus quadrangularis* was the highest followed by water

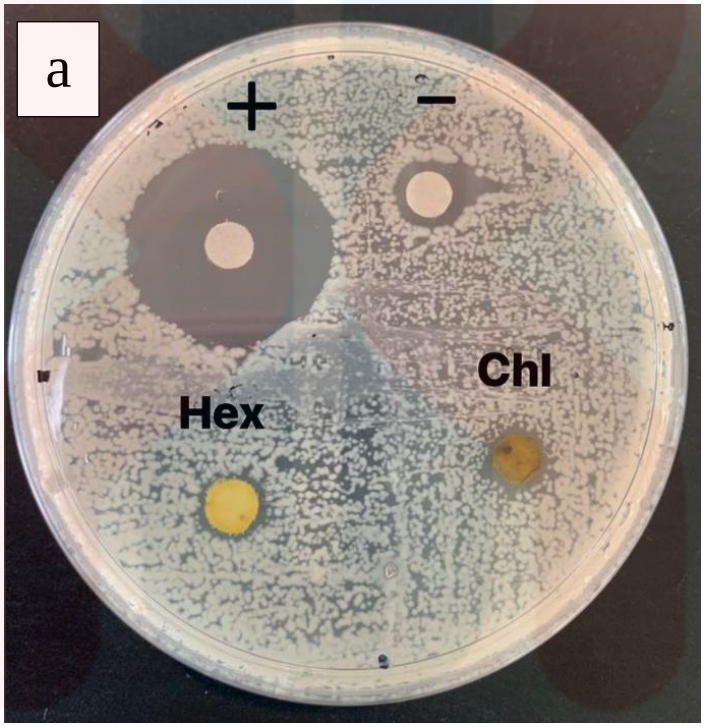
extract. Therefore, it can be concluded the usage of methanol as an extractant solvent could recover greater extractable compounds from a variety of medicinal plants.

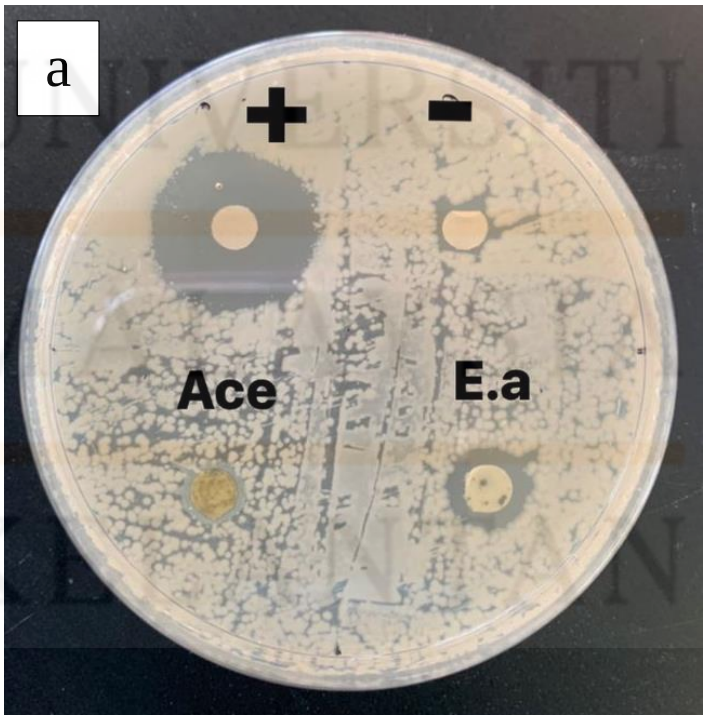
4.2 Bacterial inhibitory potential of different extracts of *C. nutans* leaf

4.2.1 Disc diffusion susceptibility test

Groups of foodborne pathogenic bacteria were selected for the disc diffusion susceptibility test. It consists of Gram-positive and Gram-negative bacteria where a total of seven bacteria were used to test the antibacterial potential of leaf extract from *C. nutans*. Five different extracts were tested against seven types of bacteria which four of them were Gram-positive bacteria (*B.* cereus, MRSA, *S. aureus* and *B. subtilis*) while another three were Gram-negative bacteria (*Y. Enterocolitica, K. pheumoniae* and *E. coli*). Twenty microlitres per disc of chloramphenicol and DMSO were used as positive and negative control, respectively. Chloramphenicol was used as referral drugs to indicate the effect of antibiotic on test microbes. The test was done three times and antibacterial activities was determined based on the mean of inhibition zone diameter ± standard deviation (mm ± SD).

The images of inhibition zone of *C. nutans* extracts against *B. cereus* and *K. pneumoniae* bacteria were illustrated in Figure 4.1





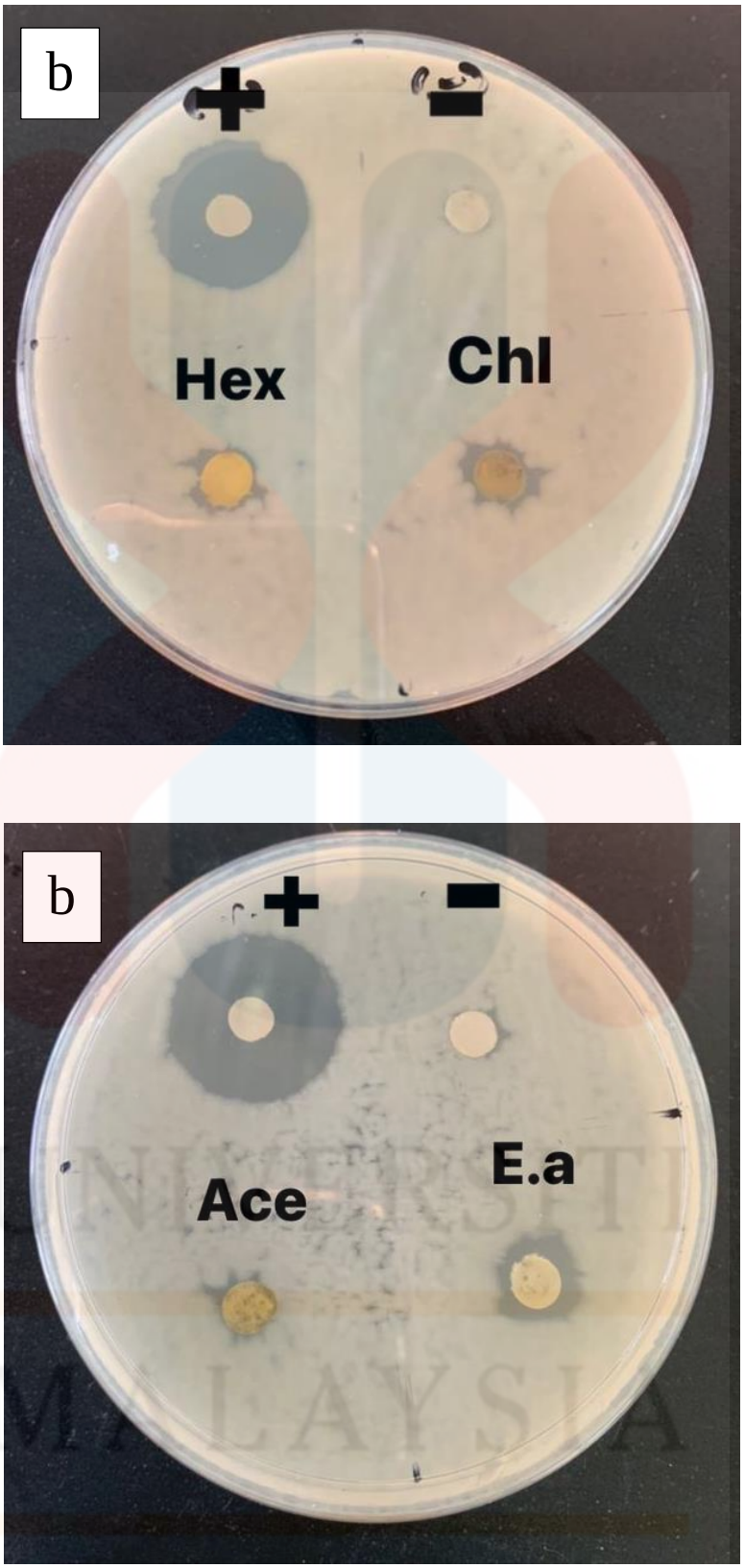


Figure 4.1 Inhibitory zone of different extracts towards *B. cereus* and *K. pneumoniae* bacteria; a: *K. pneumoniae*, b: *B. cereus*

[+ = positive control, - = negative control, ace = acetone extract, hex = hexane extract, chl = chloroform extract, e.a = ethyl acetate extract]

Table 4.2 Antibacterial activities of *C. nutans* extracts against test microorganism

Bacteria	Mean of inhibition zone diameter against test microorganism (mm ±SD)						
	Hexane	Chloroform	Acetone	Ethyl Acetate	Methanol	Positive control	Negative control
B. cereus	9.0 ±1.7	9.3 ± 1.2	8.0 ±1.0	10.7 ±1.2	-	21.4 ±0.9	-
MRSA	-	-	-	5.0 ± 0.0	-	20.0 ± 0.0	-
S. aureus	-	-	-	-	-	-	-
B. subtilis	-	8.3 ± 0.6	6.0 ± 0.0	11.0 ± 0.0	-	23.3 ± 1.6	-
Y. Enterocolitica	-	6.3 ± 0.6	-	8.7 ±0.6	-	16.3 ± 0.6	-
K. pneumoniae	8.0 ±0.0	8.7 ± 0.6	8.3 ± 0.6	11.7 ± 0.6	-	22.9 ± 2.4	-
E.coli	-	-	-	-	-	24.1 ± 0.9	-

Table 4.2 shows the antibacterial activities of *C. nutans* extracts against selected test microorganisms. Based on table 4.2, all the extracts exhibited antibacterial activities for at least two types of bacteria with the exception of methanol extract. The range of inhibition zone for Gram-positive bacteria was between 5.0 mm to 11.0mm, while for Gram-negative bacteria between 6.3mm to 11.7mm. Figure 4.2 shows a clear illustration of percentage of inhibition zone of all extracts against tested microorganism. From the graph, we can see that all five bacteria were inhibited by the extracts whereas for *S. aureus* and *E. coli* showed no inhibitory activities. The same finding from the study by (Yang et al., 2013) showed S. aureus and E. coli had lower susceptibility towards *C. nutans* extract among all tested bacteria. Among Gram-positive bacteria, S. aureus exhibited no antibacterial activities which contradicts with the findings by Bhatt & Negi (2012) where MRSA was less susceptible towards *Plectranthus amboinicus* extract as compared to other Gram-positive bacteria.. Figure 4.2 showed the percentage of inhibition towards tested microorganism. It showed that *B. cereus* and *K. pneumoniae* had the highest percentage of inhibition zone which exhibited 80% of antibacterial activities from five different extract followed by *B. subtilis* which exhibited 60% and *Y. enterocolitica* 40%. As for MRSA, it had the lowest percentage of inhibition with only 20% of all extracts.

As for comparison between each extract, hexane, chloroform, acetone, ethyl acetate and methanol, ethyl acetate extract showed inhibition zone diameter against tested bacteria with the range from 5.0 to 11.7mm which considered as intermediate susceptible while other extracts showed no to small inhibition zone with the range below 10mm. Current study was in line with the study conducted by Arullappan *et al.,* (2014) where ethyl acetate fraction was the most effective of all the solvents, with high anti-bacterial properties against B. cereus and E. coli. However, the same study was carried out on Plectranthus amboinicus, the results illustrate that superior antimicrobial activity was shown by the acetone extract (Bhatt & Negi, 2012). This disparity could be attributed to differences in extraction methods and microbes tested. Thus, ethyl acetate extract demonstrated the strongest antibacterial activity, inhibiting 5 microorganisms and producing intermediate inhibition zone.

Gram-positive bacteria appeared to be more vulnerable towards all extracts compared to Gram-negative bacteria in this study. Similarly, (Swamy et al., 2015) stated that Gram-positive bacteria was more susceptible towards solvent extracts of *L. camara* compared to Gram-negatibe bacteria. However, finding by (Lim et al., 2020) illustrate that *C. nutans* extracts were more effective against gram-negative bacteria than gram-positive bacteria in terms of antimicrobial activity.

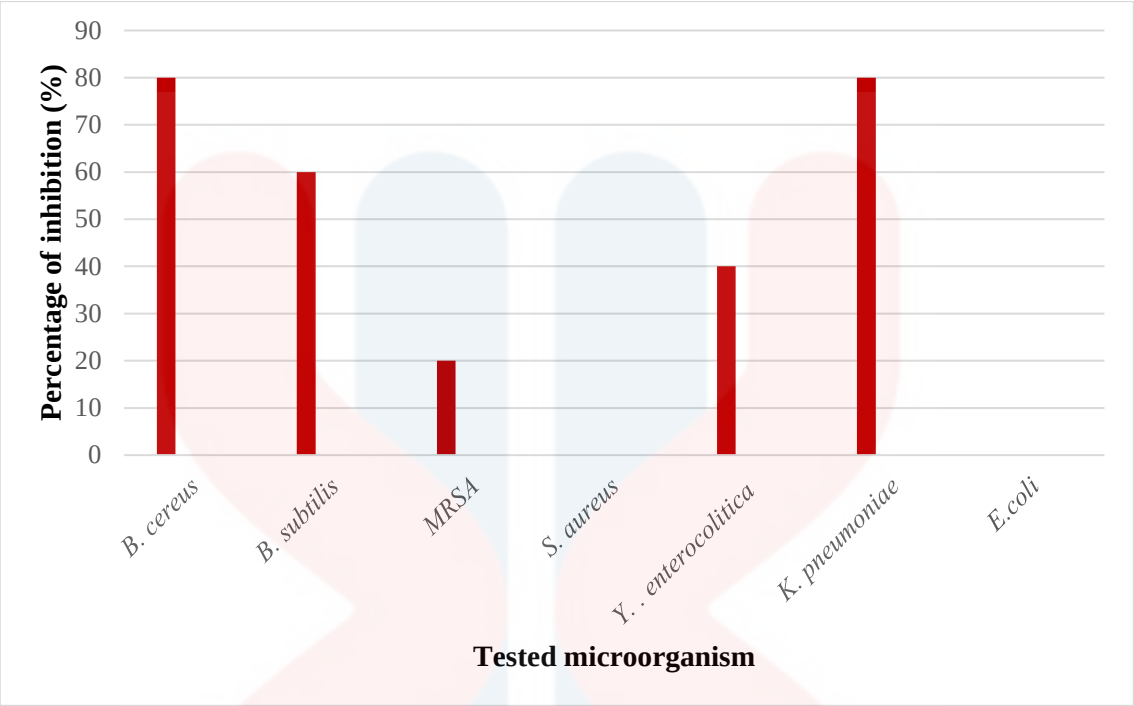


Figure 4.2 Comparison of percentage of inhibiton of *C. nutans* extracts against tested microorganism

Through the disc diffusion susceptibility test, it is possible that the difference in sensitivity between Gram-negative and Gram-positive bacteria is due to differences in cell wall structure. Bhatt *et al.*, 2013 claimed that Gram-negative bacteria are more resistant to a wide range of antibiotics and chemical medicines than Gram-positive bacteria. Differences in outer membrane of bacteria are also connected to their susceptibility to plant extracts (Breijyeh *et al.*, 2020). Generally, the cell wall of Gram-positive bacteria is mostly made up of peptidoglycan, which contributes 90% of the cell wall while Gram-negative bateria have more compilicated cell wall. Higher susceptibility on Gram-positive bacteria could be because the extracts that permeate the cell wall have easier access to the mesh-like peptidoglycan layer while the resistance towards *C. nutans* extracts by Gram-negative bacteria may contributed due to the outer membrane that made up from lipopolyssacharide which opposed entry of plant extracts. (Lim *et al.*, 2020).

From the results, ethyl acetate extract possesses the most prominent antibacterial activity against all of the microorganisms tested, which could be related to more soluble bioactive chemicals extracted in this extract. Possibily, bioactive compounds in the extract able to efficiently suppress microbial growth by binding to their cell surface. In line with the previous study, antibacterial action of plant extract is thought to be due to the cumulative effect of phenolic compounds adsorption onto the cell membrane, which causes disruption and cell leakage, as well as phenolic compounds' formation of hydroperoxides (Arumugam *et al.*, 2016).

4.3 Thin layer chromatography

Thin layer chromatography was carried out using solvent system of hexane: petroleum ether: methanol at the ratio of 1:1:1 to develop chromatograms. It was conducted to separate bioactive compounds within the five extracts (hexane, chloroform, acetone, ethyl acetate and methanol). It will determine the polarity of the compounds by separating mixed compounds into individual compound according to the principal functional group(s) each contains. Molecular classes can be classified into a range or spectrum of chromatographic polarity ranging from very polar to highly non-polar, based on their relative retention (Petrova & Sauer, 2017). As shown in Figure 4.3 and Figure 4. there were differences in distance travelled by solvent on each TLC plate.

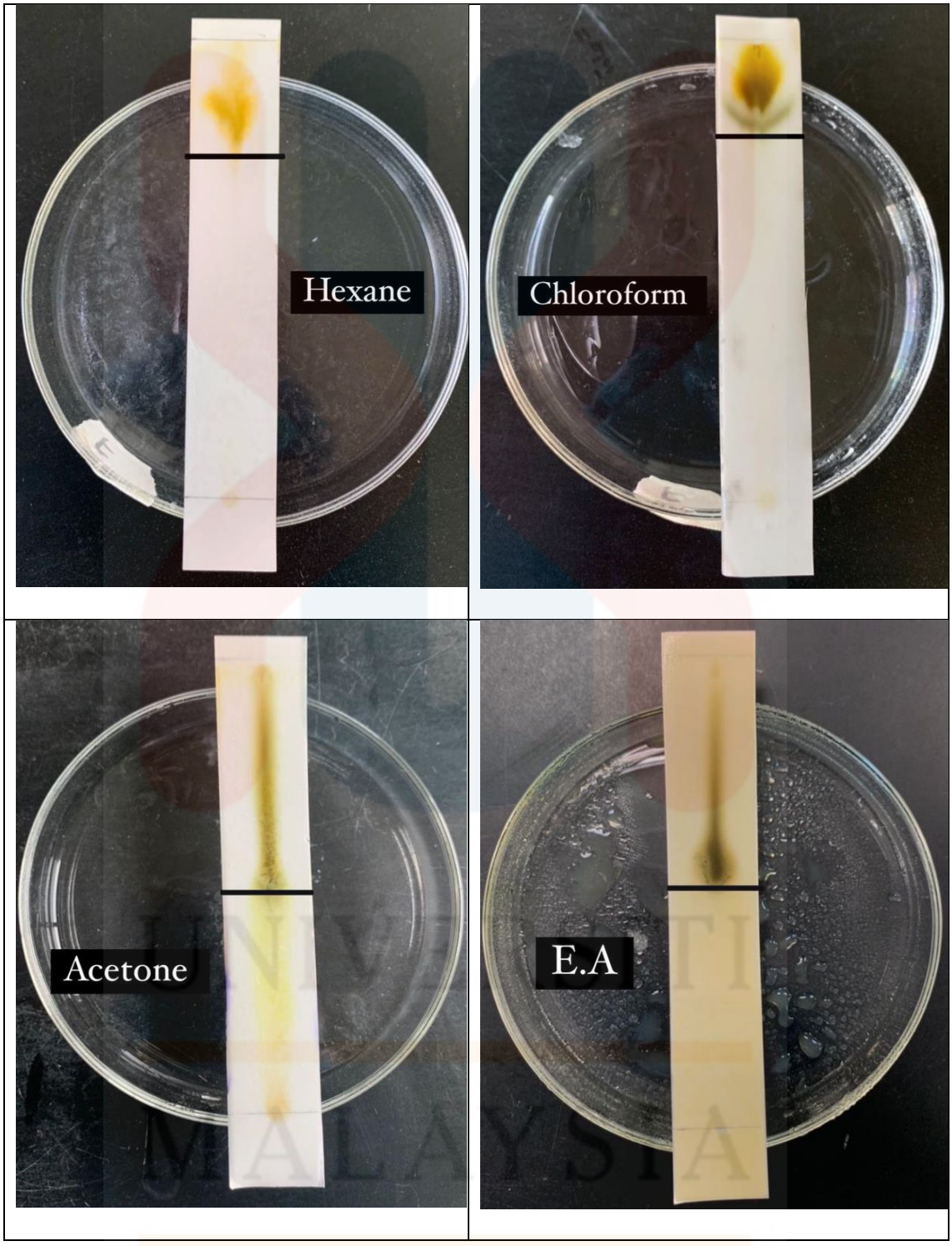


Figure 4.3 Distance travelled by solvents on each TLC plates

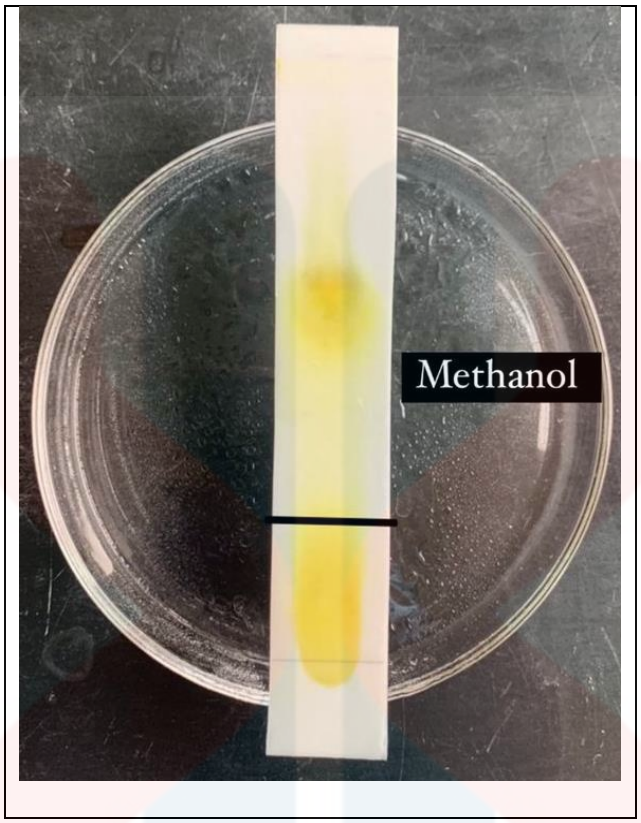


Figure 4.4 Distance travelled by methanol extract on TLC plate

Some common solvents are placed in order of relative chromatographic polarity is called an eluotropic series. Mobile phase molecules that compete effectively with analyte molecules for the attractive stationary phase sites displace these analytes, causing them to move faster through the column. It creates competition for the various compounds contained in the sample by choosing a mobile phase and a stationary phase with different polarities. Then, compounds in the sample that are similar in polarity to the stationary phase will be delayed because they are more strongly attracted to the particles. Compounds whose polarity is similar to that of the mobile phase will be preferentially attracted to it and move faster.

4.4 Detection of various compounds on TLC plate

In order to detect the presence of structural groups of compounds in the extracts (phenol, alkaloid, anthraquinone, flavonoid and lactone), several tested were done using different spray reagents. The reagent of each test was prepared and sprayed on the TLC plates or by placing the plates in a chamber. The presence of bioactive compounds was determined by observing changes in the colour of the extracts or sedimentation forms after they were treated with the appropriate reagents. The results were tabulated in Table 4.3. Figure 4.5 showed a clear illustration of chromatograms of *C. nutans* extract treated with appropriate reagents percentage of inhibition zone of all extracts against tested microorganism.

Table 4.3 Phytochemicals analysis of different extracts *C. nutans* leaves

	Preliminary chemical profiling of antibacterial compounds				
Extracts	**Phenol**	**Alkaloid**	**Flavonoid**	**Anthraquinone**	**Lactone**
Hexane	Green	Reddish brown	Yellow	-	-
Chloroform	Black	Reddish brown	Yellow	Green	Brown spot
Acetone	Green	Reddish brown	Yellow	Red	Brown spot
Ethyl acetate	Green	Reddish brown	Yellow	Red	Brown spot
Methanol	Green	-	Yellow	Green	Brown spot

Based on the data presented in Table 4.3, at least three bioactive compounds were detected in each of the extracts where hexane showed the least diverse of compound as compared to the other extracts. Chloroform, acetone and ethyl acetate had the most diverse as all the five group of compounds tested in current study present in these two extracts. In the present study, the presence of bioactive compounds was better evidenced in polar solvents extract (acetone and ethyl acetate) compared to nonpolar solvents (hexane). The differences in compound present in each extract could be attributed to the wide range of diverse phytochemical components in *C. nutans* leaves and differences in the solvent polarity. As mentioned by Iloki-Assanga et al., (2015), the polarity of extracting solvents and the solubility of each compound in the solvent used for the extraction process influence the recovery of phenolic contents in different samples. The result was also in line with previous study by Bhatt & Negi, (2012), stated acetone extract contained significantly higher total phenolics compared to the methanol or hexane extracts of *P. amboinicus.*

As compared to non-polar solvents like hexane, polar solvents were able to improve cell permeability and penetrate inside the cells, extracting more intracellular secondary metabolites, both polar and less polar compounds. (Seidel, 2012) . In general, secondary metabolites with polar features, such as flavonoid glycosides, tannins, and various alkaloids, were extracted using methanol with a high polarity index while non-polar solvents with zero polarity index, such as n-hexane, only dissolved lipophilic substances such as alkanes, waxes, colour pigments, sterols, numerous terpenoids, and alkaloids, extracting fewer secondary metabolites (Seidel, 2012).Therefore, our results showed that our C. nutans extracts contain higher constituent of polar compound rather than non- polar compound. .

CHAPTER 5

CONCLUSION & RECOMMENDATIONS

The antibacterial activity of the solvents extract of *C. nutans* leaves was evaluated and the results revealed that 4 out of 5 extracts of *C. nutans* leaf possessed antibacterial effect against test bacteria except methanol extract. The results obtained demonstrated that methanol was the least effective solvent to extract phytochemical compounds compared to chloroform, acetone, ethyl acetate, and hexanes. This might be due to the most of the compounds in the leaf of *C. nutans* is either non-polar or semi polar compounds.

The present investigation also revealed the phytochemical composition of *C. nutans* leaf extract varied with respect to different solvents. In this study, the presence of various soluble bioactive compounds in *C. nutans* extracts greatly contributed to antimicrobial activities. Thus, it shows that some of the medicinal plants used in traditional medicine are potentially effective antimicrobial agents. In comparison to Gram-negative bacteria, Gram-positive bacteria were more susceptible to the extracts. Due to limited studies on *C. nutans* extracts, further studies must be conducted to elucidate the pharmacologically active compounds from this plant.

The evaluation of antimicrobial potential and its bioactive compounds in the plants used in traditional medicine is necessary. Several *C. nutans* extracts were shown to have potential antibacterial activity against a wide spectrum of bacteria used in this study. Thus, the potential bioactive compounds in the C. nutans extracts were preliminary screened using TLC plates by spraying respective reagents to identify the group of organic compounds that may present in the samples. In the future, the phytochemical structures of these bioactive compounds should be further purified and identified them by employing high-performance liquid chromatography, Liquid chromatography–mass spectrometry or gas chromatography–mass spectrometry as it may serve as a lead to the novel compounds.

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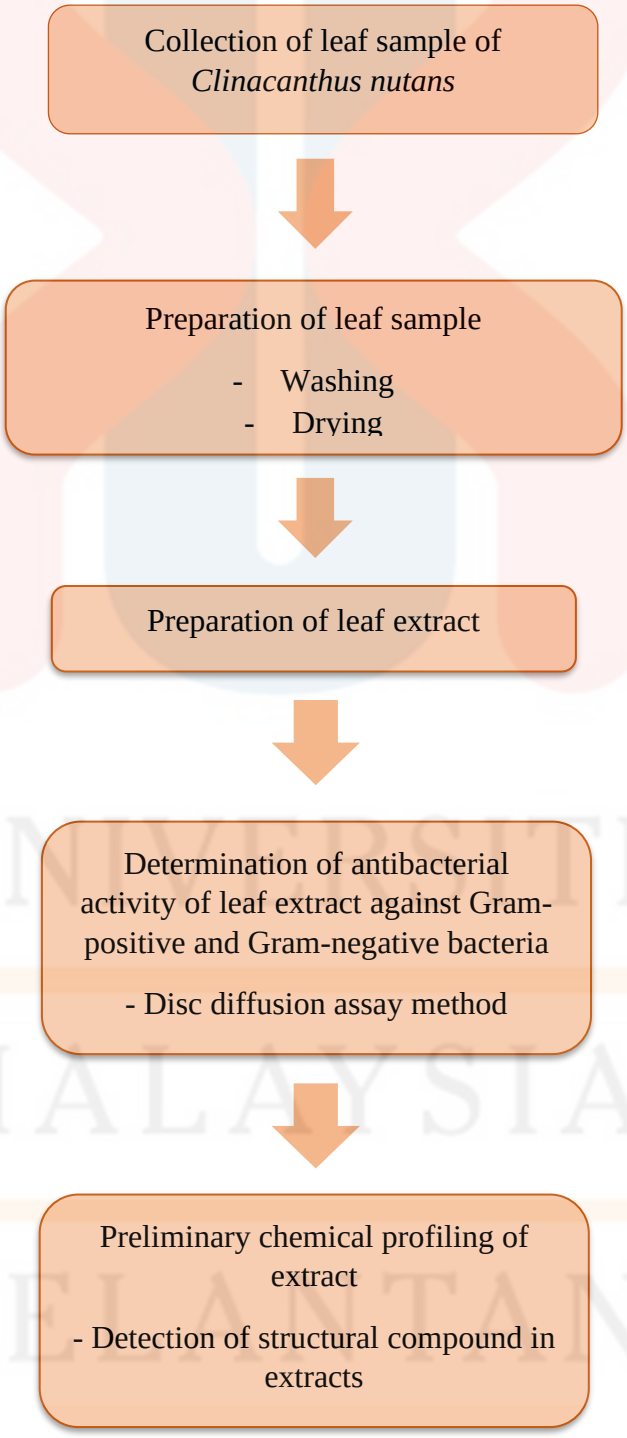
APPENDIX A

Flow chart of experiment



Gantt Chart

		2021				2022					
Month/ Activities		**3**	**4**	**5**	**6**	**9**	**10**	**11**	**12**	**1**	**2**
Title selection											
Research proposal											
Submission of research & video proposal											
Proposal presentation											
Submission of final research proposal											
Research conducted	Collection of plant sample										
	Preparation of leaf sample & extract										
	Disc diffusion susceptibility test										
	Preliminary chemical profiling of extract										
Data collection											
Result Analysis & Interpretation data											
Written thesis report											
Submission of final thesis											
Thesis presentation / Viva											