Antimicrobial activity of *Chromolaena odorata* and Screening Bioactive Compound for the Development of New Phytopharmaceutical

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research. The thesis has not been accepted for any degree and not submitted in candidature of any other degree.

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LIST OF SYMBOLS

cm	Centimetre
mm	Millimetre
%	Percentage
wt%	Weight percentage
°C	Degree celcius
min	Minute
cm^{-1}	Reciprocal centimetre
mg	Milligram
ml	Millilitre

LIST OF ABBREVIATIONS

FTIR	Fourier Transform infrared spectroscopy
HPLC	High-Performance Liquid Chromatography
NI	No Inhibition
C.odorata	*Chromolaena odorata*
E.coli	*Escherichia coli*
Staphy.aureus	*Staphylococcus aureus*

Antimicrobial Activity of Chromolaena Odorata and Screening Bioactive Compound for the Development of New Phytopharmaceutical

ABSTRACT

Generally, the usage of plants and herbs give extreme benefits for healing process in which to cure the human disease. In this present study, *Chromolaena odorata*, (*C.odorata*) was selected as the functional medicinal plant. The antimicrobial activity of *Chromolaena odorata* with different sample parts of plants against bacterial strains was investigated and screened the natural bioactive compounds in order for development of new drug for microorganism-resistance. The parts of the plant that were used are stem, leaves and root. Two different solvent namely methanol and ethanol were used in the extraction process. Two different strains which are for Gram-negative strains is *Escherichia coli* and for Gram-positive is *Staphylococcus aereus* was selected to test the antimicrobial activity. Antimicrobial activity of solvent extract was preliminarily screened by using Kirby-Bauer disc diffusion test. The bacteria are incubated in 37^{o}C within one day to get the result. There are three different extract concentration used which are 10µL, 20µL, 30µL. The diameter of the inhibition zone was measured to determine the resistance bacteria. Within the two bacteria, the gram positive bacteria which *Staphylococcus aereus* showed the positive result which shows clear zone of inhibition. While for negative bacteria which is *E.coli* also showed a clear zone of inhibition. However, compared to the other part of *C.odorata* plant, leaves part gives the best clear zone of inhibition while stem and root do not give any inhibition. Hence, in this research also was determined the amount of natural bioactive compound in that plant. The finding for this project will provided the fundamental scientific evidence of antimicrobial activity in different parts of *C.odorata* by using different solvents. Data obtained can be applied in pharmaceuticals field that able to improve the amount of medicinal plant in Malaysia methanol.

Keywords: *Chromolaena odorata, (C.odorata),* antimicrobial activities, toxicity, bioactive compound, antimicrobial susceptibility testing

Aktiviti Antimikrob dari Pokok Chromolaena Odarata dan Menentukan Kompaun Bioaktif untuk kegunaan dalam bidang Farmaseutikal

ABSTRAK

Secara umumnya, penggunaan tumbuh-tumbuhan dan herba memberi manfaat yang bagus untuk proses penyembuhan untuk menyembuhkan penyakit manusia. Dalam kajian ini, *Chromolaena odorata, (C.odorata*) telah dipilih sebagai tumbuhan ubatan yang mempunyai fungsi sedemikian. Aktiviti antimikrob *C.odorata* dengan bahagian-bahagian sampel tumbuhan yang berbeza ke atas bakteria dan juga untuk meneliti sebatian bioaktif semulajadi untuk membangunkan ubat baru untuk rintangan mikroorganisma. Bahagian sampel yang digunakan adalah batang, daun dan akar dan ia diekstrak dengan menggunakan pelarut yang berbeza iaitu metanol dan etanol. Seterusnya, aktiviti antimikrob telah diuji pada dua jenis mikrob yang berbeza iaitu untuk strain gram-negatif adalah *Escherichia coli* dan strain gram-positif adalah *Staphylococcus aereus*. Aktiviti antimikrobial ekstrak pelarut diuji dengan menggunakan ujian penyebaran cakera Kirby-Bauer. Bakteria diinkub dalam 37^{o}C dalam satu hari untuk mendapatkan hasilnya. Terdapat tiga kepekatan ekstrak yang berbeza yang digunakan iaitu 10µL, 20µL, 30µL. Diameter zon inhibisi diukur untuk menentukan bakteria rintangan. Dalam kedua-dua bakteria, bakteria gram positif iaitu *Staphylococcus aereus* menunjukkan hasil positif yang menunjukkan zon perencatan yang jelas. Manakala, bagi bakteria negatif iaitu *Escherichia coli* juga menunjukkan zon perencatan yang jelas. Walau bagaimanapun, berbanding bahagian lain dari tumbuhan *C.odorata*, bahagian daun memberikan zon halangan terbaik yang manakala batang dan akar tidak menunjukkan sebarang zon halangan. Di samping itu, dalam kajian ini juga telah menentukan jumlah kompaun bioaktif semulajadi. Penemuan untuk projek ini adalah untuk menyediakan bukti ilmiah asas aktiviti antimikrobial di bahagian-bahagian lain di *C.odorata* dengan menggunakan pelarut yang berbeza. Data yang diperolehi boleh digunakan di bidang farmaseutikal yang dapat meningkatkan jumlah kilang ubat di Malaysia.

Kata kunci: *Chromolaena odorata, (C.odorata),* aktiviti antimikrob, ketoksikan, sebatian bioaktif, ujian kerentanan antimikrob

CHAPTER 1

INTRODUCTION

1.1 Background of Study

In recent time, the use of traditional medicines has well developed in third world countries and its shows a potential to cure the diseases. In Malaysia, Burkill (1996) was reported on the extensive compilation of the commercial production of the Malaysian peninsular, statically not less than 1300 plants have been used in traditional medicines. Traditional medicine is rich in biological and pharmacological activity which is widely use in drugs medical treatment. The full acceptance of traditional medicine as an alternative form of healthcare has alarming increase in the incidence of new and re-emerging infectious diseases that have triggered the necessity to study the potential of medicinal plants as a new alternative of medicinal treatment (Vital and Rivera, 2009). Currently, statistical accounts that almost 60% diseases were recorded as a primary cause of death especially in the developing and emerging countries. Many researched about the medicinal plants has been done due to less efficiency and microorganism-resistance towards synthetic medicine as commercial drugs. According to Wiart *et al.*,2011 this medicinal plants shows a rich source of the antimicrobial agent. It was supported by Bonjor *et al.*, 2004, the investigation of different extracts found from the traditional plant as potential sources of new antimicrobial agents has led to increasing

interest of researchers due to the large quantity of plants on the earth's surfaces. A large number of plants have been extracted and semi-purified from around the world to investigate their antimicrobial activity (Suntar, 2014).

The aim of this research is to study the antimicrobial activity of the medical plant of *C.odorata.* This type of plant is a part of a family from *Asteraceae* species. *C.odorata* or commonly known as *'Pokok Kapal Terbang'* by rural residents in Malaysia is a tropical and subtropical species of flowering shrub in the sunflower family. This type of plant was rarely planted by people and it usually found by the roadside and in the jungle. People have neglected this wild plant because of the toxicity and it diffuse, scrambling shrub that is mainly a weed of plantation crops and pastures. However, based on the previous study for C. odorata in Malaysia are more focusing on antimicrobial activity on leaves part only, and only a little bit of information had divulged regarding antimicrobial acticity of medicinal plants. Plant materials have been found to be active against infectious diseases, for instance, Garcinia biflavonoid has been found to be active against a wide variety of microorganisms such as *Salmonella typhi, Escherichia coli, and Staphylococcus aureus* (Kigigha and Zige, 2013). Thus, in this research, the antimicrobial activity of the selected part of the plant and between the selected strains have been done and proved that *C.odorata* has the potential of antimicrobial activity.

In addition, from the previous study which is by Akinmoladun et al., (2007), also evaluate the relationship between the phytochemical compounds that produced by this plant. This phtochemical compound which are coumarins, tannins, steroids, saponins, terpenoids, terpenes, flavonoids and cardiac glycosides plays a role in inhibiting the growth of pathogenic microorganisms (Akinmoladun, Ibukun, & Dan-

Ologe, 2007). However, this research was investigated the chemical compounds from a different part which are stems, leaves, and root of *C.odorata*.

1.2 Problems Statement

There are very limited researches have been done towards *C. odorata* which has been proven to have an appreciable antimicrobial effect. This present study was conducted to investigate the antimicrobial activity of medicinal plant which is *C.odorata* in Malaysia, especially in Kelantan area. This plant was tested on selected strains that commonly have high infections towards human itself.

Recently, many researchers have reported that an efficacious antimicrobial compounds was found from the plant leaf, but in this research will testing on the different part of *C. odorata*. In this research was investigated the antibacterial activity of *C. odorata* extracts against bacterial human skin infection strains. Various parts of *C. odorata,* which are leaves, stem and root, were extracted with ethanol and methanol by using Soxhlet Extraction method. Antimicrobial agent derived from *C.odorata* plants will be an alternative method to reduce the antibiotic resistance of bacterial pathogens.

In addition, the previous study shows that commercial drug used nowadays is not as active due to the microorganism-resistant towards commercial medications. Hence, many researched has been done to find out another alternative method by replaced with this *C.odorata* as extracted medicinal plants for medical purposes. From the previous study, a few reports have done to investigate the effect of *C. odorata* extract on bacterial human skin infection were tested to selected strains. In this research

is focussing on the study of antimicrobial activity toward chosen pathogens which are *E.coli* and *Staphylococcus*.

1.3 Objectives

The purposes of the research are:

a) To evaluate the organoleptic of *C.odorata* as medicinal plant

b) To investigate the antimicrobial activity of *C. odorata* using the different solvent, concentration and different parts of plants

c) To determine the natural bioactive compound by HPLC-DAD Method

1.4 Scope of Study

In this present study is to identify the antimicrobial activity of the selected part of a plant and between the selected strains which are *E.coli* as gram negative strains and *Staphylococcus aureus* as gram positive strains. From that to that, there are three parts extracted the sample of *C.odorata* which are leaves, stems and root were evaluated for their antimicrobial testing against the selected strains by using different crude concentration. Besides, for the extraction process, the various solvents such as methanol and ethanol are used for testing the ability affect the rate of antimicrobial activity. This antimicrobial susceptibility was tested against selected bacterial strains by using disc diffusion method. There are two kinds' microorganisms used in this research which is supplied by the laboratory in UMK that comprises one of the Gram-positive strains and one of the Gram-negative strains.

This types of microorganism are common include infectious disease and pathogenic to the living things. However, besides testing the antimicrobial activity, this research also determined the bioactive natural compound that can be extracted from *C.odorata* that will serve as a lead for the development of new phytopharmaceuticals. Thus, the scopes of this study are to evaluate the antimicrobial activity of *C.odorata* as a medicinal plant and to determine the bioactive compound from that plant.

1.5 Significant of Study

Overall, in this research provided valuable information for new and novel antimicrobial discoveries derived from *C.Odorata* and offer a new approach of bioactive compounds to eradicate the disease-producing microorganism efficiently. The identification of antimicrobial activity in *C.odorata* becomes a vital and exciting topic to explore especially it much importance and will contribute in pharmaceutical field which is genuine medicines related to antimicrobial infection diseases. Hence, it also cause to the evolution of new drugs which based on native medicinal plants.(Hung et al., 2011)

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CHAPTER 2

LITERATURE REVIEW

Chromolaena odorata (C. odorata) is native plant and abundantly found at tropical Africa, and Southest Asia. It grown annually and widespread globally around the world which are roughly about 165 species of perennials and shrubs in the aster family. It was declared as prohibited species in Australia and became a primary target of weed surveys. Colour range of this species can be found from green colour, and it is herbaceous perennial with a bushy habit which forms a very dense thicket about 2 m high, in almost pure stands. The plant is hairy and glandular, and the leaves give off a pungent, aromatic odour when crushed. *C.odorata* which is in leaves part contains carcinogenic pyrrolizidine alkaloids, and it is toxic to organisms. Season, climate, growth phase, extraction solvent, extraction procedure, and plant material to solvent ratio are some factors that will affecting the biological activity of medicinal plants (Maji *et al.*, 2010; Kothari, Gupta, & Naraniwal, 2012).

The economic value of *C. odorata* is low due to it is unpopular plants in subtropical regions, and it also has negative sides due to the high level of nitrate that above the toxic level. Despite of the negative effect, it still has patronage from practitioners of traditional medicine. According to data analysis from World Health Organization (WHO), studied by Santos et al., (1997) several of medicines can be derived from this *C.odorata* plant. Kigigha and Zige (2013) were reported that for over 50 years, this weeds has been in Nigeria and it can be found along roadsides, waste and

fallow lands. *C.odorata* is a known toxic weed that is widespread over many parts of the world including Nigeria especially in the southern part of Nigeria. The leaves of *C.odorata* were used for wound dressing, skin infection and to help in blood coagulation. Traditional medicine that produced by the living organism is still the mainstay of about 75-80% of the world population, predominantly in the developing countries and for primary health care (Kamboj, 2000).

Nowadays, hospital-acquired infections (HAIs) are a major cause of patient morbidity and mortality, and are correlated with fomites. Fomites are reservoirs of pathogens which can infect patients by direct contact or indirectly from touching contaminated hospital environmental surfaces (Mangicaro, 2012). Previous study by Maryam *et al.,* 2014, studied at hospital in northern Nigeria, pathogenic bacteria were found on 65.7% of the samples tested for example stethoscopes, doorknobs, including *Staphy. aureus* which is 21.7% frequency, *Staph. epidermis* which has frequency 8.7%, *Streptococcus* spp. which is 8.7%, *Bacillus* spp. which is 13.0%, *E. coli* which is 26.1%, *Pseudomonas* spp. which is 8.7% and *Klebsiella* spp. which is 13.0%. Hence, obtaining new antimicrobial agents from natural resources is important to develop alternative drugs for safe and cost-effective health care because there has been a slightly high increase in the incidence of infections with drug-resistant microorganisms acquired by patients while in the hospital. This bacterial human skin infection strains are correlated with nosocomial infection, fomites and antimicrobial-resistant strains. Previous studies by Suksamrarn et al., 2004, , Coopoosamy, & Naidoo, 2011, Pisutthanan et al., 2005, have reported that *C. odoarata* plant exhibits biological activities such as has potential in antimicrobial activity. Traditionally, fresh green leaves or a decoction of *C. odorata* have been used throughout many tropical countries for the treatment of leech bite , soft tissue wounds, burn wounds, skin infection and liver diseases (Zachariades & Goodall,

2002). The interest in undertaking the study on *C. odorata* is justifiable based on the local uses of the plant for the treatment of headache, wounds and the series of scientific investigations into the various activities of the plant (Gill, 1992; Phan et al., 2001). According to Akinmoladun et al., (2007), *C. odorata* has medicinal value which is in their component contains phytochemicals such as alkaloids, tannins, flavonoids and other phenolic compounds, which produce a definite physiological action on the human body. Hence, by running systematic research for useful bioactivities from a medicinal plant, it now can consider being a rational approach to nutraceutical and drug research in future.

2.1 Morphological studies

C. odorata is a formerly *Eupatorium odoratum L.*, which is belonging to the plant family *Asteraceae* (Figure 1). Mabberley (1997) reported that about 165 species of the plant *Chromolaena* are distributed in tropical and warm temperate regions. It is a diffuse, scrambling shrub that is mainly a weed of plantation crops and pastures of southern Asia and western Africa in which it forms a bush three to seven m in height when grown in the open (Phan et al., 2001). Previous study which is resembler by Chakraborty, Rambhade, & Patil, (2011) from the Queensland Department of Agriculture (2013), reported that the common names of *C. odorata* are Siam weed, Christmas bush, and common floss flower. However, in rural residents of Malaysia, they called them as "Pokok Kapal Terbang" (Figure 2.1).

This type of plant is herbaceous perennial with a bushy habit which has many branches that become luminescent when it has an opportunity to climb on support. Their leaves are simple, opposite –decussate and without stipules. *C.odorata* has all the

characteristics of one of the world's worst invasive alien species which by it dispersed by wind, man and by many other agents. It also an incredibly fast growing plant, capable of vegetative regeneration and shows numerous genetic strains that has many close relatives.

Figure 2.1: *C. odorata* plants
Sources: Akinmoladun et al., (2007)

Table 2.1: Taxonomy of *Chromolaena odorata* plant

Scientific classification	
Domain	Eukaryota
Kingdom	Plantae
Phylum	Spermatophyta
Subphylum	Angiospermae
Class	Dicotyledonae
Order	Asterales

Family	Asteraceae
Subfamily	Asteroideae
Tribe	Eupatorieae
Genus	Chromolaena
Species	C.odorata

2.2 Phytochemical studies

2.2(a) Secondary metabolites

The bioactive compound of the medicinal plant comes from secondary metabolites compound in which it also called specific metabolites. It is also can be defined as a pathway and small molecule products of metabolism such as antibiotics and pigments that are not vital for organism's survival. Many plants have been used for testing the antimicrobial activity because of their antimicrobial activity. It is because the compounds synthesized in the secondary metabolism of the plant which are phenolic compounds which are a part of the essential oils, as well as tannin (Saxena et al., 1994). Plants produce phytochemicals that include alkaloids, phenolics, terpenoids, glycosides and so on. (Naidoo, Coopoosamy, & Naidoo, 2011). The antimicrobial traits from extracted part of *C. odorata* plant were correlated to the amount of both total phenolic and flavonoid compounds.

2.2(b) Toxicity

It is believed that *C.odorata* contains a significant amount of toxic poison. According to Omokhua, et al., (2017) the plant can be poisonous to animal thus it not recommended for livestock feed as it has an exceptionally high level of nitrate which is 5 to 6 times higher than the permissible toxic level in the leaves and young shoots. Furthermore, there is also some chemicals composition like alkaloid, tannin and saponin which lead to the toxicity of the plant. It had examined to the cattle feeding on this plant, and it has died due to tissue anoxia. Besides, the previous study by Asomugha, Ezejiofor, Okafor and Ijeh, (2015) reported that the ethanolic plant extract had greater concentrations of alkaloids, glycosides, sterols, allicin, tannins, terpenes and flavonoids, the aqueous extract had a greater concentration of protease inhibitors and saponins. The greater concentrations of protease inhibitors and saponins that are well known potentially toxic substances may also contribute to this greater toxicity observed with the aqueous extract.

2.3 Pharmacological studies

In term of pharmacological, *C. odorata* have been claimed that it brings various advantages include as traditional medicine, biopesticide and others. It is very important to develop such a safe antimicrobial agents because of the use of medicinal plants is increasing due to the development of microorganism-resistance to antibiotics. Back to the previous study, (Phan *et al.*, 2001) reported that a formulation prepared from the aqueous extract of the leaves of *C. odorata*, in Vietnam, has been licensed for clinical

use. The researchers are excitedly focussing their attention to complementary medicine searching for new ways to develop better drugs against microbial infections (King, Vital, & Rivera, 2009).

C. odorata for examples is the plants have been used because of their antimicrobial traits, which are due to compounds synthesised as secondary metabolites of the plant which contains phytochemicals such as alkaloids, tannins, flavonoids and other phenolic compounds (Akinmoladun *et al.,* 2007). *C. odorata* has been reported to have antispasmodic, antiprotozoal, antitrypanosomal, antibacterial and antihypertensive activeties. It has also been reported to possess anti-inflammatory, astringent, diuretic and hepatotropic activities (Weniger and Robinean, 1988; Iwu, 1993)

Previous study by Hanphanphoom & Krajangsang, (2016) was reported that the efficiency of antibacterial activity of *C. odorata* extracts from the different part of the plant which is the stem and leaves that were tested against bacterial human skin infection strains. The previous study has been conducted by Vital & Rivera, (2009); Maji *et al.,* (2010); Naidoo *et al.*,(2011); Eze *et al.,* (2013) on the antimicrobial activity of *C.odorata* plant extract have been restricted to clinical diarrheal strains such as *B. cereus*, *E. coli*, *Kleb. oxytoca*, *Salmonella enterica*, *Salmonella* Typhimurium, *Shigella sonnei* and *Vibrio cholera*, and skin infections with bacteria such as *Staph. aureus* and *Staph. Epidermidis.*

2.4 Extraction Method

Extraction is the separation process of active compound in order to produce pure extract form by using standard extraction procedures such as maceration process or

Soxhlet extractor and others. In this present study, Soxhlet extractor was used to extract the sample. For application of Soxhlet extractor, it will take almost 48 hours of extraction with temperature 80 ºC depending on the solvent properties (Sukanya *et al.*, 2011). Crude drug or extract that might be in a various form such as decoctions, infusions, fluid extracts, dry extracts and tinctures was formed at the end of product. To produce a dry extract, the sample must undergo evaporation process by using rotary evaporator. By evaporation (R-200 rotary evaporator) the filtrates were concentrated and frozen in a freeze dryer. The crude extract usually will be stored in airtight container and being autoclave in order to perform the sterile condition to the crude that will be used in next process and it was stored at 4^{o}C as stated by Naidoo *et.,al* (2011).

Two different solvents that were used in this extraction process which are methanol and ethanol. Ethanol and methanol which are highly polar organic solvents are typically used for extraction of bioactive compounds from plants. The polarity of ethanol and methanol plays an important role in increasing phenolic solubility (Naczk & Shahidi, 2006). Alkaloids, flavonoid and some phenols are polar of secondary compounds that contain in medicinal plant (Delahaye et al., 2009). From the previous study reported that leaf extracts gave significantly higher percentage yields compared to stem extracts for all extraction solvents. This research was done using twelve samples of *C. odorata* extracts were collected from leaf, stem and root plant parts, and were used for water, ethanol, methanol and hexane extractions (Hanphanphoom & Krajangsang, 2016). Generally, solvent type, plant material to solvent ratio, time of extraction, and particle sizes of the plant will affect the extraction efficiency (Tiwari, Kumar, M. Kaur, G. Kaur, & H. Kaur, 2011). Tiwari *et al.*, (2011) also reported the rate of extraction can be enhanced by enlarging the surface area and using 1:10 (w/v) which is a sample to solvent ratio.

2.5 Pathogenic Bacteria

The pathogenic bacteria used to test the antimicrobial activity of the plant extracts were two strains which are *Escherichia coli (E. coli)* for Gram-negative bacteria and *Staphylococcus aureus (S. aureus)* for Gram-positive bacteria. *E. coli* stains Gram-negative because its cell wall is composed of a thin peptidoglycan layer and an outer membrane. According to Himedia, Mumbai,(India) all the tested bacteria were grown on trypticase soy broth (TSB) and incubated at 35 ± 2 ºC for 18–24 h before it being used in the future process.

Reported by Vital and Rivera, (2009) and supported by El Astal *et al.,* (2005) the Gram positive bacteria were more susceptible than gram a negative bacteria which is due to the difference in sensitivity between gram positive and gram negative which shows their morphological differences between these microorganisms meanwhile this also might be due to the fact that the cell wall of Gram positive bacteria which is less complex and lacks the natural sieve effect against large molecules due to the small pores in their cell envelope (El Astal *et al.,* 2005). According to Nikaido and Vaara (1985) Gram-negative bacteria have an outer phospholipid membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da. Gram positive bacteria should be more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer and Gerhardt, 1971). Hence, according to Anyasor et al., (2011) it also can suggest that due to the presence of flavonoids and tannins, *C. odorata* antimicrobial mode of action binds to bacterial cell wall and inhibits its biosynthesis.

2.5(a) *Escherichia coli*

Escherichia coli (Figure 2.2) is a gram-negative facultative anaerobic, rod-shaped bacterium that is commonly found in lower intestine of warm-blooded organisms which is endotherms. The harmless strains are part of the normal flora of the gut and can be benefit their host by producing vitamin K_2 (Bentley et al., 2017). Majority *E.coli* strain are harmless to our body, serotypes may cause food poisoning.

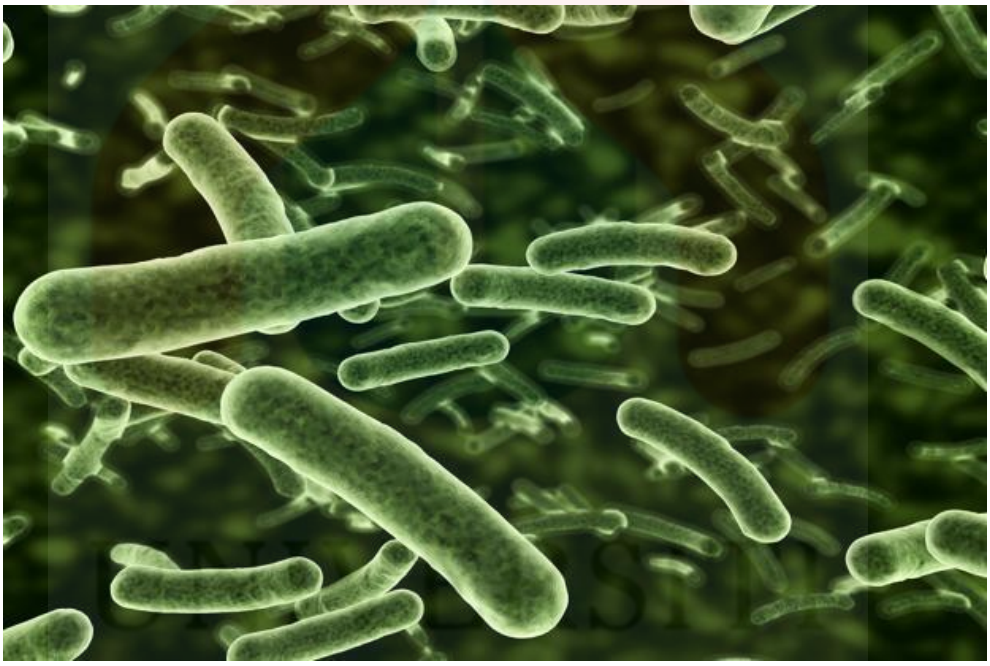


Figure 2.2*: Escherichia coli*
Source : Akinmoladun et al., (2007)

2.5.2 *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive pathogen, round-shaped bacterium and it is a usual member of the microbiota of the body that can cause a variety of diseases,

ranging from kindly skin infections to life-threatening endocarditis and toxic shock syndrome(Chang et al., 2017). The nasal membrane and skin of warm-blooded animals including humans is the main habitats of this bacterium, which they also act as a primary line of protection against infection. However, when this pathogen enters the underlying tissues, innate host defense primarily mediated by macrophages plays a pivotal role. Generally, during active infection, macrophages and other lymphocytes use toxic reactive oxygen species such as hydrogen peroxide, superoxide, and hydroxyl radicals to destroy the phagocytosed bacteria.(Chang et al., 2017)

Figure 2.3: *Staphylococcus aureus*
Source: Akinmoladun et al., (2007),

2.6 Antimicrobial Susceptibility with standard antibiotics

In this antimicrobial susceptibility, the Kirby-Bauer disc diffusion method is preferred for antimicrobial susceptibility testing. The techniques are important due to there will be some bacteria which are resistant to survive in more than one antibiotics agent. The uses of filter disc impregnated with the known concentration of antibiotic agent are the fundamental principle for Kirby-Bauer test. Then, the disc will be placed on the agar plate that contains the inoculated of microorganism strain. At the incubation period, the antibiotics will spread away to the surrounding area of agar that shows the clearance zone formation. That clearance zone is also known as the inhibition zone that displays the microorganisms are susceptible to antibiotics agents. The plate with the antibiotic disc is then incubated at 37^{o}C for 2hours to observe the zone of inhibition produced by the antibiotics (Naidoo *et al*., 2011). Thus, to observe the distance antimicrobial, the diameter zone inhibition can be measured. Generally, the sensitivity of microbe towards the antibiotic and the rate of diffusion of the antibiotic through the agar and depth of agar are the factors that influence the sizes of inhibition zone.

However, the antimicrobial susceptibility also can be evaluated by using standard antimicrobial agents like Ampicillin, Penicillin G and Kanamycin were tested against microorganisms. By using standard antibiogram chart, the inhibition zone is measured, and their sensitivity pattern was compared, and the result was tabulated according to susceptibility.

2.7 Phytochemical screening by High-Performance Liquid Chromatography (HPLC)

Previous phytochemical investigations of this *C.odorata* plant described the presence of flavonoids, phenolics, alkaloids, terpenoids, and essential oil. Based on previous study, it was reported that ethanol leaf extract, followed by water, methanol and hexane showed the maximum content of total phenols. Furthermore, *C. odorata* has about fourteen different flavonoids that can be identify from the latest being which are 5, 7-dihydroxy-6, 4'- dimethoxyflavanone (Pisutthanan et al., 2006). Besides, from Suksamrarn et al. (2004) flower extract contains flavonoids and flavones hence, root extract includes triterpenes, poriferasterol, octadecane, butyrospermol acetate, bis(2-ethylhexyl) phthalate, chrysophanol and physcion while stem extract has not studied(Suksamrarn *et al.*, 2004). The ethanolic leaf extract showed the highest flavonoid and phenols contents compared with other plant parts and solvents (Hung *et al.*, 2011).

Hence, the previous study prove that methanolic extracted are better to extract the active ingredients of the plant parts compared to other solvents. The methanol extracts contain alkaloids, coumarins and tannins (Okemo, 1996). According to Baez et al., (1998), the presence of these flavonoids could contribute to the antimicrobial activity in *C. odorata* . The conditions of phytochemical analysis by using HPLC machine were set as for mobile phase (methanol: 1% acetic acid), retention time (20 min), and flow rate (1.000 mL/min). The analytical column used in this study was a Hypersil Gold C18, size 4.6×150 mm (GL Sciences, Tokyo, Japan). Gallic acid and Trolox were used as standards for phenolic contents, and rutin and quercetin for flavonoid contents (Hanphanphoom & Krajangsang, 2016).

2.7(a) Secondary metabolites

i. Alkaloids

Alkaloids is the largest group of secondary chemical constituents made largely of ammonia compound with nitrogen bases which synthesis by the amino acid with various radicals of hydrogen atoms in peptide ring which is mostly contain oxygen. The basicity in based on the structure of the molecule and the position of the functional group(Pandith et al., 2013) . Figure 2.4 shows the examples of alkaloids which are morphine, heroin and codeine.

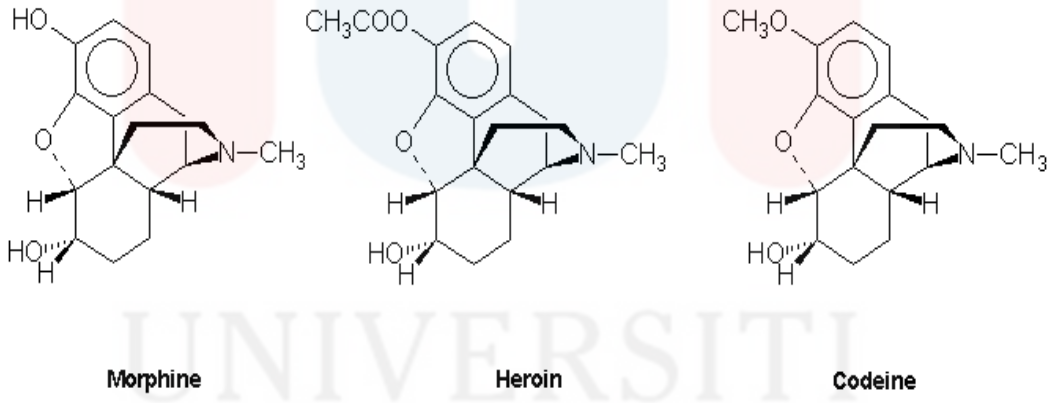


Figure 2.4: Examples of alkaloids

ii. Flavonoids

Flavonoids are one of the important polyphenols which can be found among the plant flora. The structure of the flavonoids contain more than one of benzene ring and it

mostly use an antioxidants or free radical scavengers(Hung et al., 2011). Figure 2.5 shows an example of flavonoids.

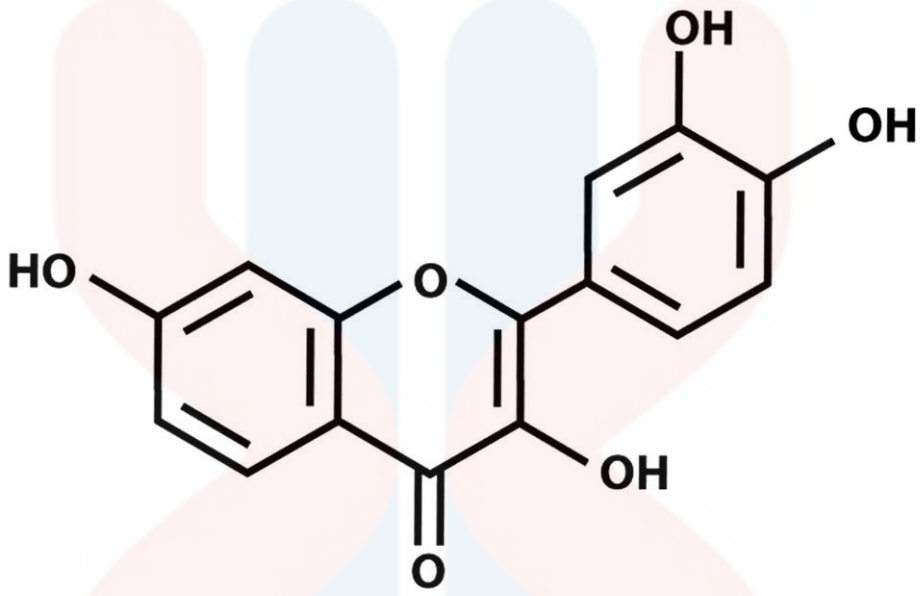


Figure 2.5: Structure of Flavone

iii. Phenolics

Phenolics, phenol or polyphenolics are the natural color pigment from the fruits of the plants. Phenolics synthesized from the phenylalanine via the action of phenylalanine Ammonia Lyase (PAL). It can be found in plants which is mainly for human pathogenic infection(Doughari et al., 2009). The examples of phenolics are Gallic acid, Rutin, Resveratrol, Naringin, Hesperidin, Caffeic acid and Chlorogenic acid (Figure 2.6)(Doughari et al., 2009)

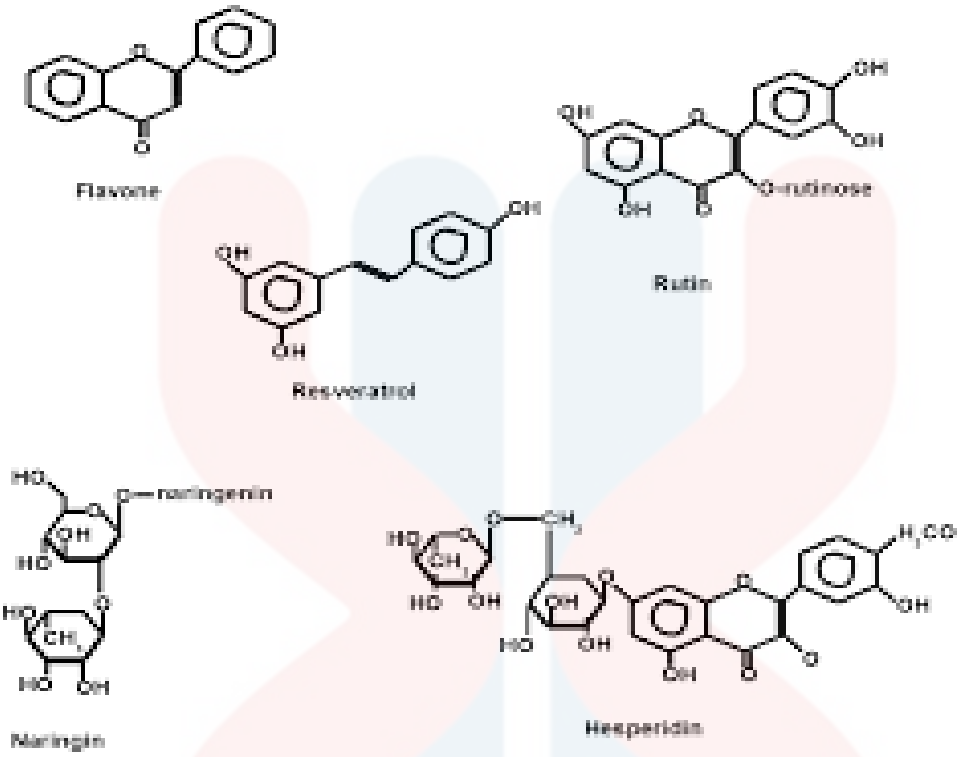


Figure 2.6: Basic structure of pharmacological important plant derived Phenolic

2.8 Identification of organic molecular groups and compounds by using Fourier-transform infrared spectroscopy (FTIR)

FTIR is particularly useful for identification of organic molecular groups and compounds due to the range of functional groups, side chains and cross-links involved, all of which will have characteristic vibration frequencies in the infra-red range. FTIR is perhaps the most powerful tool for identifying the types of chemical bonds which is functional groups present in compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. FTIR results show that the intensity of the peak absorbance of the O–H

functional group decreased with aging but the intensity of the peak absorbance of the C–H and C=O functional groups increased with aging.

From the previous study that reported by Pednekar and Raman (2013) carried out the FTIR spectroscopic analysis of methanolic leaf extract of *Ampelocissus latifolia* for antimicrobial compounds using the FTIR spectroscopic analysis. A survey of literature revealed that the lack of studied of FTIR analysis on the functional groups of *C. odorata.* According to Iqbal Ahamad *et al*. (2006), while studying the *in vitro* efficacy of bioactive extracts of 15 medicinal plants against ES L producing multi drug resistant bacteria, it was detected major groups of compounds as the most active fraction of plants extracts by infrared spectroscopy.

The various functional groups observed in the different extracts probably indicate the presence of carbohydrates, carotenoid, glycogen, amino acids, amides, starch, calotropin, calotropogenin, phosphates, lipids, glycogen and cellulose. Among the functional groups observed in the extracts, OH group was found to be present uniformly only in the methanol extracts of all plants. As OH group has got the ability of forming hydrogen bonding capacity, presence of OH group particularly in methanol extract of leaf of all the plants probably indicates the higher potential of methanol extract towards inhibitory activity against microorganisms. Such a higher antimicrobial activity of methanol extracts of leaf of all those four plants have been already demonstrated (Ashokkumar and Ramaswamy, 2013).

Figure 2.7: Fourier Transform Infrared Spectroscopy (FTIR)

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1(a) Chemical/Reagents

Ethanol, methanol, distilled water, Trypticase Soy Agar (TSA) powder, Ampicilin, Dimethyl Sulphoxide (DMSO)

3.1(b) Instrument and apparatus

Blender, a rotary evaporator (BUCHI), vacuum pump, rotary shaker, filter tunnel, sterile petri dishes, aluminium foil, refrigerator, incubator, autoclave machine, desiccator, centrifuge machine, Soxhlet apparatus, HPLC-DAD (SHIMADZU, Japan).

3.2 Methodology

3.2(a) Collection of plant sample

Disease-free, fresh and green *Chromolaena Odorata* was collected from housing area at Kampung Pasir Dusun which is located in Jeli area of Kelantan state. The plant has been exiled between the desired parts which are leaves, stems and roots.

3.2(b) Organoleptic evaluation of *C. odorata*

Organoleptic evaluate various sensory parameters of *C.odorata* plant parts such as their , appearance of the plant parts mainly size and shape colour, external texture, fracture whether it is granular, splintery, or smooth and external markings, for instance, furrow, wrinkles, ridges, annular, outgrowth between the plant parts. Besides, the evaluation also makes for the fragrance, test for odour for example in term of aromatic, balsamic, camphoraceous, spicy, pleasant, irritating and it taste whether it is sweet, bitter, sour, astringent, pungent, acidic, alkaline.

3.2(c) Preparation of sample

The plant sample is being washed by using tap water, rinsed with distilled water and dried under shade (lgwe, 2015). The sample was dried in drying oven at temperature of 80 ºC for 24 hours (Oko et al., 2017). And after using drying oven, to make sure the maximum dried, the sample was being dried under sunlight for three days. After drying, the samples were cut into small pieces before grounded. The small pieces of samples were grounded by using a blender until all of them become into

powder form. The *C.odorata* powder was kept in the desiccator for the future extraction process.

3.2(d) Extraction

300 mL of methanol (MeOH) and Ethanol (EtOH) is measured and mixed with 30g grinded samples for each sample respectively. The exact ratios will be 1:10 which is 1g of sample to 10 mL of solvent. By using Soxhlet extractor, the grinded powder of sample was extracted with different solvents at 80 ºC for 24 hours. After that, by using a rotary evaporator, the supernatant has been concentrated to the best value.

3.2(e) Microorganism strains

The microorganisms that will be used for this research are *Escherichia coli* for Gram-negative bacteria and *Staphylococcus aureus* for Gram-positive bacteria. These strains will be provided by FIAT's laboratory and lecturers who had the stock culture. According to Himedia, Mumbai,(India) by using trypticase soy broth (TSB), the tested bacteria were grown and incubated at 35 ± 2 ºC for 18–24 h before it being used in the future process.

3.2(f) Preparation of medium

In order to make trypticase soy agar (TSA) medium, 40g of Mueller-Hinton agar powder will be swirled in 1L of distilled water. Then, the pH will be checked to make sure the suitable pH for bacterial to take place their activity. At room temperature, the medium is let to be cooled. After that, in order make it sterile, the medium has been autoclaved for 121 ºC for 15 minutes. The agar was poured into the petri dish and it is allowed to solidify, and by using parafilm, the petri dish is being sealed to avoid the air to enter the plate. Then, it has been stored at 2 ºC to 8 ºC in the refrigerator. Hence, according to Ismail et al. (2016), the agar plates were incubated at 30 ºC to 35 ºC for checking its sterility.

3.2(g) Susceptibility testing with Standard Antibiotics

By using 0.5 McFarland standards, the broth inoculums has been standardised by diluting technique with sterile Trypticase Soy Broth (TSB) and the optical density measurement at 600 nm wave length required for this step. Absorbance reading was fixed to be within the range of 0.08 to 0.13 (equivalent to approximately 1.5 x 108 CFU/mL).

The plant extracts were prepared at a concentration of 0.005g dissolved in 5mL Dimethyl Sulphoxide (DMSO) and it was stored to be used in the next step. The DMSO acts as a negative control. Meanwhile, for Ampicilin, it was prepared by added 1g of Ampicillin with 20mL distilled water. The Ampicillin stock is stored in freezer with

temperature -18^{o}C as it is a bit light sensitive. Ampicillin is act as positive control in this testing.

The standardised broth inoculum has been swabbed on Trypticase Soy Agar (TSA) by using a sterile cotton swab. 10µL of the solvent extract which are ethanol and methanol were impregnated into a standard empty antibiotic disc (6mm diameter). The 6mm sterile paper disc was loaded with Ampicilin as a positive control and DMSO which act as negative control, and with different concentration plants extract of 20µL, µL and 30µL. Then the disc was inoculated on the TSA plates one by one. Agar plate was incubated at 37 ºC for 24 hours(Omokhua et al., 2017). The inhibition zone form was measured by using the calliper. The test was performed in triplicates, and the average was measured.

3.2(h) Phytochemical screening by High- Performance Liquid Chromatography (HPLC)

By using high-performance liquid chromatography (HPLC) with UV-vis diode array detector (DAD), the content of phenol and flavonoid was analysed. The mobile phase used for this analysis was methanol and 1% acetic acid with the ratio of 70:10(Hanphanphoom & Krajangsang, 2016). The volume of injection was 2 µl for the flavonoid screening and 10 µl for phenolic screening. The flowrate used was 0.800mL/min at the wavelength 280-360nm, and the maximum temperature was 40ºC (Al-shaalan, 2014). For standard HPLC, Gallic acid was used for phenolic contents, and quercetin for flavonoid contents.

3.2(i) Identification of organic molecular groups and compounds by using Fourier-transform infrared spectroscopy (FTIR)

Functional groups of *C.odorata* were obtained using the Fourier-transform infrared spectroscopy (FTIR) test. Infrared spectrum was obtained through infrared solution software which presented information in transmittance mode. The results obtained were in range of $400cm^{-1}$ to $4000cm^{-1}$ for both extracted and not extracted samples. Based on the results obtained, the functional groups and behaviour of the samples were classified and discussed.

3.2(j) Analytical data

The measurement of inhibition zones and absorbance readings were obtained from disc diffusion method, and HPLC analysis.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Organoleptic evaluation of *Chromolaena odorata*.

The evaluation of the pharmacognostic character of the medicinal plant *C. odorata* was performed to test their sensory parameters (Table 4.1).

Table 4.1: Organoleptic evaluation of *C.dorata*

Parameter	***C.odorata***
Colour	Green
Odour	Raw, Pungent when crushed
Appearance	Dark green when powdered
Taste	Bitter

4.2 Disc diffusion assay

In this study, the different part of *Chromolaena odorata (C.odorata)* plants which are leaves, stems and roots have been extracted by using two different solvents which are methanol and ethanol against the gram-positive bacteria and gram-negative bacteria. The antimicrobial test was done by using disc diffusion method on agar plates with two different types of human bacteria which are *Staphylococcus aureus* and *Escherichia coli*. The *C.odorata* plants were tested using disc diffusion assay which by measuring the diameter of inhibition zone (mm). Clearing zone indicates that incubation after incubated at 37^{o}C for 24 hours. Results obtained in the present study relieved that *C.odorata* possess potential antimicrobial activity. The diameter of inhibition zone was tabulated in the table 4.2.

Table 4.2: Zone of inhibition of different parts of *C.odorata* plant which are leaves, stems and roots with methanol and ethanol extracts against *Staphylococcus aureus*

Parts of *C.odorata* plant	**Zone of inhibition (mm)**			
	Sample extracts		Control	
	Ethanol	Methanol	Negative (DMSO)	Positive (Ampicillin)
Leaves	10.00±0.50	13.00±0.50	6.00±0.00	15.00
Stems	NI	NI	6.00±0.00	15.00
Roots	NI	NI	6.00±0.00	15.00

"NI" = No Inhibition

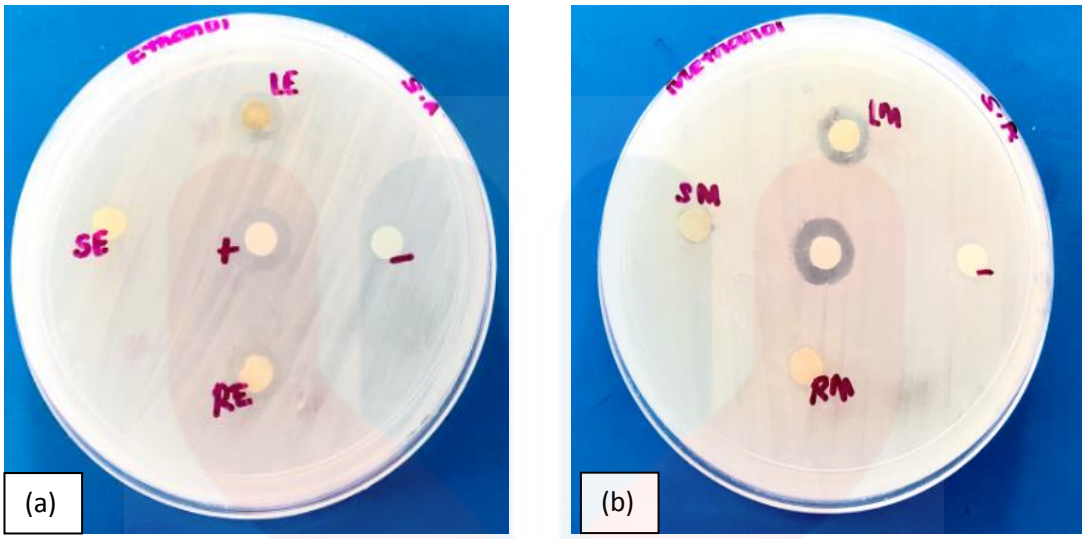


Figure 4.2: Zone of inhibition of different parts of *C.odorata* plant which are leaves, stems and roots with ethanol extract (a) and methanol extracts (b) against *Staphylococcus Aureus*

Table 4.3: Zone of inhibition of different parts of *C.odorata* plant which are leaves, stems and roots with methanol and ethanol extracts against *Escherichia coli*

Parts of *C.odorata* plant	**Zone of inhibition (mm)**			
	Sample extracts		Control	
	Ethanol	Methanol	Negative (DMSO)	Positive (Ampicillin)
Leaves	10.00±0.50	12.00±0.50	6.00±0.00	13.00
Stems	NI	NI	6.00±0.00	13.00
Roots	NI	NI	6.00±0.00	13.00

"NI" = No Inhibition

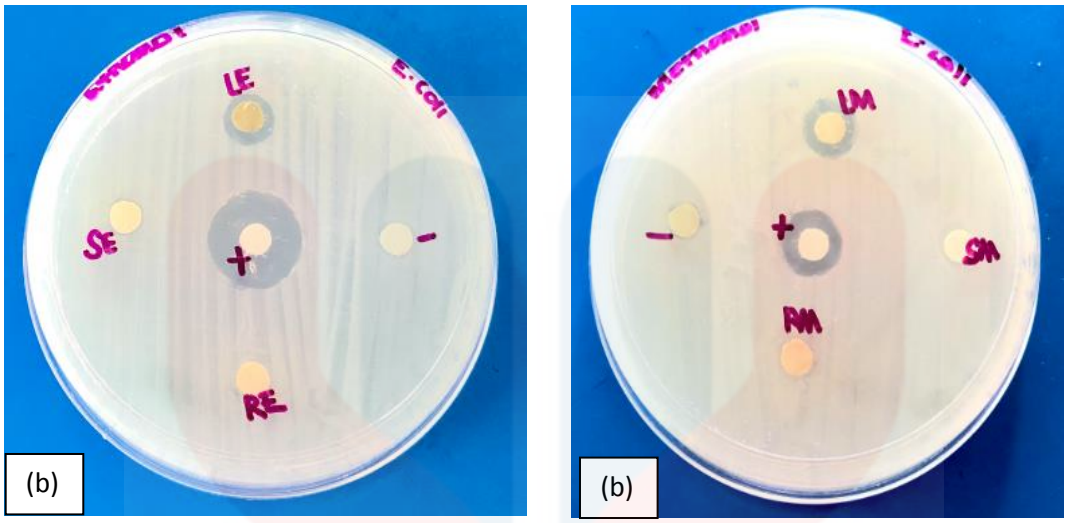


Figure 4.3: Zone of inhibition of different parts of *C.odorata* plant which are leaves, stems and roots with ethanol (a) extracts and methanol extracts (b) against *Escherichia coli*

The diameter of inhibition zone of *C.odorata* with different solvent extract against two bacteria was shown in the Table 4.2 and Table 4.3. *C.odorata* showed inhibition against the bacteria *Staphylococcus aureus* and *Escherichia coli*. The result were similar to the observation that is done by (Hanphanphoom & Krajangsang, 2016) where the plant extracts showed the antimicrobial activity. Furthermore, the results obtained shows that gram-positive bacteria exhibited better antimicrobial activity against gram-negative bacteria. This is due to the outer phospholipid membrane of gram-negative bacteria has lipopolysaccharide (LPS) components that make it impermeable to lipophilic solutes. However, gram-positive bacteria is not effective permeability barrier due to their cell wall only has an outer peptidoglycan layer, which (Vital & Rivera, 2009).

This present results were similar to previous research on the effect of *C. odorata* extracts against pathogens. Leaf extracts demonstrated the best antimicrobial activity, however stem and root extracts has no antimicrobial activity at all. Methanol is the best solvent extraction for antimicrobial activity, and it was followed by ethanol. Most of the antimicrobial activities found in the ethanol and methanol leaf extracts were against

gram-positive bacteria (*B. subtilis*, *B. cereus*, *Staph. aureus*) and gram-negative bacteria (*E. coli*) (Vital & Rivera, 2009; Naidoo et al., 2011; Mondal et al., 2012). The results show that the methanol extract of *C. odorata* showed more inhibitory effect than the other plant extracts. This tends to show that the active ingredients of the plant parts are better extracted with methanol than other solvents. The methanol extracts contain alkaloids, flavonoids, phenolics and tannins (Okemo, 1996). Coumarins, flavonoids, phenolics and tannins have antibacterial and antihelminthic properties, (Eloff, 1998). Cowan (1999) found that methanol was more efficient than acetone in extracting phytochemicals from plant materials.

From the result that was tabulated, Leaf extracts in ethanol and methanol exhibited strong inhibitory effects against *Staph. aureus* with inhibition zones in the ranges of 10.00±0.50mm and 13.00±0.50mm respectively. In gram negative bacteria, the ethanol and methanol leaf extracts affected *E.coli* with inhibition zones ranging from 10.00±0.50 and 12.00±0.50mm respectively. Gram positive bacteria have better antimicrobial activity than gram-negative bacteria like what has been discussed above. Hence, for the ethanolic stem extract and methanolic stem extract, there are no inhibition zone can be observed. The same result goes to ethanolic root extract and methanolic root extract. The effects on antimicrobial activity were associated with the plant part and extraction solvent. The leaf was absorbed by polar organic solvents more than the other plant parts which are stems and roots.

Moreover, methanol is the best extraction solvent for antimicrobial activity and followed by ethanol. In terms of antimicrobial activity, the results show that ethanol and methanol were the most suitable extraction solvents because of their high polar organic solvents that are typically used for extraction of phytochemical compounds from plants. Methanolic leaf extract and ethanolic leaf extracts gave high extraction yields and high

contents of both phenols and flavonoids. Ethanolic and methanolic leaf extracts also exhibited good antimicrobial activity against the gram-positive bacteria *Staph. aureus* and the gram-negative bacteria *E.coli*. The purified compound from methanolic and ethanolic extract of *C.odorata* consistently showed good inhibitory effect on above mentioned bacteria. These results demonstrated that *C.odorata* has the potential for inhibiting the growth of above mentioned bacteria at low concentrations, even though, FTIR spectral data of the purified *C. odorata* compound showed the presence of phenolic groups (Table 4.8).

Table 4.4: Zone of inhibition of different parts of *C.odorata* plant which are leaves, stems and roots with methanol and ethanol extracts with different concentration against *Staphy. aureus*

Parts of *C.odorata*	**Sample extract**	**Concentration (µL)**	**Zone of inhibition (mm)**	**Controls Negative (DMSO) Positive (Ampicillin) (mm)**	
Leaves	Ethanol	10	9.00	Positive	12.00
		20	11.00	Negative	0.00
		30	13.00		
	Methanol	10	8.00	Positive	13.00
		20	9.00	Negative	0.00
		30	10.00		
Stems	Ethanol	10	NI	Positive	12.00
		20	NI	Negative	0.00
		30	NI		
	Methanol	10	NI	Positive	10.00
		20	NI	Negative	0.00
		30	NI		
Roots	Ethanol	10	NI	Positive	12.00
		20	NI	Negative	0.00
		30	NI		
	Methanol	10	NI	Positive	12.00
		20	NI	Negative	0.00
		30	NI		

"NI" = No Inhibition

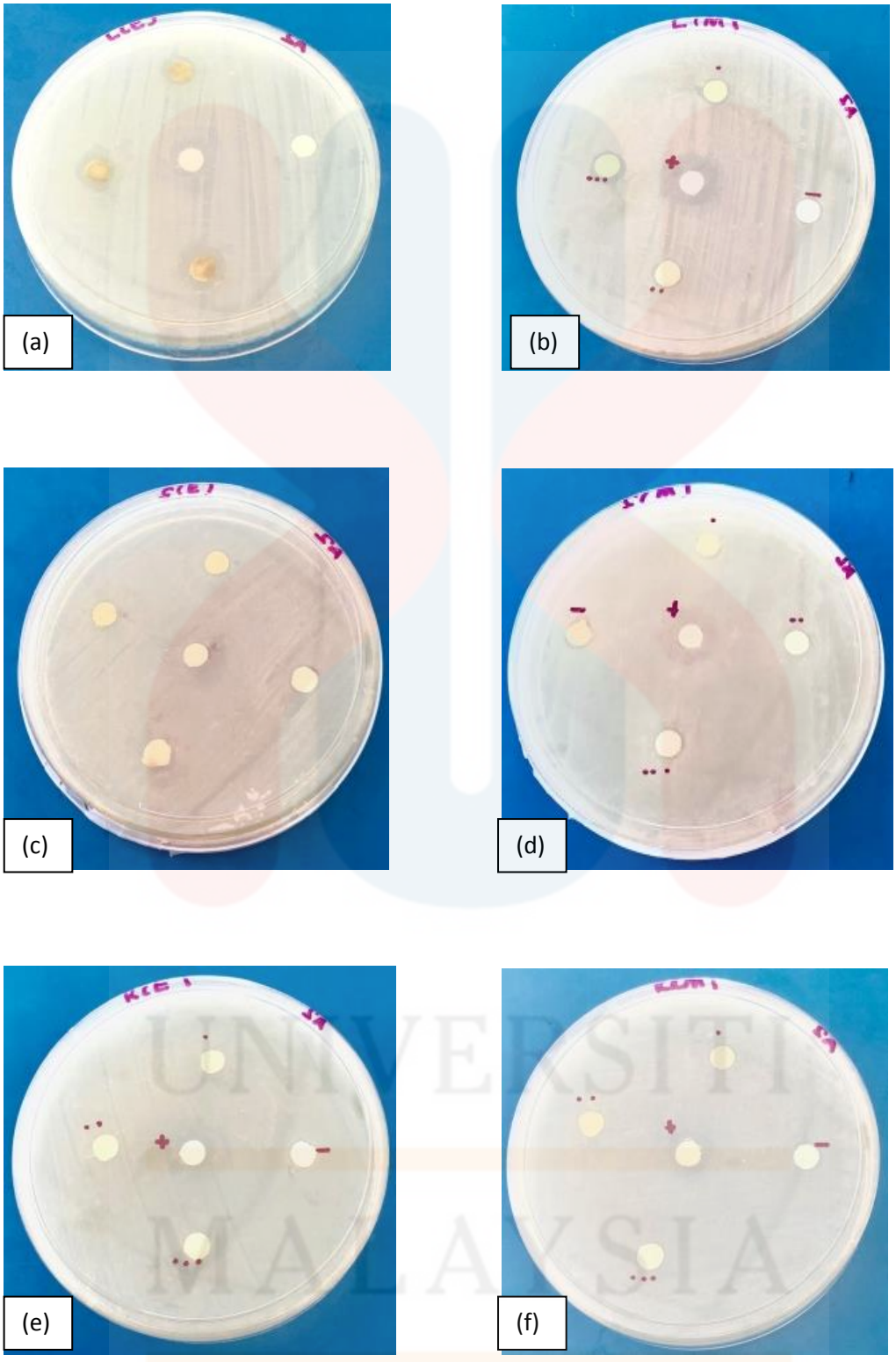


Figure 4.4: Zone of inhibition of different parts of *C.odorata* plant which are ethanolic leaves extract (a), methanolic leaves extract (b), ethanolic stem extract (c), methanolic stem extract (d), ethanolic root extract (e) and methanolic root extract (f) with methanol and ethanol extracts with different concentration against *Staphylococcus Aureus*

From the result tabulated for different concentration (Table 4.4), ethanolic leaves extraction (a) give the highest zone of inhibition which is 9.00mm, 11.00mm and 13.00mm with the concentration of 10µl, 20µl and 30µl respectively. Meanwhile, for methanolic leaves extraction (b), it illustrate the diameter inhibition zone which are 8.00mm, 9.00mm and 10.00mm with the concentration of 10µl, 20µl and 30µl respectively. However, for stem part, the ethanolic stem extract (c) and methanolic stem extract (d) did not show any zone of inhibition and the same result obtained for root part which is ethanolic root extract (e) and methanolic root extract (f) did not shows any zone of inhibition. This might due to lack of phytochemicals content like what had been proved below (Table 4.7).

Table 4.5 Zone of inhibition of different parts of *C.odorata* plant which are leaves, stems and roots with methanol and ethanol extracts with different concentration against *Escherichia coli*

Parts of C.odorata	**Sample extract**	**Concentration (µL)**	**Zone of inhibition**	**Controls**	
				Negative (DMSO)	**Positive (Ampicillin)**
Leaves	Ethanol	10	7.00	Positive	13.00
		20	8.00	Negative	0.00
		30	10.00		
	Methanol	10	8.00	Positive	13.00
		20	10.00	Negative	0.00
		30	13.00		
Stems	Ethanol	10	NI	Positive	12.00
		20	NI	Negative	0.00
		30	NI		
	Methanol	10	NI	Positive	13.00
		20	NI	Negative	0.00
		30	NI		
Roots	Ethanol	10	NI	Positive	12.00
		20	NI	Negative	0.00
		30	NI		
	Methanol	10	NI	Positive	12.00
		20	NI	Negative	0.00
		30	NI		

"NI" = No Inhibition

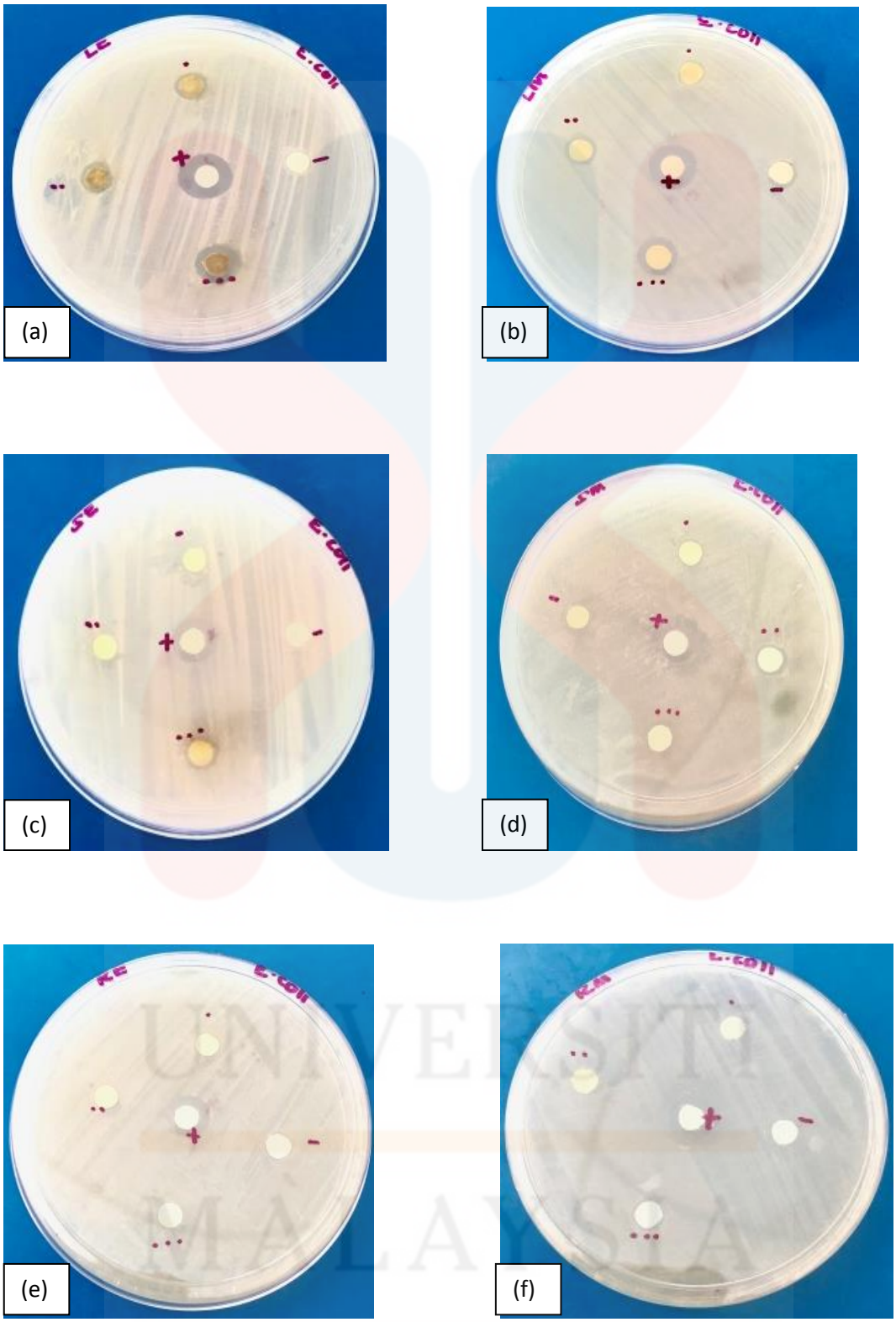


Figure 4.5: Zone of inhibition of different parts of *C.odorata* plant which are ethanolic leaves extract (a), methanolic leaves extract (b), ethanolic stem extract (c), methanolic stem extract (d), ethanolic root extract (e) and methanolic root extract (f) with methanol and ethanol extracts with different concentration against *Escherichia coli*

From table 4.5, methanolic leaves extraction (b) give the highest zone of inhibition which is 8.00mm, 10.00mm and 13.00mm with the concentration of 10µl, 20µl and 30µl respectively. Meanwhile, for ethanolic leaves extraction (a), it illustrate the diameter inhibition zone which are 7.00mm, 8.00mm and 10.00mm with the concentration of 10µl, 20µl and 30µl respectively. However, for stem part, the ethanolic stem extract (c) and methanolic stem extract (d) did not show any zone of inhibition and the same result obtained for root part which is ethanolic root extract (e) and methanolic root extract (f) did not shows any zone of inhibition. This might due to lack of phytochemicals content like what had been proved below (Table 4.7). The results show that the methanol extract of *C. odorata* (b) showed more inhibitory effect than the other plant extracts.

4.3 Content of Phenols and Flavonoids

Evaluation of total phenolic and flavonoid contents was determined for three plant parts which are leaves, stems and root with two different solvents which are ethanol and methanol. Some phenolic content and flavonoid content could be identified by means of chromatographic comparison with standards (HPLC-retention times, UV spectra generated by the diode array detector. For this analysis, Gallic acid was used as standards for phenolic contents, and quercetin for flavonoid contents. The injection volume for Gallic acid is 10µl and for quercetin is 2µl. this testing was run with flowrate of 0.800mL/min at the wavelength 254nm.

Table 4.6: Retention times of each standard component with their UV wavelength

Standard component	Retention time (min)	UV wavelength (nm)
Gallic acid	3.36	254
Quercetin	3.57	254

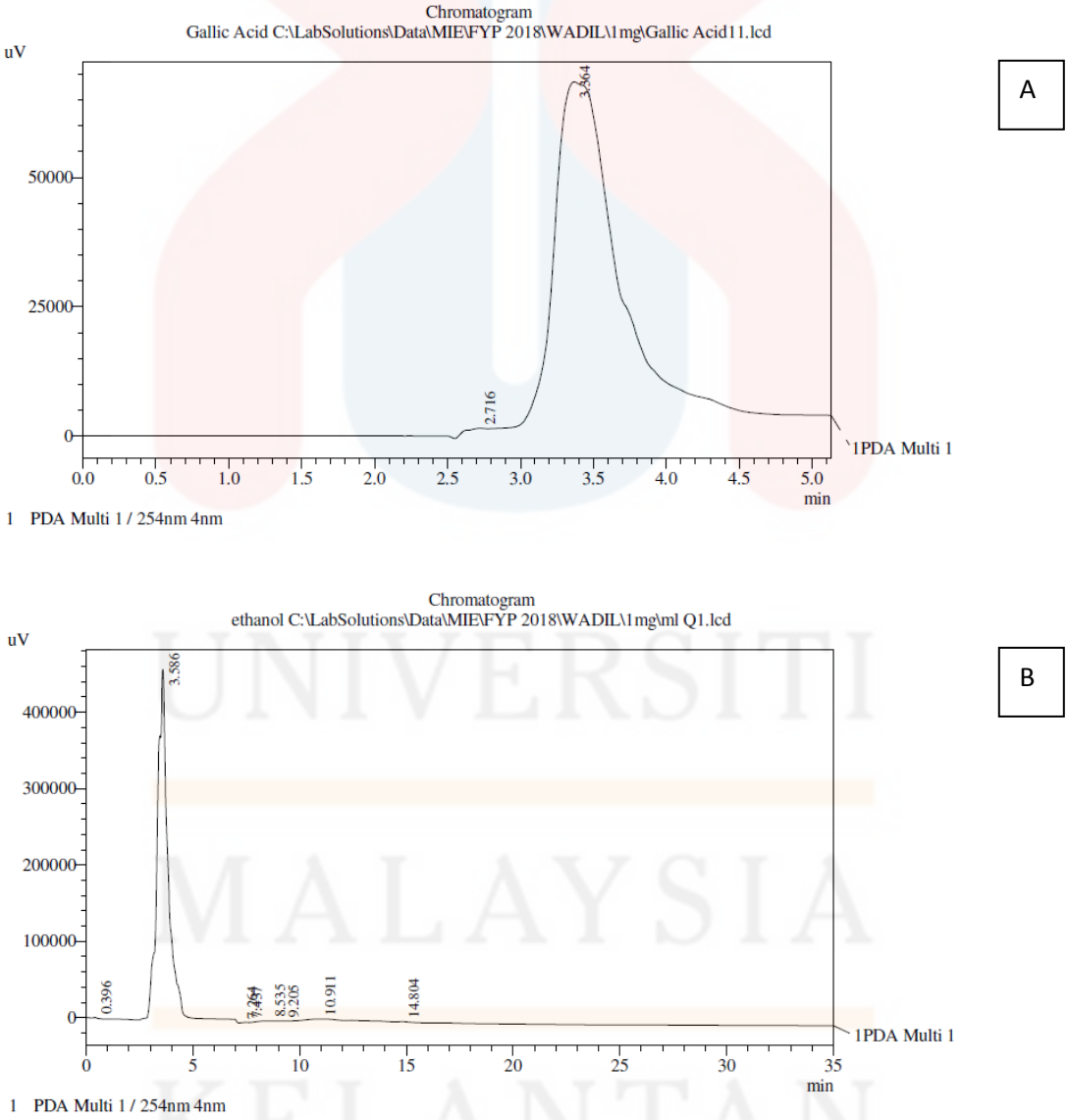


Figure 4.5: Chromatogram of retention time of standard phenolic content which is Gallic acid (A) and standard flavonoid content which is quercetin (B)

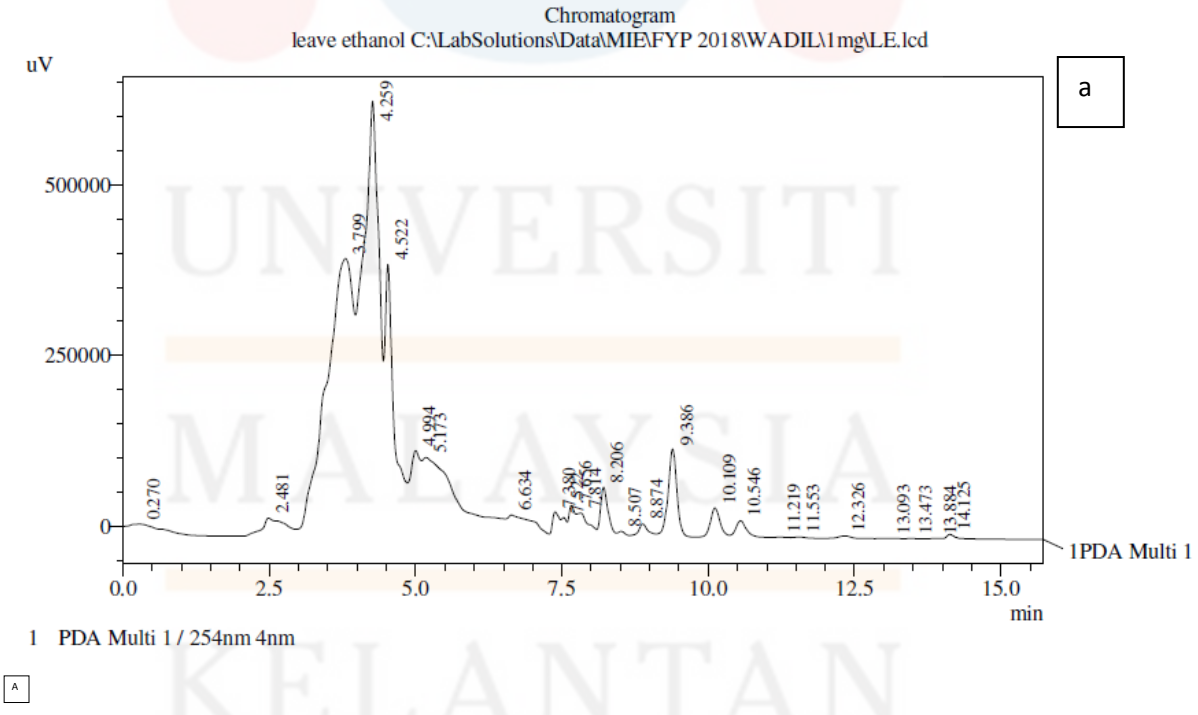
Based on the HPLC using a UV-vis diode array detector (DAD) analysis, the retention time for standard phenolic content which is Gallic acid is at minute 3.36 (A) at the UV wavelength 254nm. The injection volume10µl with flowrate 0.800mL/min. However, for standard flavonoid content, the retention time is at minute 3.57 (B) with the injection volume 2µl. For the result that was tabulated below, the phenolic content for ethanolic leaves extract (a) was detected at retentiom time 3.363 and flavonoid content also detected at retention time 3.565. Next, for methanolic leaves extract (b), phenolic content was detected and flavonoid content also detected. For stem extract, phenolic content was found meanwhile for flavonoid content was not detected in ethanolic stem extract (c). For methanolic stem extract (d), both phytochemicals content did not found. However, for root extract, ethanolic root extract (e) did not show any phytochemicals content while for methanolic root extract (f), phenolic content was detected and flavonoid content also detected at retention time 3.654.

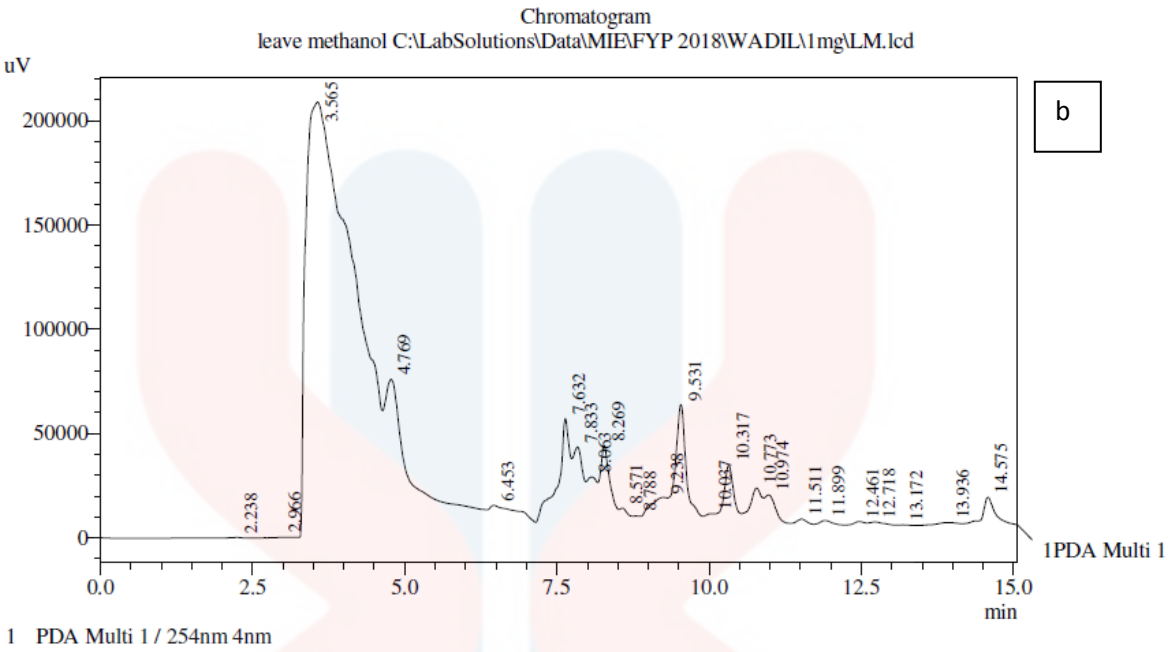
Phenolic contents and flavonoid content were found in almost all previous reports on *C. odorata* leaf extracts. However, Mondal *et al.* (2012) reported on the flavonoid contents found in *C. odorata* leaf extract but not the total phenolic contents. In this present study, only ethanolic leaves extract and methanolic leaves extract showed both phenolic content (gallic acid) and flavonoid content (quercetin) that were detected. But, for stem extract, only ethanolic extract showed the present of phenolic content and methanolic content show the flavonoid content. For root extract, only methanolic root extract showed the present of phenolic content and flavonoid content.

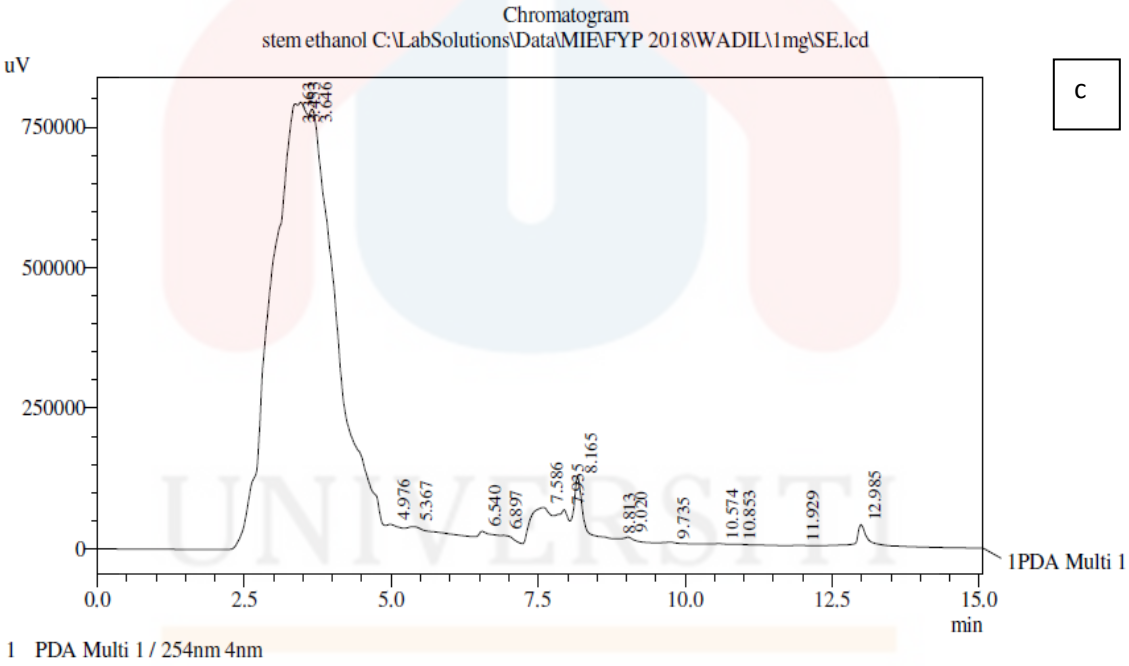
The amount of flavonoid and phenol in the plant extract can directly correlate with its antimicrobial activity. *C.odorata* plant has been used because of their antimicrobial traits, which are due to compounds synthesized as secondary metabolites of plant. This present results indicated that the ethanolic leaves extract and methanolic

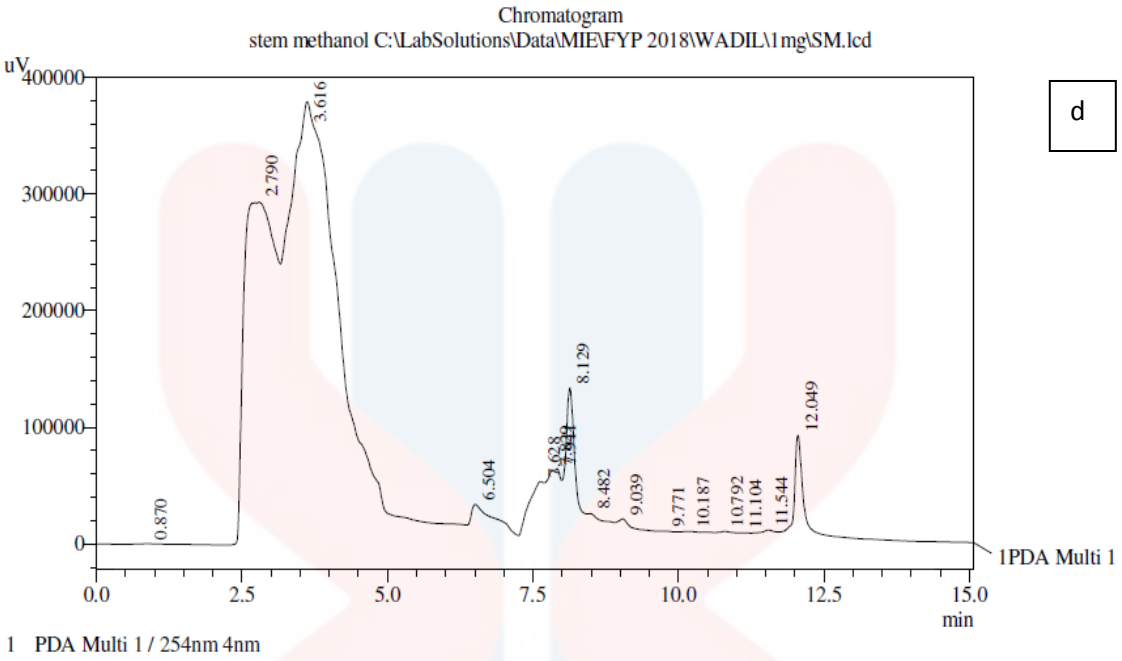
leaves extracts of *C. odorata* showed antimicrobial activity, as they exhibited the phenolic and flavonoid contents (Table 4.7)

Phenolic are the largest group of phytochemicals and have been said to account for most of the antimicrobial and antioxidant activity of plant extracts (Thabrew et al., 1998). The polar secondary compounds from medical plants contain alkaloids, flavonoid and some phenols that show antimicrobial activity (Tiwari et al., 2011, Delahaye et al., 2009). This antimicrobial compound of *C. odorata* is effective in leading the inhibition of cell wall biosynthesis by inhibiting the growth of pathogens by binding to the bacterial cell wall, which is (Anyasor et al., 2011; Lavanya & Brahmaprakash, 2011). Flavonoids are a major group of plant phenolic compounds that act as antioxidant and antimicrobial that is forms a complex with extracellular soluble proteins, bacterial cell wall and also disrupts the cell membrane.









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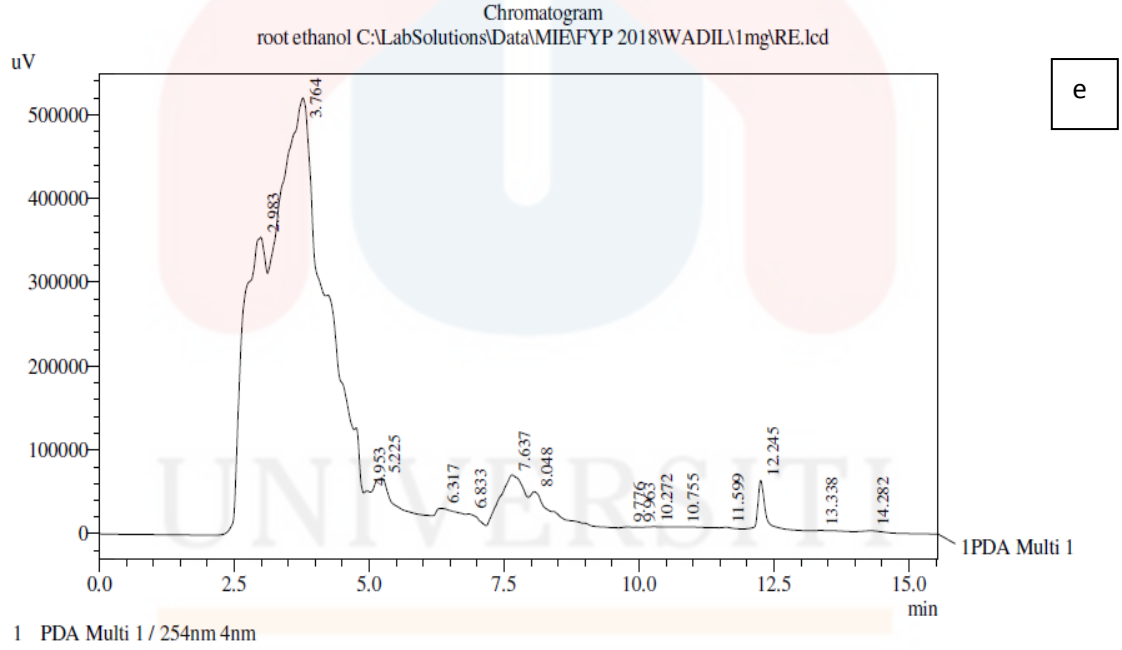

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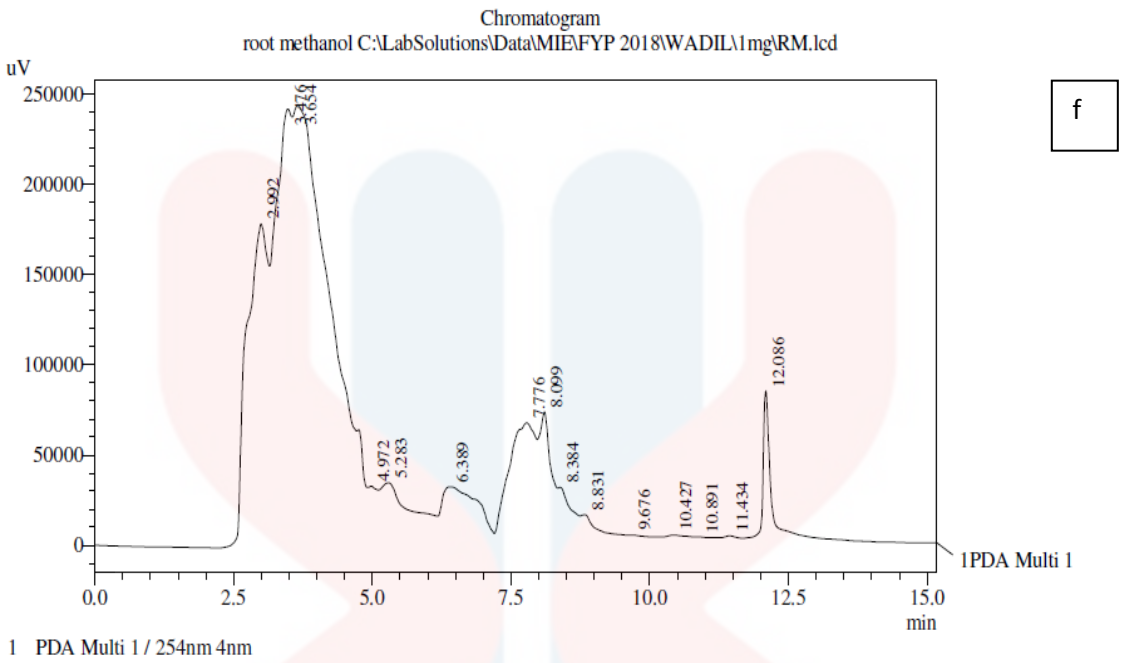

f

Figure 4.6: Chromatogram of retention time of different part of C.odorata which are ethanolic leaves extract (a), methanolic leave extract (b), ethanolic stem extract (c), methanolic stem extract (d), ethanolic root extract (e), methanolic root extract (f)

Table 4.7: Phytochemical analysis of leaves, stems, roots, of C.odorata extracted using different solvents

Plant part	**Solvent**	**Phenol**	**Flavonoid**
		Gallic acid	Quercetin
Leaves	Ethanol	Detected	Detected
	Methanol	Detected	Detected
Stems	Ethanol	Detected	Not Detected
	Methanol	Not Detected	Not Detected
Roots	Ethanol	Not Detected	Not Detected
	Methanol	Detected	Detected

4.4 Fourier transforms infrared spectroscopy (FTIR) Analysis

The Fourier transforms infrared spectroscopy (FTIR) was conducted to study the characteristics of the *C.odorata*, before and after chemical treatment with ethanol and methanol. The FTIR spectra of the untreated, ethanol treated and methanol treated *C.odorata* plant are shown in Figure 4.5 and Figure 4.6 respectively. The broad absorption band at 3335 cm^{-1} - 3852 cm^{-1} which appeared in all spectra were attributed to the H- bonded and O-H stretching structure that mostly contained major functional groups of phenols, alcohols and waters.

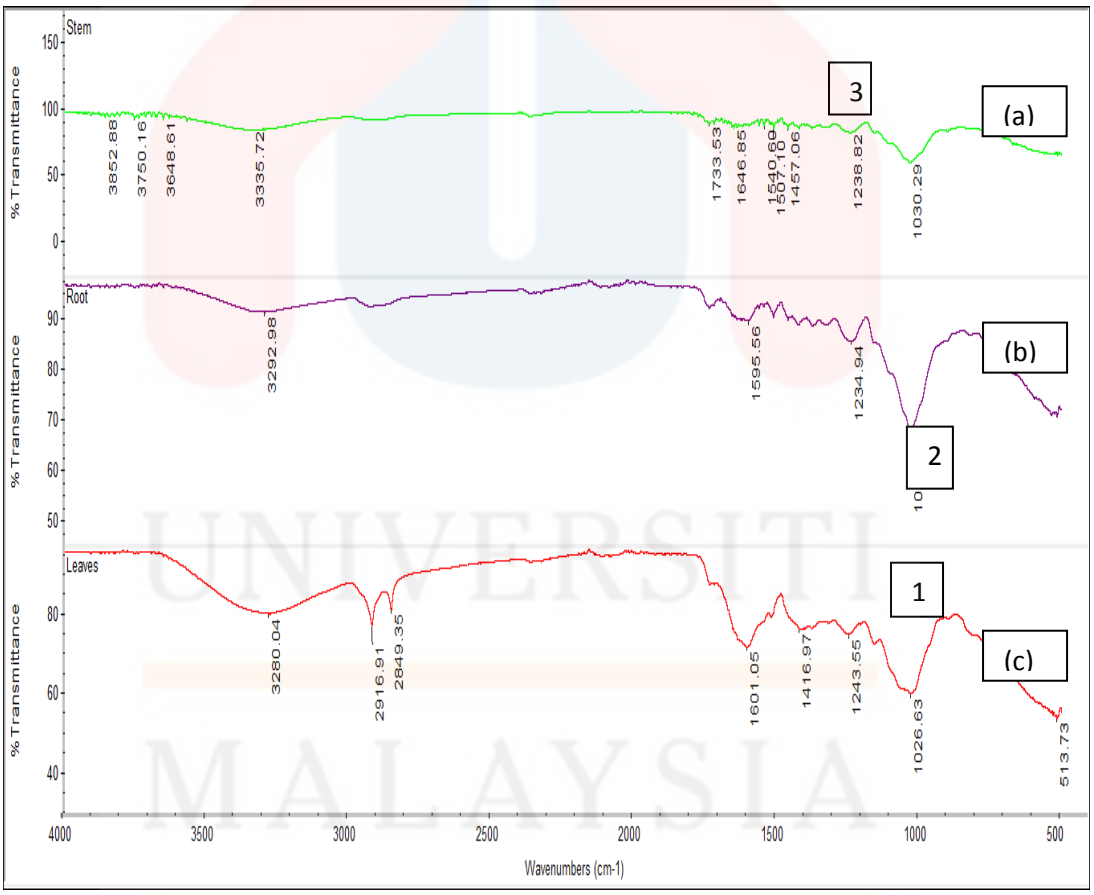

Figure 4.8: FTIR spectra of untreated different parts of *C.odorata* plant which are stems (a), root (b) and leaves (c).

Table 4.8: IR Spectrum peak number, wave number and functional groups of untreated *C.odorata* plant

Peak number	Wave number (cm^{-1})	Functional group
1	1243.55	C-O stretching and –OH deformation vibrations in alcohol and phenol
2	1027.98	Inorganic phosphate
3	1467.06	Aliphatic hydrocarbon

From the IR spectrum, at peak 1200cm^{-1} it illustrate primary aliphatic alcohol. Alcohols are compounds which contain the hydroxyl (-OH) group. These compounds are classified as primary, secondary or tertiary according to the number of other carbon atoms attached to the oxygen bound carbon. Alcohols contain the very polar -OH group. This allows hydrogen bonding between molecules in the condensed phase. Due to this hydrogen bonding the boiling points of alcohols are much higher than the corresponding alkane with the same number of carbon atoms. Hence, from figure 4.5, the peaks labelled (1) which is at 1243.55cm^{-1}, it show that C-O stretching and –OH deformation vibrations in alcohol and phenol. Phenols are a class of aromatic compounds in which one or more hydroxy groups are directly attached to the benzene ring. In addition to phenol, there are several important phenol families including the cresols and xylenols. At peak 1027.98, it shows the functional group of inorganic phosphate. Inorganic phosphates have very characteristic spectra. There are two strong bands at around 1000 cm^{-1} and 550 cm^{-1}. There are also typically water bands around 3400 cm^{-1} and 1640 cm^{-1}. The example spectra were run as nujol mulls. The CH absorptions due to the mulling oil have been blanked for clarity. For the third peak number, the Aliphatic groups are found in many compounds that the infrared spectroscopist at peak 1467.06 which is labelled with (3). The most important vibrational modes are the C-H stretching around

3000 cm^{-1} and the -CH deformation modes around 1460 cm^{-1} and 1380 cm^{-1}. The atoms directly attached to the aliphatic groups may result in significant shifts from the standard frequencies. In particular adjacent atoms with high electro negativity will shift the band locations to higher frequencies.

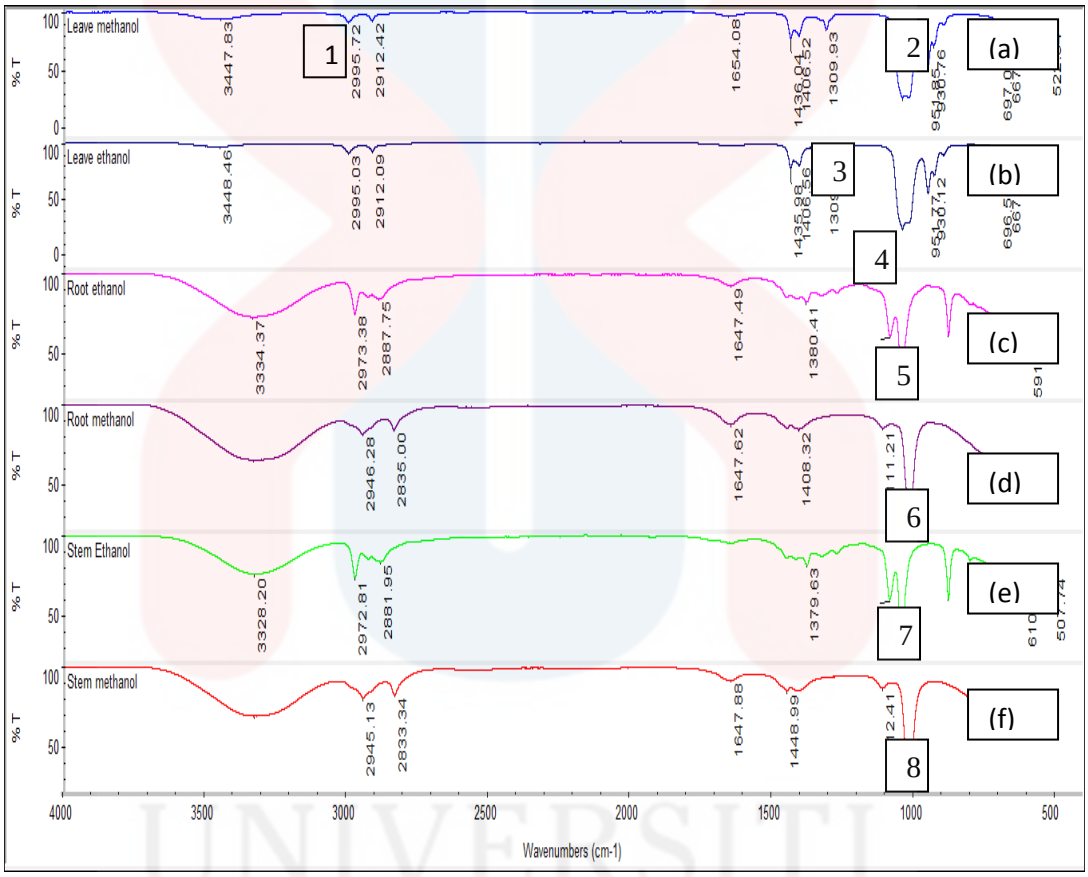

Figure 4.6: FTIR spectra of treated different parts of *C.odorata* plants which are ethanolic leaves extract (a), methanolic leaves extract (b), ethanolic root extract (c), methanolic root extract (d), ethanolic stem extract (e) and methanolic stem extract (f)

Table 4.9: IR Spectrum peak number, wave number and functional groups of treated different part of *C.odorata* plant with methanol and ethanol

Part of *C.odorata*	Solvent	Peak number	Wave number (cm^{-1})	Functional group
Leaves	Ethanol	1	2995.72	Aliphatic group C-H stretching
		2	1309.93	Inorganic phosphate
	Methanol	3	1309.43	Inorganic phosphate
		4	1200.00	C-O stretching and -OH deformation vibrations in alcohol and phenol
Root	Ethanol	5	1050.00	Alcohol functional group -OH stretching frequencies , Free -OH form
	Methanol	6	1015.87	Primary Aliphatic Alcohol
Stem	Ethanol	7	1044.99	Primary aliphatic alcohol
	Methanol	8	1018.82	Aliphatic Sulfur-Oxygen Groups

The small peak at 3852.88 cm^{-1} for untreated *C.odorata* plant and 3448.46 cm^{-1} for treated *C.odorata* plant were attributed to the C-H stretching and O-H stretching bond structure for functional group of alkanes (cellulose and lignin) and carboxylic acids. The small peak in the region of the C-H stretching bond structure includes the functional group of methyl (CH_3), methylene (CH_2) and aliphatic saturated. From the tabulated data, aliphatic group (1) were found in ethanolic leaves extract (a) at wave number 2995.72cm^{-1}. Inorganic phosphate also found at the wave number 1309.93. It showed the same result as untreated leaves of *C.odorata* which the inorganic phosphate also found in the both sample. The example spectra were run as nujol mulls. The CH absorptions due to the mulling oil have been blanked for clarity. For methanolic leaves extract (b), it also found the aliphatic group (3) and at wave number 1200.00cm^{-1} (4),

alcohols functional group was found. Alcohols are compounds which contain the hydroxyl (-OH) group which contain the very polar -OH group. This result illustrates the same functional group that was found in untreated *C.odorata* plant (Table 4.8) which it shows that C-O stretching and –OH deformation vibrations in alcohol and phenol. For root treated with alcohol (c) and methanol extract (d), it showed the functional group of primary aliphatic alcohol labelled with (5) and (6). However for ethanolic stem extract (e), the highest peak is at wave number 1044.99cm^{-1} showed that primary aliphatic alcohol (7). For methanolic stem extract, aliphatic Sulfur-Oxygen Groups was obtained at the wave number 1018.82cm^{-1}.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The antimicrobial assessment of *C.odorata* against the selected pathogen reveals that the phytoconstituents has high antimicrobial property which was compared and confirmed with commercial antimicrobial agents. Since the drug resistance nature of the pyogens increases day by day this herbal remedies will serve as an alternative medicine without side effects. These extracts were used as medicine for pathogenic infection instead of using synthetic antibiotics and drugs. These *C.odorata* plants can be used to discover bioactive natural products that serve as lead for the development of new phytopharmaceuticals.

In comparing the antimicrobial activities of different plant parts and extraction solvents, it was found that the most successful extraction procedure for *C. odorata* leaf was by using ethanol followed by methanol solvents by using Soxhlet extraction method. The ethanolic and methanolic leaf extracts gave high extraction yields and high contents of both total phenols and flavonoids. Ethanolic and methanolic leaf extracts also exhibited good antimicrobial activity against the gram-positive bacteria *Staphylococcus aureus* and the gram-negative bacteria which is *Escherichia coli*. These plant extracts were also more active against gram-positive bacteria than gram-negative

bacteria since it shows the best zone of inhibition towards the gram-positive bacteria. The antimicrobial activity of *C. odorata* extracts was correlated with the amount of both total phenol and flavonoid compounds. The results obtained suggest that the ethanolic and methanolic leaf extracts could be used as a potential natural source for drug development to treat bacterial skin infections.

On this background, the biotechnological potential of *C.odorata* in terms of production of phenolic compound inhibiting both clinical and phytopathogenic bacteria is noteworthy. Results obtained in the present investigation indicated that *C. odoratum* produced a stable phenolic and flavonoid compound with active antimicrobial activity.

5.2 Recommendations

For the antimicrobial activity, a good suggestion for the *C.odorata* extracts to use different concentration of plant extracts, to determine how the concentration of the plants extract will affect the inhibition zone of bacteria. Besides, for the extraction process, despite of using the Soxhlet extractor, we can try other types of extraction for example maceration extraction in order to compare the yield of extraction.

Furthermore, for phytochemical analysis, is good that before the experiment is carry out, it is better to remove the chlorophyll which present in the leaf of plants. This is because, the chlorophyll which present will affect the result of the experiment. Hence, the toxic compounds in the plant could be removed through appropriate extraction and processing methods making extracts and products from the plant safe for the utilization of animal and human. *C.odorata* can be commercialized as effective natural remedies.

The active compound that present in the plant need to be isolated, purified, studied and characterized in order to have in depth knowledge of the activity of the plant extracts against diseases. Besides, the products are necessary to go through the safety test of the herb for pharmaceutical applications.

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