Antimicrobial Potential of Leaf Extract of *Chromolaena odorata*

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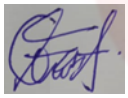
DECLARATION

I hereby declare that this work embodied in this report is the result of my own research except for the excerpt as cited in the references.



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ABSTRACT

Since ancient times, medicinal plants have been used widely by people to cure numerous diseases and infections. Biological active compounds such as alkaloids and flavonoids found in these plants are believed to possess antimicrobial properties. Hence, this study was aimed to investigate the antimicrobial potential of leaf extract of *Chromolaena odorata* isolated from the sequencially solvent extraction which were hexane, chloroform, acetone, ethyl acetate and methanol. The powdered leaves sample of *C. odorata* was successively extracted with different organic solvents according to the increasing polarity to produce crude extracts. These extracts were then screened for antimicrobial activity against selected Gram-positive and Gram-negative bacteria, viz, *Stapylococcus aureus, Methilin-resistant Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Escherichia coli, Klebsiella pheumoniae as well as Yersinia Enterocolitica*. The results indicated that the methanol leaf extract of *C. odorata* had the highest percentage of extraction yield compared to others solvent. Besides, the antimicrobial activities from different extracts indicated that they were easier to inhibit Gram-positive bacteria compared to Gram-negative bacteria due to the different composition of bacterial cell wall. In conclusion, the identification of bioactive compounds responsible for antimicrobial activity of leaf extract of *C. odorata* can lead to the development of novel antimicrobial agents as medicinal plants have been acknowledged to harbour diverse bioactive compounds. This study will contribute to the development of natural antimicrobial agent that can replace the existing of antibiotic where the bacteria have become resistance to them.

Keywords: medicinal plants, antimicrobial properties, *C. odorata,* crude extracts, antimicrobial activity.

Potensi Antimikrob bagi Ekstrak Daun *Chromolaena odorata*

ABSTRAK

Sejak zaman purba, tumbuhan ubatan telah digunakan secara meluas oleh orang ramai untuk menyembuhkan pelbagai penyakit dan jangkitan. Sebatian aktif biologi seperti alkaloid dan flavonoid yang terdapat dalam tumbuhan ini dipercayai mempunyai sifat antimikrob. Oleh itu, kajian ini bertujuan untuk mengkaji potensi antimikrob ekstrak daun *Chromolaena odorata* yang diasingkan daripada pengekstrakan pelarut mengikut urutan iaitu heksana, kloroform, aseton, etil asetat dan metanol. Sampel daun serbuk *C. odorata* diekstrak berturut-turut dengan pelarut organik yang berbeza mengikut kekutuban yang semakin meningkat untuk menghasilkan ekstrak mentah. Ekstrak ini kemudiannya disaring untuk aktiviti antimikrobial terhadap bakteria Gram-positif dan Gram-negatif terpilih, iaitu *Stapylococcus aureus, Methilin-resistant Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Escherichia coli, Klebsiella pheumoniae* serta *Yersinia Enterocolitica*. Keputusan menunjukkan bahawa ekstrak daun metanol *C. odorata* mempunyai peratusan hasil pengekstrakan yang paling tinggi berbanding pelarut lain. Selain itu, aktiviti antimikrob daripada ekstrak yang berbeza menunjukkan bahawa mereka lebih mudah untuk menghalang bakteria Gram-positif berbanding bakteria Gram-negatif disebabkan oleh komposisi dinding sel bakteria yang berbeza. Kesimpulannya, pengenalpastian sebatian bioaktif yang bertanggungjawab terhadap aktiviti antimikrob ekstrak daun *C. odorata* boleh membawa kepada pembangunan agen antimikrob baru kerana tumbuhan ubatan telah diakui mempunyai sebatian bioaktif yang pelbagai. Kajian ini akan menyumbang kepada pembangunan agen antimikrob semulajadi yang boleh menggantikan antibiotik sedia ada di mana bakteria telah menjadi rintangan kepada mereka.

Kata kunci: tumbuhan ubatan, sifat antimikrob, *C. odorata*, ekstrak mentah, aktiviti antimikrob

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LIST OF ABBREVIATIONS

NO.	ABBREVIATION	MEANING
1.	WHO	World Health Organization
2.	TCIM	Traditional, Complementary and Integrative Medicine
3.	R&D	Research & Development
4.	SA	*Staphylococcus aureus*
5.	SSTI	Skin and Soft Tissue Infections
6.	CAP	Community-Acquired Pneumonia
7.	cUTI	Complicated Urinary Tract Infections
8.	cIAI	Complicated Intra-Abdominal Infections
9.	MRSA	*Methilin-resistant Staphylococcus aureus*
10.	VRE	Vancomycin-resistant enterococci
11.	CRE	Carbapenem-resistant-Enterobacterales
12.	UV	Ultra Violet
13.	NAG	N-acetylglucosamine
14.	NAM	N-acetylmuramic
15.	LPS	Lipopolysaccharide
16.	(GC-MS)	Chromatography-mass Spectrometry
17.	NA	Nutrient Agar
18.	MHA	Mueller Hinton Agar

19.	DMSO	Dimetyl sulfide
20.	SD	Standard Deviation
21.	TLC	Thin Layer Chromatography
22.	$AlCl_3$	Aluminium chloride
23.	NaOH	Sodium hydroxide
24.	HCl	Hydrocloric acid
25.	KOH	Potassium hydroxide
26.	$FeCl_3$	Ferric chloride
27.	DNA	Deoxyribonucleic acid
28.	FFA	Free Fatty Acid
29.	TEC	Total Extractable Component
30.	BC	*Bacillus cereus*
31.	BS	*Bacillus subtilis*
32.	KP	*Klebsiella pheumoniae*
33.	*E. coli*	*Escherichia coli*
34.	YE	*Yersinia Enterocolitica*

LIST OF SYMBOLS

NO.	SYMBOL	MEANING
1.	%	Percentage
2.	m	Meter
3.	cm	Centimetre
4.	°C	Degree Celsius
5.	MHz	Megahertz
6.	GHz	Gigahertz
7.	g	Gram
8.	ml	Millilitre
9.	CFU/mL	Colony Forming Unit per millilitre
10.	°	Degree
11.	mg	Miligram
12.	Mg/mL	Miligram per millilitre
13.	mm	Millimetre
14.	μL	Microlitre
15.	μg	Microgram
16.	v/v	Volume per volume
17.	nm	Nanometre
18.	μg/mL	Microgram per millilitre
19.	w/v	Weight per volume

20.	-	No inhibition zone
21.	+	Small inhibition zone
22.	++	Medium inhibition zone
23.	+++	Large inhibition zone
24.	-	Absent
25.	+	Present

CHAPTER 1

INTRODUCTION

1.1 Research Background

Malaysian biodiversity encompasses variuos kind of medicinal plant species where they can adapt successfully in this country due to the favourable environment and suitable climate, allowing them to preserve their value. These medicinal plants which derived from various kind of flora are believed to have therapeutic properties and have been used by many people around the world as an alternative medicine. Medicinal plant has been used by people for a long time ago as a traditional medicine since there is lack of alternative medicine at that time. Besides, medicinal plant has commonly been used as a herbal medicine due to the occurrence of adverse effect of synthetic drugs in treating illness and infections. According to World Health Organization (WHO), it said that herbal medicines that is developed by medicinal

plants are becoming increasingly popular around the world since many people especially those who live in local area turning to these crops for treatment of a variety of health problems. Other than that, this traditional medicine is actually apart of TCIM (Traditional, Complementary and Integrative Medicine) and therapies that incorporates the use of traditional herbs, metaphysical and traditional healing, as well as other medicinal procedures that function to avoid from getting diseases and is considered as an ancient type of healthcare (Kenu, 2021). Moreover, majority of medicinal plants possess antimicrobial properties as they able to generate bioactive compund and contain a wide range of phytochemical as well such as alkaloids, tannins, terpenoids and flavonoids.

Chromolaena odorata is one of the local medicinal plant that possess the therapeutic characteristics and positive pharmacological impact towards living organisms. This plant species have many benefits and considered to be a significant weed in plantation crops around the world like palm oil, rubber and coconut. It is because *C. odorata* has the potential to be a nutrient sink, provide a consistent foundation of organic matter and nutrients to the crops. Moreover, it can also be used as green manure and has a positive impact on exchangeable K levels. Another benefits of *C. odorata* to the plantation crop is it can responds to acidic soils better as a fallow plant than other leguminous plants. *C. odorata* has an impact on ecological structure, species composition and it has been suggested that the long-term use of these weeds as an organic mulch in the establishment of young banana plantations should be encouraged in order to maximise their effectiveness. The roles of *C. odorata* that provides some positive effects on human other than become a traditional medicinal plant is some portions of this plant such as leaves that possess antimicrobial properties is used to manage wounds, skin infections and they also have anticancer and antiinflammatory as well that is able to guarantee human health.

Moreover, the leaf part of *C.odorata* can be used for extraction purpose to evaluate the antimicrobial properties that is able to manage wound healing and skin infections which is actually act as antimicrobial agent. In other word, the leaves of *C. odorata* have the ability to produce therapeutically active compounds that could be used as building blocks for the production of potent and safe drugs. Several phytochemicals that were discovered in *C. odorata* is flavonoids, saponins, alkaloids and tannins where all of them are known as secondary metabolites (Usunomena, 2016). Secondary metabolites can be defined as small organic molecules that was formed by an organism where those molecules are not required for its growth and reproduction. A variety of secondary metabolites derived from medicinal plants can act as active wound healers. Furthermore, the most common type of diseases that can be cured by this plant species is diabetes, diarrhoea, tooth ache and colitis. For example, the root extract of *C. odorata* is able to improve glucose control and lowering glycated albumin levels, which are responsible for the higher levels of albumin in diabetic patients (Saidu, 2019). Next, some bioactive compounds consist in *C. odorata* is stigmasterol, quercetin and scutellarein tetramethyl ether.

Figure 1.1**:** Leaves of *C. odorata*

1.2 Problem Statement

Today, antibiotic resistance has become a major clinical and public health concern in most people's lifetimes because the development of bacterial resistance is considered to be a significant issue since humans struggle to combat them even by using antibiotics. Several bacterias have been reported to develop antibiotic resistance where it is actually occurs when the antibiotics are being used in a very large amount and is known to be uncontrolled. Besides, antimicrobial resistance is on the rise as a result of changes in the natural environment, which have transformed the population dynamics of microorganisms, including the determination of healthy quality, putting human health at risk and making it difficult to anticipate.

Other than that, the occurrence of antimicrobial resistance is actually due to the changes of microorganisms itself like bacteria, fungi and viruses which make the drugs that is used to treat some infections become ineffective. Nowadays, antimicrobial resistance has emerged as a main issue in veterinary medicine, especially when it comes to bacterial pathogens that affect both humans and animals. In aquaculture, it employs a lot of antimicrobials to manage bacterial infections which then create a problem where the bacteria have perfected a number of techniques for avoiding the antimicrobial agents' inhibitory effects and they become resist to them (Schwarz, 2016).

Furthermore, antimicrobial resistance also has give some impacts on human health since the aquaculture industry has use antimicrobials extensively and it has placed a selective strain on drug-resistant bacteria and transferable resistance genes in fish parasites that contributes to the production of horizontal, which can transmit resistance genes to human pathogens from the aquatic environment and directly enter the human body. The presence of antimicrobial resistance in human pathogens will restricts the therapeutic options available in human infections and this issue must be avoided to lower the risk of human health.

According to this issue, the use of medicinal plant is very important in order to produce new bioactive compounds obtained from the leaves parts which is actually able to manage various kind of disease problems. This is because the leaf extract of medicinal plant like *C. odorata* will produce some bioactive compounds such as alkaloid and other bioactive compounds that can act as sources of natural antimicrobial agent. In addition, it is considered to be more safer to human compared to synthethic antibiotics because it is derived from natural products. Since Malaysia is one of the world's most biodiverse nations, there are many medicinal plants that have yet to be discovered, including an impressive of antimicrobial potential of leaf extract of *C. odorata* that is capable of producing novel bioactive

compounds. Next, these natural bioactive compounds have a variety of biochemical structures that could contribute to the creation of new antimicrobial agents. In order to resolve antimicrobial resistance, further experiments and studies about the antimicrobial potential of leaf extract of medicinal plant as antimicrobial agents should be performed.

1.3 Hypothesis

Leaf extraction of *C. odorata* was forecasted to have an antimicrobial activity against a few selected of Gram-negative and Gram-positive bacteria.

1.4 Scope of Study

The present study was focusing on the antimicrobial activity of the leaf of local medicinal plant which is *C. odorata*. The antimicrobial potential of this leaf extract was evaluated by using several selected pathogenic Gram-negative and Gram-positive bacteria.

1.5 Significance of Study

Many research on natural products involving medicinal plant such as *C. odorata* that have been proven to possess a significant pharmacological activity have been conducted recently. Thus, the current study was conducted as a part of ongoing investigation to determine the antimicrobial potential of leaf extract of *C. odorata* using disc diffusion assay method. Besides, majority of medicinal plants are being an important foundations in order to undergo an extraction especially on its leaf with the aim to identify the antimicrobial potential of that plant in order to develop natural antimicrobial agent. The natural antibiotic is more efficient because it possess less adverse side effect to human health. Furthermore, the results of this study may provide guidance to all the pharmaceutical corporations in the production of natural antimicrobial agents derived from medicinal plants in order to combat the existing of antimicrobial resistance.

1.6 Research Objectives

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

2. To investigate the antimicrobial potential of five different extracts of *C. odorata* leaves on the selected Gram-positive and Gram-negative bacteria using disc diffusion assay.
3. To preliminary screen the group of compound present in the leaves extract of *C. odorata*.

CHAPTER 2

LITERATURE REVIEW

2.1 The History of Antibiotic Resistant and an Emerging Crisis of Antimicrobial Resistance Phenomenon

Antibiotic resistance occurs when bacteria and fungi acquire the ability to withstand antibiotics that were intended to destroy them where it indicates that the germs are not destroyed and keep to produce. It will be hard or incredible to manage several infections when the microorganism becomes resistant to antibiotic and that problem often necessitate longer hospital stays, extra follow-up doctor appointments and the bill for managing it will be more expensive. Besides, the meaning of antibiotic resistance is not imply that the body has developed resistance to antibiotics but it is where that particular bacteria or microbe have developed resistance to the antibiotics intended to destroy them.

Moreover, antibiotic resistance was identified proximately after the first extensive clinical trials of penicillin in the early 1940s. Mass manufacturing of penicillin is one of the largest war effort when the drug was commonly used by some armies as well as civilian population on the ancient time. Multiple strains of antibiotic-resistant bacteria were chosen as a result of prolonged exposure to antibiotic agents. *Methicillin-resistant Staphylococcus aureus* and *Penicillin-resistant Enterococcus* are the two types of bacteria that are resilient to antibiotics where both of them are found to be tuberculosis drug and rifampicin resistant (Morier, 2021).

However, antibiotic resistance is actually does not oocurs for providing negative effects but it also provides a good alternative regarding the use of antibiotic in order to ensure that is will be effective in treating a particular disease on human that comes from different ages. A study conducted by Benedict Hayhoe *et al.,* (2013) found that antibiotics can help prevent severe side effects from acute otitis media such as mastoiditis where antibiotics appear to be most effective in children under the age of two years old, who have bilateral acute otitis media, where four episodes must be managed to achieve one additional beneficial outcome (NNTB), and in children of all ages who have otorrhoea (NNTB 3).

Furthermore, the occurrence of crisis regarding the phenomenon of antimicrobial resistance has increaase the number of infectious diseases that is actually hard to be treated. This crisis has gives some negative effects on a large number of newborns where most of them are die every year and cannot survive due to an emergent of antibiotic resistant bacteria as well as there is a shortage of effective antibiotics. The drawbacks also give impacts on human health, with major consequences for poverty reduction and injustice, as well as food safety. Besides, this crisis will lead to a large number of human population to fall into extreme poverty each year, particularly on 2050 (Avafia, 2019). Antimicrobial resistance oocurs due

to a number of factors which involves a shortage of sufficient health care, improper antibiotic use in humans and food animals, inadequate water and there is deficiencies in R&D for critical health technologies.

2.2 Development of Antimicrobial Agents

Antimicrobial agent can be defined as a broad term that refers to medications, chemicals, and other constituents that destroy or delay the development of microbes such as bacteria, fungi and viruses where the example of antimicrobial agent is including antiviral drugs, antiparasitic drugs as well as antibacterial drugs. In the other word, antimicrobial agent is known as any of a wide range of chemical compounds and physical agents that is actually being used to kill or inhibit the growth of microorganisms. The development of antimicrobial agent is actually due to the increasing number of people that have been infected by many diseases (Burki, 2021).

Microorganisms were discovered to be the cause of a number of infectious diseases that have plagued mankind since the dawn of time and as the primary therapeutic technique, chemotherapy aimed at the causative species was established. A study conducted by Ehrlich in 1910 found that salvarsan which is known as a synthetic compund is the earliest antimicrobial agent that has been developed in order to overcome the problems of infectious diseases. In 1928, another antimicrobial agent which is penicilin that is used as antibiotic was revealed by Fleming since he discovered that there is *S. aureus* (SA) in a region nearby a contaminated blue mold in culture dishes.

Other than that, the detection of the first selective antimicrobial agent in a past few decades was a watershed moment in medical and public health history where the following development of antimicrobial therapy was primarily focused on finding drugs that were successful against microbial species that were resistant to the drugs in use at the time. Besides, these new medications that is known to have a very good effect on human health since it can treats many seriuos diseases are considered to possess the capability of saving lives (Melo, 2021).

Next, since antibiotics are derived from microorganisms, some microbial species have developed the ability to neutralise them where the antibiotic resistance has emerged among these strains. In addition, the antimicrobial agent is actually being developed when the antimicrobials at subtherapeutic levels are routinely used in animal feeds which will provides negative effects to people since it is endangering human health by contributing significantly to the reservoir of antimicrobial-resistant bacteria that could be transmitted to humans (Floris, 2020). The use of those antimicrobials is actually for feed quality enhancement as well as to increase the growth rate of that substances.

Table 2.1**:** Antibiotics Approved Since 2010 (Floris, 2020).

Antimicrobial Agent	**Approval Year**	**Indication(s)**	**Coverage**
Ceftaroline (Teflaro)	2010	SSTI, CAP	MRSA
Dalbavancin (Dalvance)	2014	SSTI	MRSA
Tedizolid (Sivextro)	2014	SSTI	MRSA, VRE
Oritavancin (Orbactiv)	2014	SSTI	MRSA, VRE
Delafloxacin (Baxdela)	2017	SSTI, CAP	MRSA
Meropenem-vaborbactam (Vabomere)	2017	cUTI	CRE
Plazomicin (Zemdri)	2018	cUTI	CRE, aminoglycoside-resistant Enterobacteriaceae
Cefiderocol (Fetroja)	2019	cUTI	CRE, *Acinetobacter*, *Pseudomonas*
Omadacycline (Nuzyra)	2019	SSTI, CAP	MRSA, VRE, *Pseudomonas*, *Klebsiella*
Imipenem-cilastatin-Relebactam (Recarbrio)	2019	cUTI, cIAI	CRE, *Pseudomonas*

2.3 Medicinal Plant Extract as a Source of Antimicrobial Agent

Plants and plant products have been used for their medicinal properties since the dawn of time beause most of the medicinal plants that come from various types are able to possess the potential of bioactive compounds which can be used to treat many infectious diseases on human. Hence, many scientists and researchers have been working on developing plant products as a main source of antimicrobial agents because it will contributes to the production of highly effective traditional medicines. It is because natural products derived from medicinal plants is known to be more safer since it provides less negative side effects to people (Menrad, 2018).

One example of medicinal plants that are mostly being used by many people for the disease treatments is garlic due to its therapeutic benefits. The scientific name of garlic is known as *Allium sativum L.* and it is considered to be the major used of traditional medicine since many bioactive compounds such as Allicin have been found in that plant. Allicin is known as one of the organosulfur compound that is believed to have a sharp odour and is formed when garlic is squashed or sliced. Next, the production of allicin from chopped garlic will produce an enzyme which is alliinase enzyme that will metabolize alliin to allicin. Besides, the enzyme produced is known to have an important role in order to treat some deseases which is including high blood pressure, diabetes and tuberculosis as well.

The other benefits of garlic is women who ate a diet that is high in allium vegetables is actually having less osteoarthritis and there is a possibility of developing therapies for the disease using compounds contained in garlic (Newman, 2017). Compounds that found in garlic is actually able to be used as new antimicrobial agents since the biological activity of

garlic encompasses antioxidant, anti-diabetic and also anticancer which can lower the blood pressure and decreasing the total cholesterol. Furthermore, garlic also can act as antibiotic since it is usually considered as an excellent cure for many health problems. Besides, it is good for infections in the intestines and for getting rid of parasites in the intestines. Garlic also can be combined with prescription antibiotics to help sustain the drug and reduce possible side effects (McLaughlin, 2021).

Other than that, leaf extraction of medicinal plant such as *C. odorata* is actually able to act as antimicrobial agent since it possess the antimicrobial properties when the extract being analyzed for phytochemical components on the selected Gram-negative and Gram-positive bacteria. The prove of medicinal plant extract as antimicrobial agent can be seen when most of them are commonly used to treat a variety of infections. Besides, it is also used to treat wounds since it consists the characteristic of wound-healing, hence, it allows people to have a better health (V, 2017). Furthermore, since the leaf extract of *C .odorata* is found to be more effective against the chosen pyogens such as *Pseudomonas aeruginosa* and *Staphlylococcus epidermidis*, it shows that antimicrobial properties of phytoconstituents in that plants are strong and contribute them to serve as antimicrobial agent where this herbal plant can be used as a non-addictive alternative to conventional medicine. Next, these plants can also be used to find bioactive natural products which contribute to the growth of new phytopharmaceuticals.

In addition, the other example of medicinal plant that can benefits living organisms is *Aloe vera* where it is usually being used as a traditional medicine in several countires especially in Egypt and China. *Aloe vera* is actually can be defined as a medicinal plant which is normally safe to use it straight from the plant in order to cure a variety of ailments during the ancient time. The transparent gel present in *Aloe vera* leaves is used in *Aloe vera* creams,

gels, and ointments where these products can be used topically to treat a variety of skin problems (Cronkleton, 2018). Besides, Aloe is also available in capsule or liquid form for internal consumption to encourage health and well-being. *Aloe vera* is known as a short-stemmed shrub that is often called as 'wonder plant'. The genus of this flowering medicinal plant is Aloe that consists of around 500 species and they can be found in abundance in North Africa. Moreover, like the other types of medicinal plant, *Aloe vera* also contains a huge amount of bioactive compounds which is including saponins and lignins and this actually proved that *Aloe vera* also act as a source of antimicrobial agent nowadays.

Furthermore, antibacterial and anti-inflammatory properties are among the biological functions of *Aloe vera*. It is also known for its soothing properties, as well as its ability to reduce the effects of UV radiation on the skin. Besides, *Aloe vera* that comes in different form such as *Aloe vera* cream, *Aloe vera* gel or placebo is having different rates of effectiveness where a study conducted by Maret Rossi in 2018 found that the *Aloe vera* extract 0.5% in hydrophilic cream is more effective in treating some diseases as it was linked to a statistically significant decrease in healing time and there is a larger number of patients who were healed compared to *Aloe vera* extract 0.5% in a hydrophilic placebo group (Rossi, 2018).

2.4 Chromolaena odorata

C. odorata which is also called as *Eupotorium odoratum* is being classified in many categories such as family, genus, scientific name and taxonomic name which is as shown in

Table 2.4 below. Family and genus are both in the scientific classification of Kingdom of *C. odorata* and the origin of this medicinal plant is Florida, Americas and Texas as well in United State. Besides, within the sunflower family, there are two different climate adaptation which is tropical and subtropical flowering shrubs.

Table 2.2**:** Scientific classification of *C. odorata* (Al Sayed, 2017).

Scientific name	*Chromolaena odorata*
Taxonomic name	*Chromolaena odorata* (Linn) King & Robinson
Family	*Asteraceae (Compositae)*
Genus	*Chromolaena*
Synonym	*Eupatorium odoratum*
Organism type	Herb (54,64)
Habitat	Agricultural areas, natural forests, planted forests, range/grasslands, riparian zones,ruderal/disturbed, scrub/shrublands(74)
Common names	Siam weeds (64,65,66,67,69,80) : Triffed weeds (64,65): Jack in the bush (64,65): Pokok kapal terbang/aeroplane plant (69,80): Pokok Jerman (69)

C. odorata which is also called as *Eupatorium odoratum* is actually being classified in the kingdom of Plantae and its genus is Chromolaena DC. *C. odorata* also have been named in English such as Siam weed, Jack in the Bush as well as baby tea. *C. odorata* is considered to be a low-growing perennial shrub with the common height of three to seven metres. Moreover, *C. odorata* is known as a poisonous plant with extremely high amounts of nitrate in young plants, which are five to six times higher than those harmful to animals in young plants (Al Sayed, 2017). Chromolaena gets its name from the Greek word chromolaena, that brings the meaning of "colour and when crushed, the leaves emit a strong odour, earning the species name "odorata." Besides, since *C. odorata* consists of 900 genera and 13,000 species, it is known as a member of the largest family of flowering plants and the number of its genus is about 1,200 species which is comes in three categories including small herbs, shrubs or subshrubs. In several countries such as Vietnam, *C. odorata* provides many benefits to people where they can use the leaf part of that medicinal plant in order to treat some dieseases such as skin infections, insect repellent and also to treat leech bites because it contains therapeutic properties.

This medicinal plant has been presented in several countries such as Thailand, west Africa and Malaysia as well. Moreover, *C. odorata* creates a close network and uses competition to stifle the growth of different harvests. Next, the properties of *C. odorata* is the stems dry out and easily burn during the dry season, but the stumps stay alive and grow quickly, hence, covering the ground during the long period of rainy day (Zahara, 2019). In addition, the species of *C. odorata* is mostly found in the south-eastern United States, Mexico, the Caribbean, and tropical South America. Furthermore, Tropical and southern Africa, tropical Asia, and several tropical oceanic islands with mild climates are among the places where *C. odorata* has become naturalised. The habitat of this medicinal plant species

includes both tropical and subtropical weed that can be found in riparian zones which is most well-known as riverbanks and it is also initiate in waste fields, roadsides and abandoned pastures as well.

The features of *C. odorata* is it has an upright or spreading shrub that forms thickets and normally grows in a range between 1.5 to 3 m tall but the height of this plant can extent until 6-20 m when it climbs over the other taller plants. Other than that, the colour of the slender stems of *C. odorata* are yellowish-green and slightly hairy. These stems is actually can reach a length more than 7 metres and most of them are typically derived from the plant's long-lived rootstock. They have a lot of branches, and the lateral branches in the leaf forks are typically formed in pairs which is called as axils. Next, the leaves of *C. odorata* is arranged oppositely by the size of 5-12 cm long and 3-7 cm wide. These leaves grow on stalks (petioles) up to 6 cm long and when being crushed, they emit a strong odour.

2.5 Basic Extraction For Medicinal Plant Extract

Basic extraction for medicinal plant extract is involving several procedures which is the collection of pyogenic pathogen such as *P. aeruginosa* and *S. aureues*. For the extraction purpose, the collected leaves of medicinal plant will be isolated by using disc diffusion assay in order to evaluate the antimicrobial potential of that plants. Besides, the dried plant extract also will be isolated for detecting the existence of phytoconstituents such as alkaloids, saponins and terponoids. Then, the growth inhibition of that plant extract will includes the use of agar well diffusion method.

Moreover, extraction of medicinal plant can be defined as the use of a method to remove several compounds such as terpenoids, alkaloids and flavonoids where all of these compounds are known as secondary metabolites. Those compounds is actually being removed from inactive material by using suitable solvent and a standard extraction technique. In order to further an experiment of medicinal plant extract, the preparation of that plant itself is the most important stage in obtaining a high-quality research result, where it includes extracting and determining the quality and quantity of bioactive constituents before moving on to the biological testing (Haque, 2020).

Besides, the selection of a suitable solvent, techniques of extraction, phytochemical screening procedures, fractionation processes and detection methods are all important steps in obtaining a high-quality bioactive molecule. Solvents that are usually being used in medicinal plant extract is actually comes in different polarity which is polar solvent, intermediate polar and nonpolar. Water and alcohol are two types of solvents that have been classified as polar solvent while ether and chloroform is known as nonpolar solvent. Next, there is also intermediate polar such as acetone.

Furthermore, the basic extraction for an experiment on medicinal plant is involving the step of collecting the leaf part of plants properly and on time, having it authenticated by an expert, drying the leaves within the required time which is for about one week as well as grinding the leaves according to the needed amount. Next, the bioactive compound is extracted, fractionated, and isolated, if necessary and it also encompasses of determining the amount and nature of bioactive compounds. Besides, basic extraction for medicinal plant extract involves several techniques which is the first one is maceration where this technique is very simple and effective when working with thermolabile plant matter. In maceration technique, a particular part of medicinal plant such as leaves, stem bark or root bark is put in

a jar or container and menstruum will be poured over it until the drug material is fully coated before closing the jar. Menstruum is known as a solution that is used for an effective extraction process. Next, the container will be stored for about three days.

Furthermore, the next technique is followed by infusion which is ideal for extracting bioactive constituents with a high solubility where it is already soluble. The drug substance will be grinded to get them into powdered form and put it in a clean beaker or bottles. The extraction solvent either hot or cold then will be poured into the drug material with an appropriate ratio of 4:1 or 16:1. The third procedure is digestion where it includes the use of moderate heat in order to undergo the extraction process. The selected solvent for extraction will be poured into a clean beaker before adding the content of powdered drug. The mixture then will be put in an oven with a moderate temperature which is about 50°C in order to ensure that the secondary metabolites will be removed by reducing the viscosity of extraction solvent. Besides, decoction extract is known to has a high concentration of water-soluble impurities and it is actually not suitable for both thermolabile or volatile materials. In a process of decoction, it involves the use of constant hot extraction method with a specific volume of water as a solvent.

In addition, percolation is considered to be more effective compared to maceration since it is known as a continuous phase where the saturated solvent is continuously replaced by fresh solvent. In this method, a larger amount of solvent is added and the mixture of powdered plant material is held for few hours. Next, the Soxhlet extraction technique combines the benefits of refux extraction and percolation by involving the use of refux and syphoning principles to ensure that the herb is always being extract with fresh solvent (Zhang, 2018). There is some benefites initiate in percolation phase which is a larger volume of medication can be extracted with less solvent and there is no need for filtration. Next,

thermolabile products that is known to be ineffective in this phase is one of the limitation. The other basic extraction techniques of medicinal plant extract is Microwave-assisted extraction where it is more sophisticated extraction techniques that is use to prepare medicinal plants and it employs a dipole rotation as well as ionic transfer mechanism. Microwaves release electromagnectic radiation with frequencies ranging from 300 MHz to 300 GHz and wavelengths ranging from 1 cm to 1 m (Altemimi, 2017).

2.6 Cell Envelope of Gram-positive and Gram-negative Bacteria

Gram-positive bacteria have cell walls that are physically different from Gram-negative bacteria's cell walls which is peptidoglycan is the main ingredient in bacterial cell walls. Peptidoglycan can be defined as a woven-like macromolecule that is made up of sugars and amino acids. The amino sugar component is made up of N-acetylglucosamine (NAG) and N-acetylmuramic acid molecules that alternate (NAM). Besides, the NAM tetrapeptides are commonly cross-linked with a peptide interbridge and full cross-linking. All of this adds up to a cell wall that is extraordinarily robust and the peptidoglycan is actually protects microorganisms while also defining their form (Bailey, 2020).

In addition, the peptidoglycan layer of Gram-positive cell wall is made up of numerous layers where the thick layers of peptidoglycan support the cell membrane while also providing a site for other molecules to adhere (Bailey, 2020). Gram-positive bacteria are

also able to retain the majority of the crystal violet dye during Gram staining, giving them a purple appearance (Steward, 2021). Moreover, teichoic acid is another component of a Gram-positive cell wall that is actually known as a glycopolymer contained inside the peptidoglycan layers. Teichoic acid is thought to play a number of vital roles in the cell, including generating the cell's net negative charge, which is required for the production of a proton motive force (Bailey, 2020). Teichoic acid chains extending from the plasma membrane through the peptidoglycan cell wall are also found in Gram positive cell walls. These sugar-based polymers help keep cells in form and aid in cell division. Teichoic acid also aids in the infection and illness of Gram-positive bacteria (Steward, 2021).

Other than that, Gram-negative bacterial cell wall also consists of peptidoglycan, as same as the Gram-positive bacteria, but the peptidoglycan is a single thin layer compared to the dense layers found in Gram-positive cells. During Gram staining, this thin layer absorbs the pink colour of the counterstain rather than retaining the initial crystal violet dye and the Gram-negative bacteria's cell walls are actually more complicated than Gram-positive bacteria's cell walls (Bailey, 2020). Next, a gel-like matrix called periplasmic space is found between the plasma membrane and the thin peptidoglycan layer. Gram-negative bacteria have an exterior membrane layer that is separate from the peptidoglycan cell wall, which is not present in Gram-positive bacteria. Murein lipoproteins are membrane proteins that connect the cell wall to the outside membrane.

Furthermore, the existence of a plasma membrane outside of the peptidoglycan layers which is known as the outer membrane, is the most striking feature of the Gram-negative cell wall. The outer membrane is made up of a lipid bilayer with polar heads, fatty acid tails and integral proteins, similar to the cell membrane. The presence of big molecules known as

lipopolysaccharide (LPS), which are embedded into the outside membrane and extend from the cell into the environment, distinguishes it from the cell membrane (Steward, 2021). LPS is a big glycolipid complex that protects bacteria from hazardous environmental chemicals. The O-antigen or O-polysaccharide, which represents the structure's outermost section is one of three components that make up LPS. The LPS is then anchored into the outer membrane by the core polysaccharide and lipid A. The function of LPS is contributing to the cell's net negative charge and giving protection against some chemical compounds by physically restricting access to other portions of the cell wall (Steward, 2021).

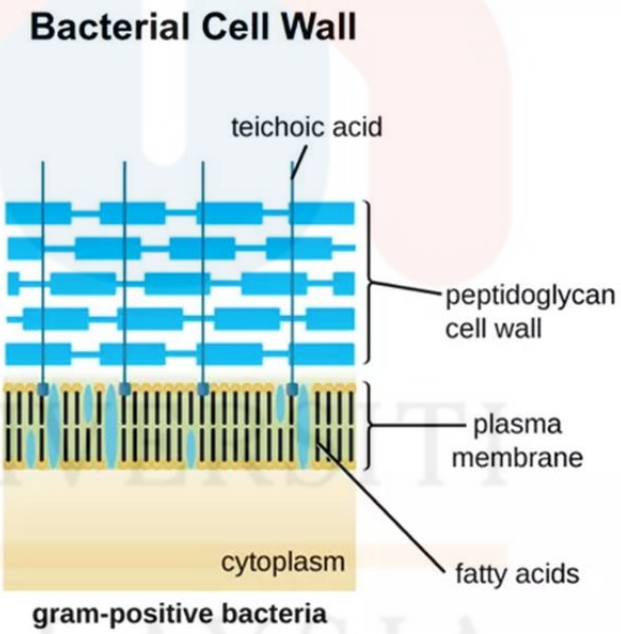

Figure 2.1: Gram-positive bacterial cell wall

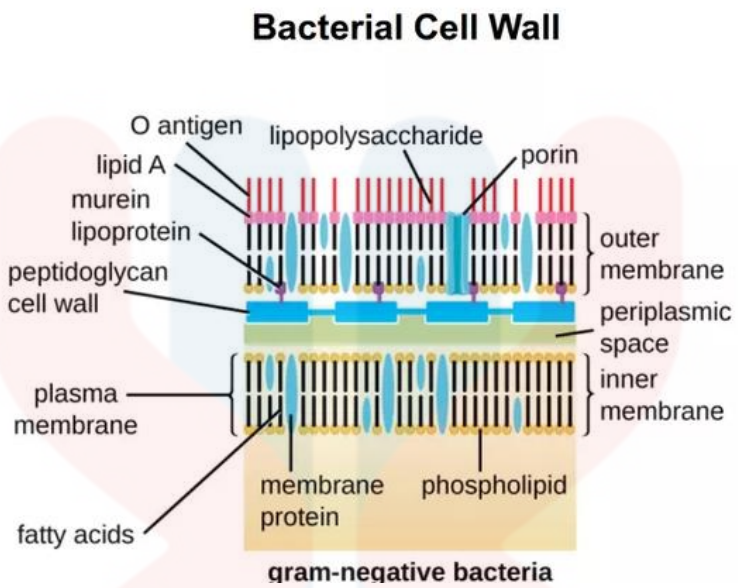

Figure 2.2: Gram-negative bacterial cell wall

2.7 Identification of Bioactive Compound in the Plant Leaves Extract

Despite all of the advantages of current synthetic medicines, people continue to choose plant-based natural treatments to synthetic medicines. Because of the contribution of many beneficial phytoconstituents contained in different plant parts, most medicinal plants are unique in their potential to treat and cure diverse human problems. Various medicinal plants have been screened for their leaf extract for a long time and are still being screened presently. For instance, medicinal plants of *Avicennia alba* that have long been used in folk medicine to cure a variety of ailments, especially in order to treat various kinds of human illnesses. The major bioactive compounds of *Avicennia alba* that are classified as secondary

metabolites includes alkaloids, phenols and steroids. These bioactive compounds were thought to have anti-inflammatory properties as well as the ability to treat cancer (G.Eswaraiah *et al*., 2020).

Besides, another medicinal plant that has been identified for its bioactive compound from the leaves part was jambolan *Syzygium sp.* that is known to be as a potential hypoglycemic and also slimming agent which is effective in weight lost. There are several types of bioactive compounds have been reported containing in the leaves of *Syzygium sp.* which are tannins, flavonoids and alkaloids that has been proposed as a viable treatment option for diabetes and obesity (Candido, 2019). Furthermore, medicinal plants of *Palmaria palmate* which known as Red algae, one of a type of aquatic plant that belongs to the eukaryotic algae family. This plant species has been identified for their antioxidant activity, that was believed to lowered oxidation with the presence of hydroxyl and carbonyl, where both are known as functional groups. According to the findings, both 9.68 g of ascorbic acid and 10.3 g of total polyphenol can diminish activity in 1 mg of dulse extracts by the same amount (Watson, 2017).

Besides, the gas chromatography-mass spectrometry (GC–MS) technique was used in an investigation to detect and identify phytochemical substances found in the medicinal plant named *Amomum nilgiricum,* which belongs to the Zingiberaceae family. This plant has been collected to test the antibacterial, antioxidant as well as antidiabetic properties of its leaves extracts where a total of 25 phytochemical substances were discovered. The finding indicated that serverogenin acetate, 2,4-dimethyl-1,3-dioxane, and (1,3-13C2) propanedioic acid had the most antibacterial, antifungal, antioxidant, as well as antidiabetic activities of the phytochemical substances (Konappa, 2020). Several biological compounds found in the leaves extract of *A. nilgiricum* are phenols, flavonoids and alkaloids.

2.8 Preliminary Chemical Profiling of Antibacterial Compounds

Plain TLC plate pieces generated in solvent systems of methanol: ethyl acetate: petroleum ether with ratio 1:1:1 (v/v) were used in detection of several structural groups of compounds such as flavonoid, alkaloid, anthraquinone, phenol and also lactone. Flavonoids are an essential component in a number of nutraceutical, pharmacological, medical and cosmetic uses because they are linked to a wide range of health-promoting benefits. Besides, alkaloids are a large collection of chemical compounds that contain a nitrogen atom in their structure which can cause alkalinity in these substances (Kurek, 2019).

Anthraquinone is an organic substances found in some plants which are made up of simple anthrones or bianthrones chemically. Anthraquinones are employed in the production of dyes, pigments, and pharmaceuticals (Bolen, 2020). Quinones are the most common type of natural quinones, and their derivatives make up the majority of them. This group also includes benzoquinones and naphthoquinones. With over 700 chemicals identified, anthraquinones are the biggest group of natural pigments. They can be found in all sections of the plant, including the roots, rhizomes, fruits and flowers. The majority of these compounds are made up of the same basic structure which is 9,10 anthracenedione that is known as a tricyclic aromatic organic compound (Diaz, 2018).

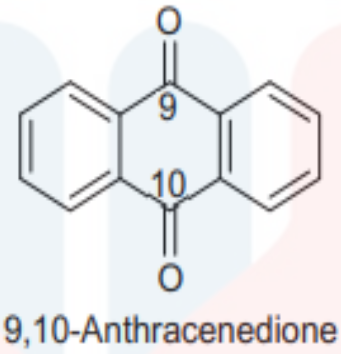

Figure 2.3: Chemical structure of 9,10 anthracenedione

Next, phenolic chemicals, which are classified into phenolic acids and polyphenols are a major family of secondary metabolites found in plants. These substances can be discovered in combination with mono and polysaccharides, connected to one or more phenolic groups, or as derivatives such ester or methyl esters (Minatel, 2017). A lactone which is also known as a cyclic ester can be defined as a carboxylic acid ester in which the ligand on the carbonyl carbon and the one on the oxygen atom are connected by one or more carbon chains (Gunawardena, 2020).

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of Plant Material

The leaves of *C. odorata* were selected from the fresh plant that shows no signs of infectious plant diseases such as leaf rust, black knot and even stem rust which is usually caused by infection of fungal. It is very important for the extraction plant's operation to allow the plant to function properly in order to ensure that the leaves are capable of producing bioactive compounds that will enhance antimicrobial activity. The leaves sample of *C. odorata* were chosen manually by hand picking method at Kampung Kubang Kacang in Kota Bharu. The fresh leaves sample that have been collected then were washed carefully under slow running water to remove any dust or contaminants on the surface of the leaves on both

front and back side of leaves. The next step is those cleaned leaves were left for about one week in a suitable plastic container to allow them to be dried at a room temperature which is 37°C and is not being put under the direct sun. The dried leaves will change in structure when they became shrink and brittle. Due to that situation, the colour of leaves will be changed from green to dark brownish.

The dried leaves of *C. odorata* then were cut into small pieces to make it easier for the grinding process to turn the plant materials into a fine powder form, which then were kept in a desiccator. This procedure is critical to avoid moisture contamination and nutrient loss due to fungal infestation. In addition, the reason for grinding the plant material is in order to improve the surface interaction between plant samples and the selected extraction solvent since particle size is reduced. The smaller particle size of plant material is very important to enhance an effective extraction process by allowing the solvent to come into contact with the target analytes. Lastly, the powdered leaves were kept in a zip lock plastic bag and in order to avoid them from being contaminated, the plastic bag were placed into a desiccator since it is able to prevent moisture loss of the prepared plant materials.

3.2 Preparation of Extract

Extraction can be defined as the process of separating a particular part of medicinal plant by using the selected solvent and being done by following the correct procedures. All extraction process are designed to isolate the plant metabolites that are soluble from the insoluble cellular marc. Before conducting the extraction, the prepared plant material that have been turned into powdered form were dried again in an oven at appropriate temperature for about 40°C within the recommended time at least 4 hours. The powdered materials were weighed accurately according to the needed amount which is started with 75 g, then the powdered leaves sample were extracted with the selected solvents which are hexane, chloroform, acetone, ethyl acetate and methanol.

The amount of 75 g of powdered leaves sample that have been weighed were soaked in 1.5 L solvent for about 72 hours. Next, the mixture was filtered using Whatman no.1 filter paper in order to separate the solvent and biomass. After that, the biomass was dried first before proceeding to further extract by using successive solvent where the process starting from drying the powdered leaves material until the step of extraction with five different solvents in increasing polarity were repeated until all residual marc were exhaustively extracted. The filtered solvent were concentrated in a rotary evaporator to allow them to get dry in order to obtain crude extract. Lastly, all those five different extracts obtained were kept in an airtight container at a suitable temperature which is -20°C until further use.

3.3 Screening for Antimicrobial Activity

3.3.1 Test Microorganisms

In this study, the antimicrobial activities of the leaves extract of *C. odorata* were tested on two different types of bacteria which is Gram-positive and Gram-negative bacteria:

Gram-positive bacteria

I. *Staphylococcus aureus*
II. *Methilin-resistant Staphylococcus aureus*
III. *Bacillus subtilis*
IV. *Bacillus cereus*

Gram-negative bacteria

I. *Escherichia coli*
II. *Klebsiella pheumoniae*
III. *Yersinia Enterocolitica*

3.4 Preparation of Test Inoculum and Seeded Agar Plate

A loopful of a pure bacterial colony were selected from a bacterial culture on nutrient agar (NA) where the age of bacterial culture is known about 24 hours old. Next, the bacterial culture were suspended in a sterile physiological saline (0.85% sodium chloride) solution where the required volume is 5 ml. The resulting suspension was vortex for uniform mixing and the turbidity of the suspension was visually calibrated to meet 0.5 McFarland requirements (approximately 1.5 x 108 CFU/mL). Besides, a sterile cotton swab was dipped into the bacterial suspension and pushed firmly against the inside wall of the universal container to ensure that any excess inoculum is removed. The cotton swab was then streaking over the entire Mueller Hinton Agar (MHA) surface three times with a rotation angle of 60° to ensure that the bacterial inoculum is distributed uniformly.

3.5 Preparation of Extract Solution

A total of 20 mg extracts were put in 0.5 ml dimethyl sulfoxide (DMSO). After the extract has been fully dissolved, 0.5 ml of sterile distilled water was applied to the extract in order to yield a stock with a concentration of 20 mg/mL (the concentration of DMSO in the

extract stock solution was 50%). Next, the extract solution was filtered through a sterile nylon membrane with the use of a pore size of 0.2 m.

3.6 Preparation of Susceptibility Test

For sterilisation, Whatman no. 1 filter papers with a size of 0.14 mm thickness were punched into a 6 mm diameter disc and were autoclaved for 15 minutes at 121° C. The next step is 10 µL of extract solution (20 mg/mL) were pipetted onto sterile discs and allowed to air dry for a few minutes before being impregnated with another 10 µL of extract to create a disc with 0.4 mg of extract and 1% DMSO. After that, the discs were left air dried before being placed on the agar plates that have been seeded with test microorganisms.

3.6.1 Disc Diffusion Susceptibility Test

Sterile Whatman antibiotic discs were put on the surface of inoculated medium. The negative control which is methanol was included for solvent effect detection while 30 µg per

discs (20 µL of 1.5 mg/ mL) chloramphenicol were functioned as positive control for bacteria. Next, the plates were incubated at appropriate temperature which is 37°C in a range of time between 16 to 18 hours. Furthermore, the diameter of the inhibition zones that formed around the discs were measured and the experiment was repeated three times which is beginning with the first stage and ending with the diameter measurement of the transparent or clear inhibition zones. The data obtained were expressed as mean ± SD.

3.7 Statistical Analysis

All of the tests were repeated three times in total, and the average zone of inhibition of test extracts in comparison to the negative control were measured using Microsoft Excel 2007.

3.8 Interpretation of Result

After 24 hours of incubation, the development of an inhibition zone around the agar plugs by Gram-positive and Gram-negative bacteria was examined and measured for three times. Besides, for the positive control, chloramphenicol (30 µg/mL) was used for bacteria and the zone inhibition -, +, ++, and +++ were registered as the results.

- = No inhibition zone

+ = Small inhibition zone (≤10 mm)

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

+++ = Large inhibition zone (≥ 21 mm)

3.9 Preliminary Chemical Profiling of Extract

3.9.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography is a technique for separating non-volatile mixture. The function of TLC is to track the development of a reaction and also verify a substance's purity. TLC may not only easily separate components from a mixture, but it can also assist in determining the identity of the compounds by using developing reagents in a short amount of time (Marathe, 2016). TLC is a popular analytical tool due to its ease of use, low cost, high sensitivity and rapidity of separation. Besides, TLC was used to create spots that are well defined and separated. It has been carried out by referring to the method in Hadzic *et al.*, (2019). Normal phase chromatography was utilised in the experiment.

Moreover, the TLC was cut into strips with a size of 2 cm × 12 cm and lines of 1.5 cm were draw across the TLC plate which is from the bottom and lines of 0.5 cm were draw from the top by using pencil. The TLC plates were activated in an oven at 80°C for about 30 minutes and they were ready to be used after being cooled at room temperature. Next, each extract was dissolved in its extracting solvent which were hexane, chloroform, acetone, ethyl acetate and methanol in order to prepare a concentration of 20 mg ml^{-1} extract solution. The extract solution was drawn into a glass capillary tube, known as haematocrite. The extract solution with the amount of 1.0 µl was spotted on the baseline of the plate by using thin capillary pipettes in order to create a small round spot. The chromatograms were developed

in a closed chamber that was containing three different solvent systems which were methanol, ethyl acetate and petroleum ether.

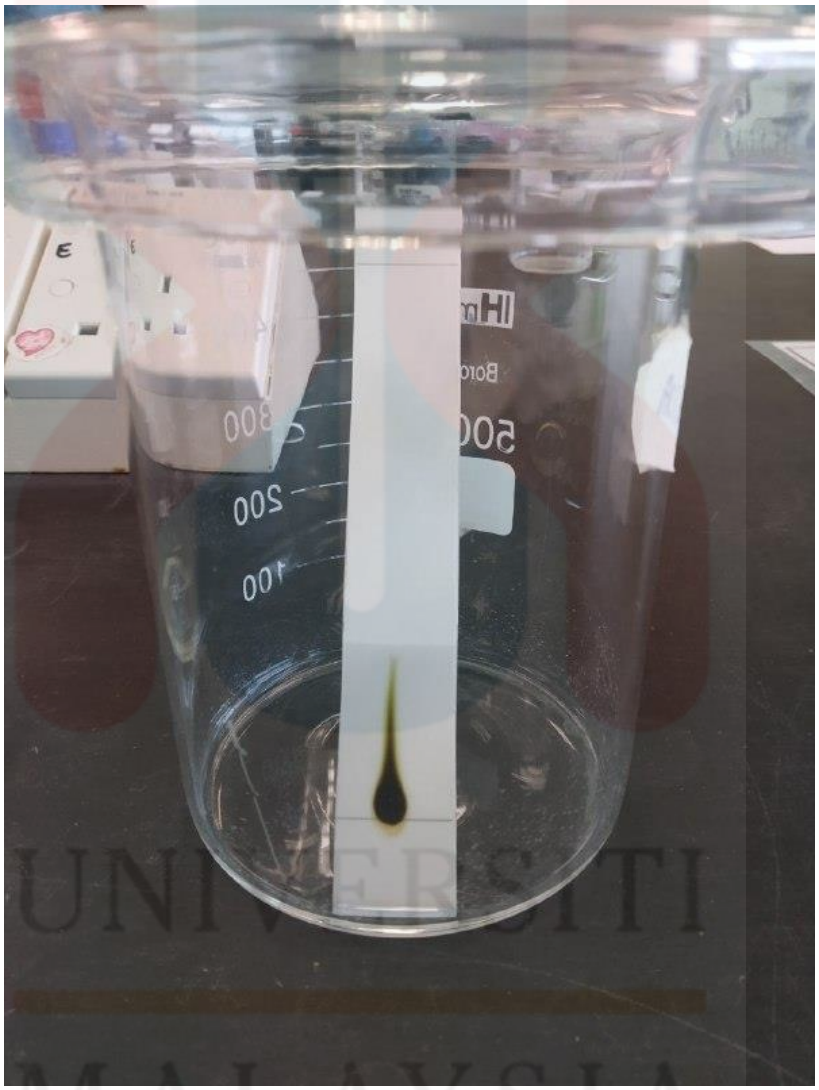

Figure 3.1: Chromatogram developed in a closed chamber.

3.9.1.1 Detection of Flavonoid

Flavonoids are soluble in water and are polyphenolic compounds with 15 carbon atoms. They are made up of two benzene rings joined by a three-carbon chain. A third middle ring is formed when one of the carbons in this chain is joined to a carbon in one of the benzine rings, either via an oxygen bridge or directly (Robertson, 2021). Moreover, flavonoids, which are regarded as vital pigments for producing the colours needed to attract pollination insects are actually abundant in plants. They are also the most frequent form of polyphenolic substance present in the human diet.

Furthermore, the presence of flavonoid was determined by spraying an increasing amount of 1M sodium hydroxide (NaOH) on the TLC plate which then appeared yellow colour and discoloured after 1M hydrochloric acid (HCl) was added (Shah, 2014). The reagent of 60% NaOH solution was prepared by filling up 60 g of NaOH with water until the volume was reached 100 ml.

3.9.1.2 Detection of Alkaloid

Alkaloids are a class of basic, secondary plant substances that usually have a N-containing hetero-cycle which possess some characteristics of colorless, crystalline and non-volatile solids. They are soluble in a polar or slightly polar organic solvents such as ethanol, ether, benzene and chloroform but not in water. Wagner's reagent was used to detect the presence of alkaloid in the five different extracts which were hexane, chloroform, acetone, ethyl acetate and methanol (Parbuntari, 2018).

Amount of 1.27 g iodine as well as 2 g potassium iodide were weight by using an analytical balance and being dissolved in 5 ml distilled water in order to prepare the Wagner's reagent. Besides, 100 ml distilled water was added to the solution. The developed TLC plate that has been placed in a closed chamber was sprayed with the prepared Wagner's reagent. The presence of alkaloid was detected with the appearance of reddish brown colour (Kandibanda, 2016).

3.9.1.3 Detection of Anthraquinone

Anthraquinones are a strong aromatic molecule with several medicinal effects, including the ability to suppress cancer growth by triggering apoptosis, relieve constipation as well as improve bowl movement. Anthracene oxidation, naphthalene oxidation, and condensation of 1, 4-naphthoquinone with butadiene are all chemical methods for producing anthraquinones (Khan, 2019).

10% methanolic potassium hydroxide (KOH) was prepared by disolving 10 g KOH in 100 ml of methanol in order to detect the presence of anthraquinone, where the reagent was sprayed to the developed TLC plate in a closed chamber. The presence of anthraquinone indicated when the original colour changed to red, green, purple or violet (Bhatt, 2019).

3.9.1.4 Detection of Phenol

Phenol is known as an organic substance which found in small amounts in a variety of home goods, including mouthwash and spray cleansers. It might be colourless or white in its purest form. Alcohols and phenols are similar in structure, but phenols create stronger hydrogen bonds. As a result, they are more water soluble than alcohols and have higher

boiling points. Phenolic compunds can be detected using freshly prepared 1% aqueous Ferric chloride ($FeCl_3$) where the reagent was prepared by weighing 1 g $FeCl_3$ and was dissolved in distilled water. The developed TLC plate in a closed chamber was sprayed with the reagent which then showed intense green, blue, purple or black colours. It showed the presence of phenol in that particular extract. The majority of phenols produce a dark-colored solution.

3.9.1.5 Detection of Lactone

Plain TLC plates were spotted with different extracts by using thin capillary pipettes which then be placed in a chamber containing iodine crystals in order to detect the presence of lactone. At normal temperature, iodine is a nonmetallic, almost black solid with a glistening crystalline look. Discrete diatomic molecules exist in the molecular lattice, as well as in the molten and gaseous phases. At room temperature, iodine has a moderate vapour pressure and, when exposed to air, steadily transforms into a deep violet vapour that irritates the eyes, nose, and throat. As a result, iodine should be weighed in a stoppered container; for the manufacture of an aqueous solution, the bottle should include a solution of potassium iodide, which lowers the vapour pressure of iodine significantly. The appearance of brown spot indicates a positive reaction which shown that the particular extract was containing compound of lactone (Christe, 2018).

CHAPTER 4

RESULT AND DISCUSSION

4.1 Preparation of Plant Material

4.1.1 Preparation of Plant Powder

The selection of plant material was very important in the extraction plant's operation for producing various bioactive compounds that will enhance antimicrobial activity. This is because the procedure was designed to produce a large amount of extraction yield in order to test the efficacy of leaf powder extracted with different polarity of solvents. Polar solvent such as alcohols, are known to extract larger levels of Free Fatty Acid (FFA) as well as undesirable items such as proteins and polysaccharides (Kay, 2018). The chosen of plant material which is to be used in this study was done based on its freshness which has no signs of infectious diseases including leaf rust or even black knot that are usually caused by infection of fungal.

In this study, the healthy *C. odorata* plants were collected from the area which are free from any fungacides for identifying of its antimicrobial activity. This step is critical for ensuring that the extraction yield obtained are devoid of contaminants and unwanted microorganisms.

The leaves sample of *C. odorata* took about one week to fully dried, which was free from any moisture content. The drying process was performed in a room temperature to avoid from direct sunlight that may damaged the plant sample due to high temperatures from the sun. The best way to dry the plant material while preserving chlorophyll content and internal composition was at 37°C in a room temperature. Besides, the fresh leaves sample were washed carefully under slow running water with the aim to maintain their freshness and also to remove any dust or contaminants on the surface of the leaves on both front and back side. The drying technique was used to remove 100% of moisture content from the leaves in order to enable it to be used in this study. The signs of dry leaves have been indicated by changes in structure where they became wrinkled and brittled as well as were reduced in the green colour intensity where the colour of the leaves changed from green to dark brownish.

The dried leaves sample of *C. odorata* were cut into smaller pieces which then had been ground until the small particle powder form was obtained. It is in order to increase the surface interaction between plant material and the selected solvents. This action indicated that the extraction of bioactive compounds increased when particle sizes were increase (Farid, 2020). The powdered leaves sample was kept in a desiccator until further to prevent contamination as well as nutrient loss due to fungal infestation. The powdered leaves sample of *C. odorata* are shown in Figure 4.1.

Figure 4.1: The dried leaves of *C. odorata* after grinding into powder form.

4.2 Preparation of Leaves Extract of *C. odorata* Using Sequential Solvent Extraction Method.

4.2.1 Effect of Different Solvent Polarity on the Extraction Yield of *C. odorata* Leaves.

In this study, the powdered leaves sample was extracted using sequential solvent extraction method according to the increasing polarity of solvent which was started with hexane, followed by chloroform, acetone, ethyl acetate and methanol. The powdered materials were weighed prior to extraction in order to get a suitable volume of solvent to soak the sample with a ratio of 1:20, w/v (Nguyen, 2019). The plant sample was macerated in the designated volume of solvent with intermittent shaking for three days in a fume hood. The

yield of plant extracts were weighed where the reading was actually increased from the least polar to the most polar solvent as shows in Table 4.1.

Table 4.1: Weight of extract yield for each solvent and percentage of Total Extractable Components (TEC).

Name of Solvents	**Weight of Extract Yield (g)**	**Weight of Samples (g)**	**Total Extractable Components (TEC) (%)**
Hexane	0.75	75.00	1.00
Chloroform	1.95	70.65	2.76
Acetone	1.54	64.35	2.39
Ethyl Acetate	0.31	62.60	0.50
Methanol	6.04	62.42	9.68

The extract yield was calculated as total extractable components (TEC) by using a specific formula as proposed by Nawaz (2020):

TEC (%) = (Weight of extract/weight of sample) × 100

The most polar solvent which is methanol resulted in the highest extraction yield (9.68%) among the four polarity solvent, which demonstrated that more polar solvents have a higher extraction efficiency (Nguyen, 2019). The extraction of potent antioxidant chemicals in polar solvents were indicated by a polarity-dependent increase in overall antioxidant

activity and reducing characteristics (Nawaz, 2020). This may be attributable to the higher solubility of proteins and carbohydrates in methanol than in hexane, chloroform, acetone and ethyl acetate. Besides, the least polar solvent which was hexane followed by chloroform also showed the increasing number of weight of extraction yield in line with the increasing of solvent polarity. The percentage of extraction yield for hexane as well as chloroform were 1.00% and 2.76%, respectively. However, the other two solvents which are acetone and ethyl acetate were resulted a decreasing percentage of extraction yield. As shows in Table 4.1, the percentage of extraction yeild for acetone is 2.39% while ethyl acetate is 0.50% where it was opposite with the fact that have been stated above since the data was slightly decreased. This is maybe due to some errors such as stirring rate and the use of volume of selected solvents during the plant extraction.

Furthermore, several parameters affecting the microextraction efficiency including stirring rate, volume of the solvent and extraction time were investigated and optimized. These factors gave an impact on the percentage of extraction yeild for both acetone as well as ethyl acetate where the weight of plant extract decreased even the polarity of that solvents increased. The first factor was the leaves extraction of sequentially solvent extract which were acetone followed by ethyl acetate maybe had a very low stirring rate and was not being mixed well during the three days of immersion which was carried out in a fume hood. The low stirring rate avoided the powdered leaves sample of *C. odorata* from constantly exposed to the selected extraction solvents, hence, lead to an ineffective extraction process since the solvents were unable to properly come into contact with the target analytes (Scott, 2015).

Other than that, the used of volume of the selected solvent also became one of the factors that was affected the percentage of both acetone and ethyl acetate extraction yield. In this experiment, the ratio of solutes to the solvents were taken into account where a

calculation was firstly made before extracting the powdered leaves sample into the particular solvents in order to increase the extraction efficiency since the plant materials were soaked in a suitable amount of solvents. However, a minor error maybe occured when pouring the solvent into a beaker containing the powdered leaves where it was significantly reduced the amount of solvent which then resulted a difference in the volume of solvent utilised compared to the calculations. Due to this error, it was decreased the percetange of extraction yield of acetone as well as ethyl acetate as shows in the Table 4.1 since the plant materials were not being extracted in an exert ratio of selected solvents.

There are a variety of solvents that can be used in the extraction process, including hexane, ethyl alcohol, and methanol as well in order to obtain the best outcome of the extracted phenolic compound with a high degree of precision. The efficacy of that solvent is actually can be determined by their various polarities, which vary from the least polar to the most polar. In this experiment, methanol is known as the most polar compared to the others solvent because of its high antioxidant efficacy, as shown by a study conducted by Koffi et al., (2016), which shown that methanol is more efficient than ethanol in extracting a significant amount of phenolic content from walnut fruit.

Figure 4.2: The extraction yield of *C. odorata* leaves from different polarity of solvent.

4.3 Antimicrobial Activity of *C. odorata* Leaves from Different Extracts

In this study, the antimicrobial activity of *C. odorata* leaves from different extracts were tested against Gram-positive and Gram-negative bacteria which are seven types of bacteria were used in this experiment. The bacteria used are Gram-postive bacteria [*Methilin-resistant S. aureus* (MRSA), *S. aureus* (SA), *B. cereus* (BC) and *B. Subtilis* (BS)] and Gram-negative bacteria [*K. pheumoniae* (KP), *Y. Enterocolitica* (YE) and *E. coli* (EC)]. In order to evaluate the antimicrobial activity, paper dics that have been pipetted with different extracts were placed on the surface of Mueller Hinton Agar (MHA) which have been seeded with test microorganisms. In this test, methanol was act as negative control while chloramphenicol was act as positive control. All plates of different bacteria that have been incubated for 16 to 18 hours were showed the results of antimicrobial activity by the presence of inhibition zone around the dics.

Table 4.2 demonstrated the antimicrobial activities of *C. odorata* leaves from different extracts. For the test on Gram-negative bacteria which is *Y. Enterocolitica*, data showed only hexane extract and acetone extract exhibited antimicrobial activity with small inhibition zone of 10.0 mm and 8.7 mm, respectively. The other three extracts which are chloroform extract, ethyl acetate extract and methanol extract did not show any inhibition zone which meant that there were no antimicrobial activities from those extracts.

The next test for antimicrobial activities were Gram-negative bacteria, *K. pheumoniae*. Based on Table 4.2, all the five extracts of *C. odorata* leaves which are hexane extract, chloroform extract, acetone extract, ethyl acetate extract and methanol extract exhibited significant antimicrobial activities against *K. pneumoniae*.

Table 4.2: Results of mean of inhibition zones diameter against test microorganism (mm±SD).

Bacteria	**Mean of Inhibition Zones Diameter against Test Microorganism (mm±SD)**						
	Hexane	**Chloroform**	**Acetone**	**Ethyl acete**	**Methanol**	**Positive control**	**Negative control**
Y. Enterocolitica	10.0 ± 0.0	-	8.7 ± 0.6	-	-	24.4 ± 2.7	-
K. pheumoniae	8.0 ± 1.0	8.3 ± 1.5	10.0 ± 0.0	8.7 ± 0.6	9.0 ± 0.0	26.6 ± 1.4	-
E. coli	-	-	7.7 ± 0.6	8.0 ± 0.0	8.0 ± 0.0	24.2 ± 3.3	-
B. subtilis	10.7 ± 0.6	9.0 ± 1.0	12.0 ± 0.0	11.7 ± 0.6	13.0 ± 3.5	30.6 ± 4.6	-
B. cereus	9.3 ± 0.6	8.3 ± 0.6	9.0 ± 1.0	7.7 ± 0.6	10.3 ± 0.6	20.8 ± 1.8	-
S. aureus	8.0 ± 1.0	-	8.3 ± 0.6	-	-	38.2 ± 2.1	-
MRSA	-	-	-	-	-	23.6 ± 3.4	-

The mean of inhibition zones diameter for each different extracts were 8.0 mm, 8.3 mm, 10.0 mm, 8.7 mm and 9.0 mm, respectively where acetone extract showed the highest antimicrobial activities and hexane extract had the lowest antimicrobial activities.

Table 4.2 indicates only three extracts of C. *odorata* leaves showed antimicrobial activities against the test of *E. coli* which were acetone, ethyl acetate and methanol extracts. The extracts of ethyl acetate and methanol were both showed same value of mean of inhibition zones which was 8.0±0.0 mm and 7.7±0.6 mm, respectively. Both of extracts possessed small inhibition zones since the mean was smaller than 10 mm.

Among the four types of Gram-positive bacteria, hexane and acetone extracts from the leaves of *C. odorata* were capable of inhibiting highest (three) number of test microorganisms. The results can be observed in Table 4.2 where hexane and acetone extracts showed inhibition zones towards three Gram-positive bacteria (*B. subtilis*, *B. cereus* and *S. aureus*) with a range of 8.0 – 12.0 mm. Another three extracts included chloroform, ethyl acetate and methanol extracts were only inhibit two out of four Gram-positive bacteria used in the current study (*B. subtilis* and *B. cereus*) with mean of inhibition zones diameter that was range between 8.3 mm to 13.0 mm (small and medium inhibition zone). In the test of antimicrobial activities, all extracts of *C. odorata* leaves did not show any inhibition zones against MRSA.

Based on Table 4.2, the antimicrobial activities from different extracts of *C. odorata* leaves indicated that they were more easier to inhibit Gram-positive bacteria since they were able to inhibit more microorganisms by demonstrating various small and moderate sizes of inhibition zones around the disc on the seeded agar plates. In this experiment, chloroform extracts indicated that they were not as potent as acetone extracts in inhibiting antimicrobial activities against all of the bacteria tested where the results showed the chloroform extracts

able to inhibit only three types of microorganism which were *K. pheumoniae, B. subtilis* and *B. cereus.* It only revealed small inhibition zones with means that were lower than 10 mm. Besides, MRSA was the most susceptible against all extracts since the results indicated that there had no any inhibition zones appear on the plates towards all those five different extracts.

The antimicrobial activity of the extracts were concentration dependent, with Gram-positive bacteria were the highest mean of inhibition by the extracts in this study. In general, Gram-positive bacteria were easier to be inhibited by the extracts compared to Gram-negative bacteria due to the different cell wall components in both groups of bacteria (Steward, 2021). This is because the Gram-positive bacteria consisted of thick layer of peptidoglycan which make it easier to absorb antibiotic substances that caused them to be more susceptible to the extracts while the Gram-negative bacteria was actually had an exterior membrane layer that is separate from the peptidoglycan cell wall where it was known to act as a barrier for the bioactive compounds, hence allowed them to penetrate further into the bacterial cell (Bailey, 2020).

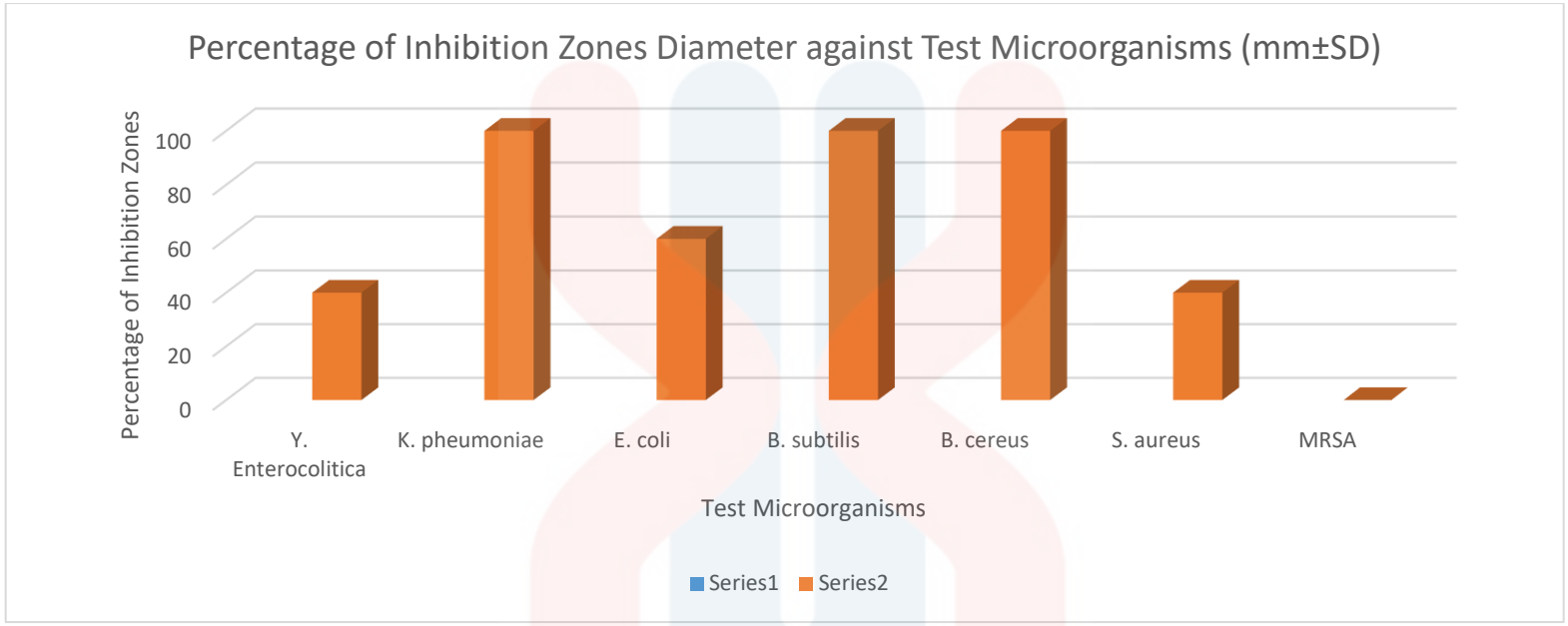

Figure 4.3: Percentage of inhibition zones diameter against test microorganisms (mm±SD).

Figure 4.3 above shows that methanol extract indicated the highest percentage of inhibition zones againts K.*pheumoniae*, *E. coli*, *B. subtilis* and *B. cereus* while MRSA had no any inhibition zones from all the five different extracts which are hexane, chloroform, acetone, ethyl acetate and methanol.

Figure 4.4: Hexane extract and chloroform extract displayed inhibitory action against *K. pheumoniae*, indicating by a clear zone around the disc.

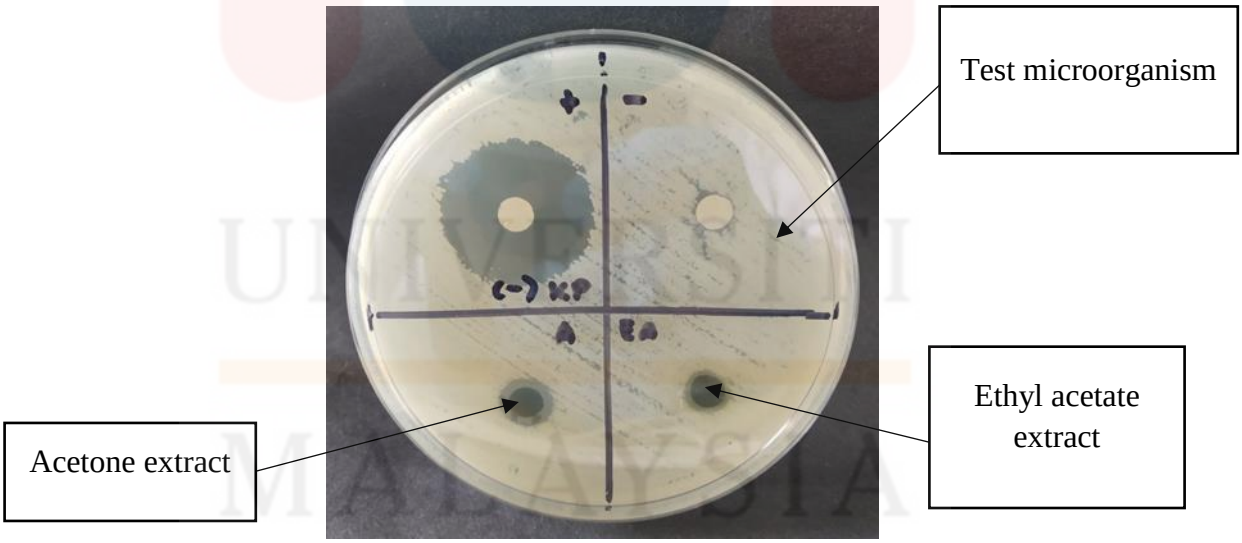

Figure 4.5: Acetone extract and ethyl acetate extract displayed inhibitory action against *K. pheumoniae*, indicating by a clear zone around the disc.

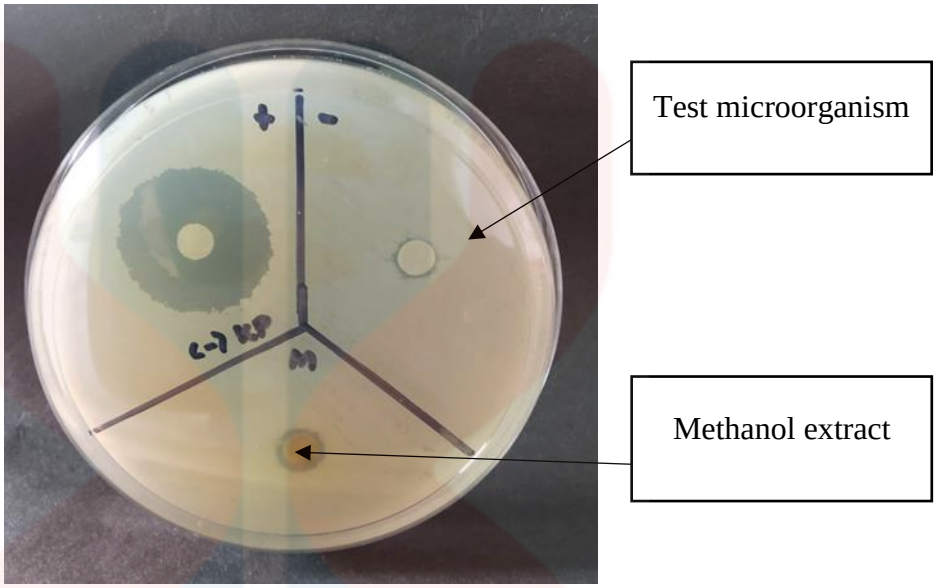

Figure 4.6: Methanol extract displayed inhibitory action against *K. pheumoniae*, indicating by a clear zone around the disc.

4.4 Preliminary Chemical Profiling of Antibacterial Compounds

4.4.1 Group of Compounds Present in the Leaves Extract of *C. odorata*

The detection of structural groups of compound such as flavonoid, alkaloid, anthraquinone, phenol and lactone were carried out by using several pieces of plain TLC plates that were generated in solvent systems of methanol:ethyl acetate:petroleum ether with a ratio 1:1:1 (v/v/v). In the detection of flavonoids, all the leaves extract of *C. odorata* from different extraction solvents (hexane, chloroform, acetone, ethyl acetate and methanol) showed the presence of flavonoid compound since the extract on the TLC plate appeared yellow colour after being sprayed with a reagent of NaOH. Then, it was discoloured after 1M hydrochloric acid (HCl) was added. According to previous study by Chaves *et al*., (2020), he stated that organic solvents like methanol are more effective at extracting flavonoids due to their polarity. The presence of flavonoid in the leaves extract of *C. odorata* provides several advantages to human as they are linked to a wide range of health-promoting benefits. This is due to their ability to control important cellular enzyme functions as well as their antioxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic capabilities (Panche, 2016).

Wagner's reagent was prepared by dissolving 1.27 g iodine and 2 g potassium iodide in 5 mL distilled water followed by the addition of 100 mL of distilled water. The leaves extracts from different extraction solvents also resulted in the presence of alkaloid when the developed TLC plate was sprayed with the prepared Wagner's reagent, where the extract

pipetted on the plain TLC plate appeared reddish brown colour (Table 4.3). Kandibanda (2016) stated that the presence of alkaloid was detected with the appearance of reddish brown colour. The alkaloid compound found in plant leaves extract plays an important role in living creatures, and it has been extremely beneficial to people for centuries, since it has been shown to have anti-inflammatory, anticancer, analgesic, neuropharmacologic, antibacterial, antifungal, and other properties. Joanna Kurek (2019) emphasized that alkaloids are important in medicine and various aspects of human life as diet elements, supplements as well as medications.

Furthermore, the presence bioactive compound of anthraquinone was detected in the extract of chloroform, ethyl acetate, acetone and methanol where there was an appearance of green colour on the developed TLC plate after being applied with 10% methanolic potassium hydroxide (KOH). The presence of anthraquinone indicated when the original colour changed to red, green, purple or violet (Bhatt, 2019).

Table 4.3: Compound groups present in the five leaves extract of *C. odorata*

Phytochemicals	**Types of Extracts**				
	Hexane	Chloroform	Acetone	Ethyl Acetate	Methanol
Alkaloid	+	+	+	+	+
Phenol	-	+	+	+	+
Lactone	+	+	+	+	+
Anthraquinone	-	+	+	+	+
Flavonoid	+	+	+	+	+

There are several benefits of anthraquinone where it was used for dyes, pigments as well as for medicinal purposes since it is known to be a powerful laxatives that can irritate both the upper and lower gastrointestinal system. Bolen (2020) stated that anthraquinones are believed to have the ability to increase the quantity of fluid in the colon while simultaneously stimulating colon contractions. In addition, the compound of anthraquinone also provides protection against various diseases such as cancer, diabetes, kidney disease and also malaria. Furthermore, as shown in Table 4.3, the hexane leaves extract showed no colour change after being sprayed with the prepared reagent, indicating that it retained its original yellow-orange colour. Hence, the leaves extract of hexane indicated that there was no anthraquinone compound contain in it.

On the other hand, phenol was seen to present in chloroform, ethyl acetate, acetone and also methanol extracts due to the apperance of green colour after being sprayed with the

reagent of 1% aqueous ferric chloride ($FeCl_3$) (Table 4.3). Phenol has been found to provide a variety of health benefits which includes as an antioxidant, cancer prevention, antimicrobial agent as well as antidiabetic agent (Carter, 2019). Antioxidants are known to exist in plant-based substances containing phenol which means that they can inhibit free radicals from reacting with other molecules in human body, reducing DNA damage as well as provides long-term health consequences. For instance, free radicals that have lost an electron may react with and destroy molecules such as DNA. When free radicals react with molecules, they might produce even more free radicals. Hence, antioxidant molecules act as a shield between free radicals and healthy molecules, replacing any missing electrons and rendering them harmless (Carter, 2019).

Besides, according to previous research by Carter (2020), phenol-based substances have been discovered to have cancer-preventive properties. It was suggested that getting phenols from a diet rich in plants that contain phenolic compounds and foods supplemented with phenols helps to boost the immune system and make cells more cancer-resistant throughout their life cycle. Next, the presence of another bioactive compound which is lactone was resulted in all five different extracts which are chloroform extract, ethyl acetate extract, acetone extract, hexane extract and also methanol extract. The presence of lactone in all five of these extracts was indicated by the appearance of brown spots on the developed TLC plate after being placed in a beaker containing iodine crystals. The appearance of brown spot indicates a positive reaction which shown that a particular extract is containing compound of lactone (Christe, 2018).

In this study, chloroform, acetone, ethyl acetate and methanol extracts indicated that they were the most effective in extracting all the tested groups of compound which are

alkaloid, phenol, lactone, anthraquinone and flavonoid. It revealed that those extracts had the most diverse compounds as all the five group of compounds tested in the current study present in these four extracts (chloroform, acetone, ethyl acetate and methanol extracts). Antioxidant substances with varying chemical properties and polarity may or may not be soluble in a given solvent. Polyphenols are commonly been found in polar extracts such as methanol and ethyl acetate because they have a better efficiency of solvation as a result of interactions (hydrogen bonds) between the polar sites of the antioxidant compounds and the solvent than nonpolar one (Thouri, 2017). On the other hand, ethanol has long been recognised as an excellent solvent for polyphenol extraction that is also safe to consume. Methanol has been found to be more effective in extracting lower molecular weight polyphenols, while aqueous acetone is better at extracting greater molecular weight flavanols.

Solvents with varying polarity can be used to isolate various groups of chemicals. The non-polar solvent such as hexane was capable to extract non-polar compounds such as alkaloids, terpenoids and fatty acid. A study conducted by Mohamed (2017) stated that hexane has a better chemical selectivity due to its non-polar nature. Based on Table 4.3, only three compounds were presented in hexane extract which are alkaloid, lactone and flavonoid which indicated that this extract had the lowest number of tested compounds compared to another four extracts. This is due to the function of hexane in the extraction which is actually to remove fats, oils and waxs that caused the extract to had the least compounds. Hexane extraction is known to produce enormous amounts of oil considerably faster and more efficiently. The used of hexane in extraction is due to several benefits where a study conducted by Prihadi (2016) stated that hexane is minimal in toxicity and does not emit any

dangerous or poisonous gases that could endanger the user as well as has no effect on the nutritional value of the food from which oil is extracted.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In the present study, the leaves of *C. odorata* were extracted by using sequential solvent extraction method according to the increasing polarity of solvents which were hexane, chloroform, acetone, ethyl acetate and methanol. All of the leaves extracts which had been macerated in different polarity of solvents were then screened for their antimicrobial activites against three Gram-negative bacteria (*Y. Enterocolitica, K. pheumoniae and E. coli)* and four Gram-positive bacteria (*Methilin-resistant S. aureus, S. aureus, B. cereus* and *B. Subtilis*). Out of the five different extracts, acetone extract of *C. odorata* leaves was exhibited the highest antimicrobial activities since it was capable of inhibiting highest (six) number of test microorganisms (*S. aureus, B. cereus, B. Subtilis, Y. Enterocolitica, K. pheumoniae* and

E. coli) while chloroform extract was exhibited the lowest potential of antimicrobial activities since it was capable of inhibiting only three types of microorganisms which were *K. pheumoniae, B. cereus and B. Subtilis*. Meanwhile, all the extracts used in the current study failed to inhibit MRSA, showing that MRSA is not susceptible to the hexane, chloroform, acetone, ethyl acetate and methanol extracts.

Next, the chemical profiling of groups of compound such as flavonoid, alkaloid, anthraquinone, phenol and lactone were carried out by using several pieces of plain TLC plates in order to create spots that are well defined and separated. Several groups of compound that have been detected in each different extracts of *C. odorata* leaves promoted various kinds of benefits to human. The presence of flavonoid by the appearance of green colour on the developed TLC plate of all five leaves extracts of *C. odorata* that was macerated in the increasing polarity of solvents provides a wide range of benefits due to their ability to control important cellular enzyme functions. In the detection of alkaloids, all the leaves extracts from different extraction solvents also resulted in the presence of alkaloid since the result appear reddish brown colour. The alkaloid compound found in the plant leaves extracts may be beneficial to people since it has been shown to have antimicrobial, anticancer and anti-inflammatory properties, which also act as diet elements and medications.

The presence of anthraquinone in the chloroform, ethyl acetate, acetone and methanol extracts of *C. odorata* leaves were indicated by the appearance of green colour on the developed TLC plate. Anthraquinone plays an important role in medication due to its powerful laxatives which can irritate upper and lower gastrointestinal system. It also may provides protection against cancer, diabetes and malaria. Moreover, the compound of phenol that was seen to be presented in all extracts (chloroform, acetone, ethyl acetate and methanol extracts) except the extract of hexane showed that it can act as antimicrobial agent as well as cancer prevention which may promotes a variety of health benefits to human. The presence of lactone compound in all extracts of *C. odorata* leaves from different solvent polarity indicated by the appearance of brown spots on the developed TLC plate after placed in a beaker containing iodine crystals.

As a conclusion, variuos compounds were detected present in the extracts used in this study, showing that these extracts possess hidden value of therapeutic elements that can be further developed as natural antimicrobial agent.

5.2 Recommendation

In this experiment, we recommended to use various plant parts such as branches, roots and flowers since all of different parts of plant will be resulted in different bioactive compounds of crude extracts. For instance, branches is known to be the most effective in

terms of crude extracts quantity where it could be beneficial in identifying the effectiveness of antimicrobial activities against Gram-positive and Gram-negative bacteria such as *S. aureus* and *K. pheumoniae*. According to previous study by Wakeel (2019), he stated that the sequence of the plant components based on the amount of extract produced was branches>leaves>flowers>roots. As previously documented in an antibacterial examination of woad plant crude extracts, these plant components have a wide range of crude extracts quantities.

Furthermore, use a combination solvents such as 1:1, v/v, n-hexane-ethyl acetate, n-hexane-ethanol, methanol-chloroform as well as acetone-water since they able to increase the crude extracts quantity where the most effective solvent is acetone-water (1:1, v/v) (Wakeel, 2019). It is because various plant sections have different amounts of crude extracts with different solvents. These solvents and plant parts have diverse antibacterial activity which is due to the different types and quantities of biological components in these extracts. In addition, different biological components have varying degrees of polarity, which can be extracted using a solvent with the proper polarity index (Wakeel, 2019).

Moreover, in term of purification of compound, use a recrystallization method in order to get the purest form of a substance with correct geometrical shapes (Nagpal, 2018). Solids dissolve more easily in heated liquids than in cold liquids. In this process, it needs to choose suitable solvents such as chloroform, water, benzene and carbon tetrachloride which are commonly employed for crystallization (Nagpal, 2018). Besides, use GC-MS method since it allows for improved sample identification, increased sensitivity as well as able to obtain a good result in a shorter time.

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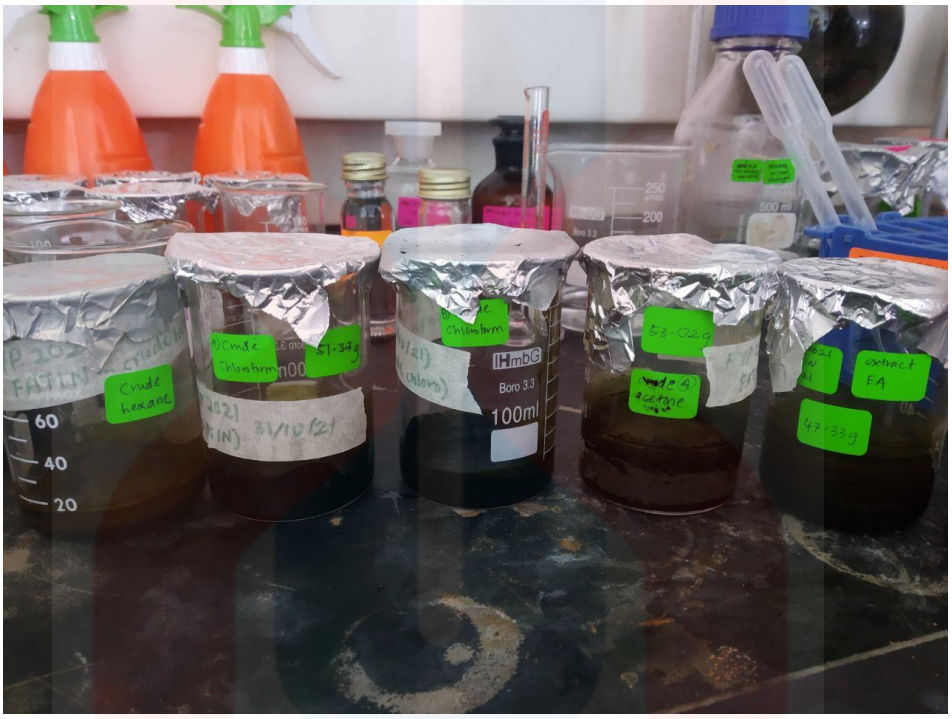
APPENDIX

Appendix A: Each different extract solutions have been evaporated to get crude extracts by using a rotary evaporator.

Appendix B: The extract solution has been filtered by using filter paper

Appendix C: Five different extracts which were from left, hexane, chloroform, acetone, ethyl acetate and methanol obtained using rotary evaporator.



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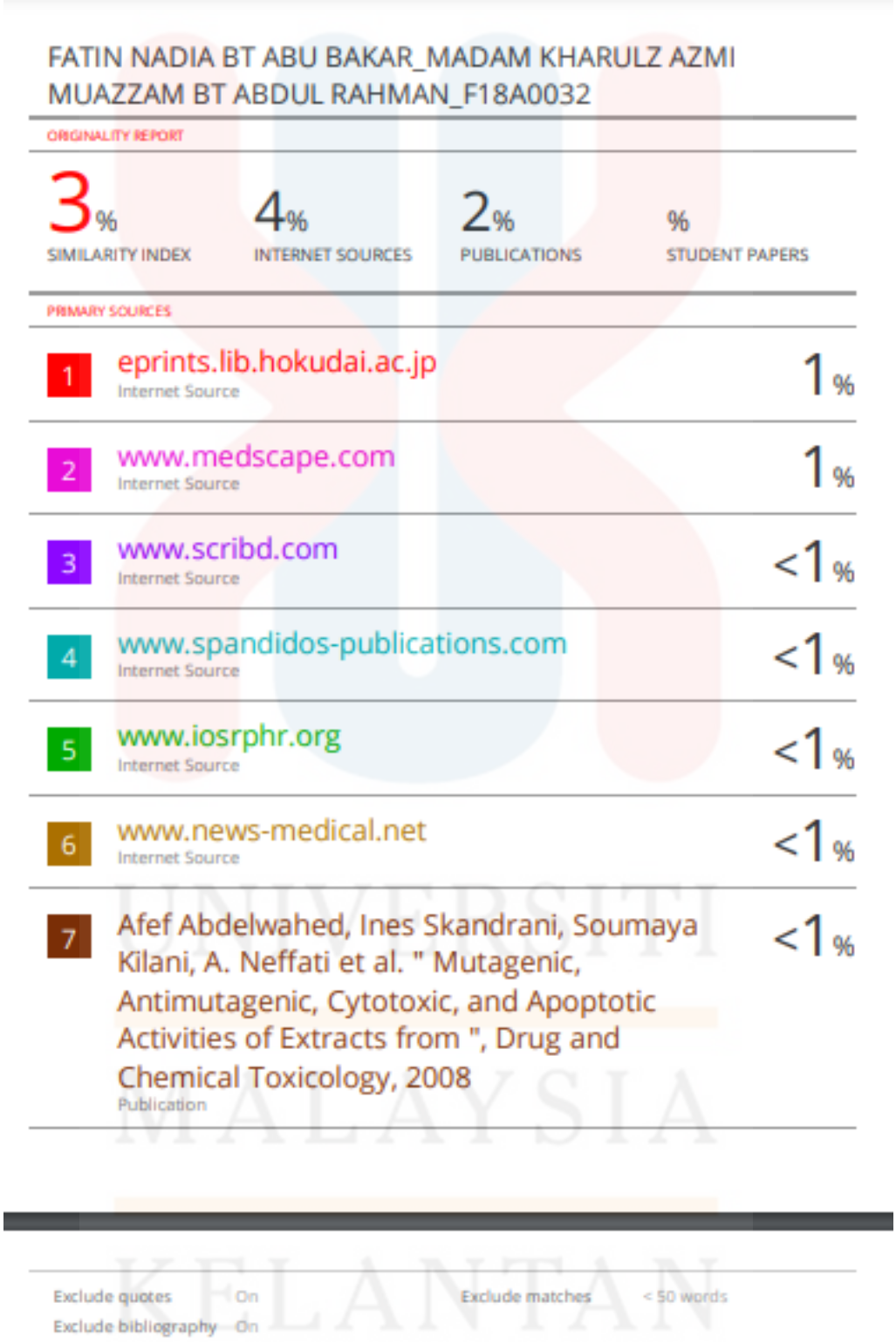

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