ANTIBACTERIAL ACTIVITY FROM TUBER OF *AMPELOCISSUS CINNAMOMEA MERR* IN THEIR SEQUENTIAL SOLVENT EXTRACT

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DECLARATION

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

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I certify that the report of this final year project entitled "Antibacterial activity from tuber of *Ampelocissus cinnamomea Merr* in their sequential solvent extract" by Tan Huey Yee, matric number F18A0235 has been examined and all the corrections recommended by examiners have been done for the Degree of Bachelor of Applied Science (Food Security) with Honours,

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Antibacterial activity from tuber of *Ampelocissus Cinnamomea Merr* in their sequential solvent extract

ABSTRACT

The current study was carried out to explore and analyze the potential of antibacterial activity in the tuber of *Ampelocissus cinnamomea* by the screening of selected Gram-positive bacteria *viz*., *S. aureus, Methicillin-resistant Staphylococcus aureus, B. cereus, B. subtilis*, and Gram-negative bacteria *viz.*, *K. pheumoniae, E. Coli*, and *Y. enterocolitica* against agar plug diffusion assay. The maceration technique was used to extract the antibacterial agent from the dried tuber of *A. cinnamomea* according to 5 sequence chemical solutions extract according to increasing polarity, which are hexane, chloroform, ethyl acetate, acetone, and methanol. Disc diffusion susceptivity test was used to examine the antibacterial activity of the extract. The results indicated that all used microorganisms were susceptible to the plant extracts. The hexane, chloroform, ethyl acetate, and acetone extracts of *A. cinnamomea* revealed a significant difference ($p < 0.05$) in this study. The result revealed that the extracts exhibit more inhibitory activities towards Gram-negative bacteria compared to Gram-positive bacteria. The highest percentage of the inhibitory potential of extract which showed the most promising antibacterial activity are ethyl acetate, acetone, and methanol as they can inhibit all the tested bacteria used in this study. Furthermore, phytochemical screening was done using preliminary screening of a group of organic compounds on TLC plates developed through a solvent system of ethyl acetate: methanol: petroleum ether with ratio 1:1:1 (v/v/v) sprayed with the respective reagent. Results of the preliminary screening of a group of compounds showed the presence of phenol, alkaloid, flavonoid, anthraquinone, and lactone. Ethyl acetate, acetone, and methanol extract of the tuber of *A. cinnamomea* showed the most diverse group of compounds as these three solvent extracts showed the presence of phenol, flavonoid, alkaloid, anthraquinone, and lactone compounds. The data from this study revealed the prominent antibacterial potential from the tuber of *A. cinnamomea* that can be used for further exploration and development of novel antibacterial from natural sources to improve human health.

Keywords: *Ampelocissus cinnamomea*, bacteria, antibacterial activity, TLC, bioactive compound

Aktiviti antibakteria daripada ubi *Ampelocissus Cinnamomea Merr* dikaji dengan menggunakan ekstrack pelarutan berurutan

ABSTRAK

Kajian semasa dijalankan untuk meneroka dan menganalisis potensi aktiviti antibakteria dalam ubi *Ampelocissus cinnamomea* dengan pemeriksaan bakteria Gram-positif terpilih iaitu *S. aureus, Methicillin-resistant Staphylococcus aureus, B. cereus, B. subtilis*, dan bakteria Gram-negatif iaitu, *K. pheumoniae, E. Coli*, and *Y. enterocolitica* terhadap ujian resapan palam agar. Teknik maserasi digunakan untuk mengekstrak agen antibakteria daripada ubi *A. cinnamomea* mengikut 5 jujukan ekstrak larutan kimia mengikut kekutuban yang semakin meningkat, iaitu hexane, chloroform, ethyl acetate, acetone, dan methanol. Ujian kepekaan resapan cakera telah digunakan untuk mengkaji aktiviti antibakteria ekstrak. Keputusan kajian ini menunjukkan bahawa semua mikroorganisma yang digunakan adalah terdedah terhadap ekstrak ubi *A. cinnamomea*. Dalam kajian ini, ekstrak hexane, chloroform, ethyl acetate, dan acetone daripada *A. cinnamomea* menunjukkan perbezaan yang ketara ($p < 0.05$). Hasilnya menunjukkan bahawa ekstrak menunjukan lebih banyak aktiviti perencatan terhadap bakteria Gram-negatif berbanding bakteria Gram-positif. Potensi perencatan ekstrak yang paling tinggi menunjukkan aktiviti antibakteria yang paling kuat ialah ethyl acetate, acetone, dan methanol kerana ekstrak ini dapat menghalang semua bakteria yang digunakan dalam kajian ini. Tambahan pula, saringan fitokimia dilakukan menggunakan sekumpulan sebatian organik pada plat TLC yang dibangunkan melalui sistem pelarut ethyl acetate: methanol: petroleum ether dengan nisbah 1:1:1 (v/v/v) yang disembur dengan reagen masing-masing. Keputusan saringan fitokimia untuk sekumpulan sebatin organic menunjukkan kehadiran phenol, alkaloid, flavonoid, anthraquinone, and lactone. Ekstrak ethyl acetate, acetone, dan methanol daripada ubi *A. cinnamomea* menunjukkan kumpulan sebatian yang paling banyak kerana ketiga-tiga ekstrak pelarut ini menunjukkan kehadiran sebatian phenol, flavonoid, alkaloid, anthraquinone, dan lactone. Data daripada kajian ini mendedahkan bahawa potensi antibakteria yang ketara daripada ubi *A. cinnamomea* boleh digunakan untuk penerokaan lanjut dan pembangunan antibakteria baru daripada sumber semula jadi untuk meningkatkan kesihatan manusia pada masa hadapan.

Kata kunci: *Ampelocissus cinnamomea*, bakteria, aktiviti antibakteria, TLC, sebatian bioaktif

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LIST OF ABBREVIATION

ANOVA	Analysis of Variance
NA	Nutrient Agar
MHA	Muller-Hilton Agar
TLC	Thin-layer chromatography
DMSO	Dimethyl sulfoxide
S. A.	*Staphylococcus aureus*
MRSA	*Methicillin-resistant Staphylococcus aureus*
B. C.	*Bacillus cereus*
B. S.	*Bacillus Subtilis*
K. P.	*Klebsiella pheumoniae*
E. Coli.	*Escherichia Coli*
Y. E.	*Yersinia enterocolitica*

LIST OF SYMBOLS

mm	Millimeter
mg	Milligram
g	Gram
µL	Microliter
mL	Millilitre
L	Litter
°C	Degree Celsius
%	Percent
-	No inhibition zone
+	Small inhibition zone
++	Medium inhibition zone
+++	Large inhibition zone
±	Plus/ minus
≤	Equal or smaller than
<	Smaller than
µ	Micro
E^o	Solvent strength parameter
v/v/v	Volume per volume per volume

CHAPTER 1

INTRODUCTION

1.1 Research Background

Plant-based antibacterial agents represent a huge unexplored resource for medicines and further research of plant antibacterial potential needs to be explored continuously (Salau & Odeleye, 2007).

Most of the medicinal plants possess antibacterial properties for example aloe vera, garlic (*Allium sativum*), ginseng (*Panax notoginseng*), potato (*Solanum tuberosum*), olive oil (*Olea europaea*), and others (Cowan, 1999). These plants are recognized as one of the promising sources of antibacterial agents in the pharmaceutical industry due to their broad spectrum of structural diversity and their wide range of pharmacological activities. The biologically active compounds that are present in plants are referred to as phytochemicals. Different parts of the plants such as leaves, flowers, barks, roots, seed coat, seed, and pulps cab are derived as the sources of phytochemicals, thereby they will be used as sources of direct medicinal agents. A very large number of secondary metabolic compounds present in the plants was described through phytochemistry.

The tuber of *Ampellocissus cinnamomea* was chosen as the sample in this research to identify its antimicrobial activity by using sequences solution extract. *A. cinnamomea* is still not well-studied in Malaysia. The extract of *A. cinnamomea* was considered to have an antimicrobial agent which can be resistant to or stop the growth of microorganisms as Malaysian being used to consume as a health supplement. Hence, this current study was carried out to examine the antimicrobial activity for the tuber of *A. cinnamomea* which may be commercialized in the future.

1.2 Problem Statement

Nowadays, the problem of antibiotic resistance is becoming one of the major threats affecting human health globally. Antibiotic resistance is a problem that the antibiotic agent loss its function to kill or inhibit bacterial growth. Examples of bacteria that are ineffective to the antibiotic are methicillin-resistant *Staphylococcus aureus* (MRSA), multi-drug-resistant *Mycobacterium tuberculosis* (MDR-TB), and penicillin-resistant *Enterococcus*. These bacteria had immune and become resistant to currently available antibiotics such as rifampicin, isoniazid, and tuberculosis drugs.

Antibacterial is used to control the spread of infection, however, new diseases are kept forming and the new resistant organism are emerging and spreading globally. This issue threatens human safety and causes pressure to treat infectious diseases. Pneumonia, foodborne disease, blood poisoning, gonorrhea, and tuberculosis that are caused by microbial infection led to the issue of antibacterial resistance showing the chance of

recovery from treatment becomes slimmer and impossible. Antibacterial resistance is accelerated by the over usage or misuse of antibiotics.

In these recent years, it had been noticed that synthetic preservation had brought a lot of negative impacts on human health concerns issue. Synthetic drugs will contain different chemical structures compared to the illegal substance that they are trying to imitate. Manufacturers of synthetic drugs constantly change their chemical structures to maintain a leading position in the law. An example of synthetic drugs is synthetic marijuana, it can be 800 times more powerful than its plant-based counterpart. The side effect after consuming synthetic drugs included having suicidal or homicidal behaviour, paranoia, extreme anxiety, hallucinations, seizures, aggression, and feeling chest pain or heart attack. While less serious effects of delusions include euphoria, sweating, inability to speak, and restlessness. Hence, a compound derived from natural posses a big potential in pharmaceutical industries as it is safer and more effective compared to synthetic antibiotics in combating the emergence of multidrug-resistant bacterial.

1.3 Hypothesis

H_0: Extract of *A. cinnamomea* tuber does not have significant different effect on antibacterial activity against selected Gram-positive and Gram-negative bacteria.

H_1: Extract of *A. cinnamomea* tuber have significant different effects on antibacterial activity against selected Gram-positive and Gram-negative bacteria.

1.4 Scop of Study

This study was focused on the antimicrobial activity of the tuber of the local medicinal plant, *A. cinnamomea*. The antibacterial potential of these tuber extracts was evaluated by using 3 selected Gram-negative and 4 Gram-positive bacterial by using the disc diffusion assay method. Furthermore, the qualitative analysis of bioactive compounds in the extracts of *A. cinnamomea* was tested using developed thin-layer chromatography (TLC) plate through the photochemical screening method by spraying the respective reagent to detect the presence of a group of organic compounds.

1.5 Significance of Study

There were many research has been conducted recently about the natural products involved in the root of medicinal plants such as ginger or ginseng which has been proven to possess a significant pharmacological activity. Thus, the current research was conducted as a part of an ongoing experiment to analyze the antibacterial potential of the sequential extract of the dried tuber from *A. cinnamomea*. The extract of the natural sources may contain the bioactive compound which was more efficient and possess fewer side effects on human health. The data from the current study identified the prominent antibacterial potential from the tuber of *A. cinnamomea* can be used for further exploration and development of novel antibacterial from natural sources to improve human health.

1.6 Objective

1. To extract the tuber of *A. cinnamomea* by using the sequential solvent extraction method.
2. To investigate the antibacterial potential of different solvent extracts from *A. cinnamomea* tuber on the selected Gram-negative and Gram-positive bacteria by using disc diffusion assay.
3. To identify the layer of chromatography profile by using all *A. cinnamomea* extracts.

CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal Plants

In Peninsular Malaysia, there are two hundred and thirteen species of plants that have been found and recorded as medicinal plants to the indigenous tribes (Milow, Malek, & Ramli, 2017). Medicinal plants act as a prospective source of antibacterial agents in each of the countries (Alviano & Alviano, 2009). Based on the research of the World Health Organization (WHO), the number of people that depend on using medicinal plants as their daily health care is around 80% of the world's population (Zakaria, Nik Rahimah, Amana, & Roslina, 2019). In developing countries, around 60 to 90% of the population are using plant-derived medicine. The medicinal plant is rich in several phytochemicals which are tannins, alkaloids, flavonoids, terpenoids, and coumarins which have been found in vitro to produce a source of antimicrobial agent. The antibacterial contained in the medicinal plant can be used for treatments of several diseases such as blood pressure, reduce the risk of cancer, and prevent cardiovascular disease (Škrovánková, Mišurcová, & Machů, 2012).

The group of plants that are applied for treatment or to possess pharmacological actions for humans and animals is grouped as medicinal plants. It is no difference in morphology between the normal plants and medicinal plants except the characteristics of certain plants to exhibit medicinal benefits. Malaysia is rich with traditional medicines and herbal plants as Malaysia is acknowledged as the world's oldest rainforest country. Malaysia was ranked 12th in the world and fourth on the list of biodiversity hotspots in Asia which is just located after India, China, and Indonesia. Malaysia possesses an estimated 12,500 species of a seed plant, and there are approximately 1,200 species were reported containing the medicinal value (Aziz, 2003).

Ginseng or *genus Panax* group is the genus of 12 species of medical herbs of the family of Araliaceae. The ginseng plant is a perennial herb with a strong taproot. The aroma of ginseng is sweet. Ginseng had been used for a long time ago to treat illnesses to improve mental performance, learning, memory, and sensory awareness. In terms of pharmacology, the effect of ginseng is non-specific and can function normally regardless of the pathological conditions. Research had suggested that red ginseng extract, crude saponin, and non-water-soluble fractions showing selective antibacterial activity against S. aureus, and non-water-soluble fractions might be used as natural antibacterial agents (Kwak, Jung, Jang, Han, & Yu, 2020).

Antibacterial is normally defined as an antibiotic, which is the main type of antimicrobial agent that is mainly used to stop the infection from the bacteria by against and inhabit bacteria to avoid it growing further. Antibacterial activity is defined as a process for all active principal agents to inhabit and kill the microbe's growth to prevent the formation of microbial colonies and disease occurring (Elmogahzy, 2020). Many well-preserved effectors have introduced antimicrobial activity from reactive nitrogen

species (RNS) and reactive oxygen species (ROS), proteases, and their inhibitors, to antimicrobial peptides (Escoubas et al., 2016).

After the first antibiotic, penicillin was introduced at a large-scale application in the 1940s, the potential of the effect for antibiotic resistance was recognized. Penicillin was a great invention in wartime as this drug is used to treat wounds. Besides, it can reduce the incidence of venereal disease among military personnel as it was particularly effective against notorious bacteria that cause gonorrhea and syphilis. However, first in 1940, the British biochemists, Sir Ernst Boris Chain and Sir Edward Penley Abraham who had discovered an enzyme were able to destroy penicillin. Four years later, the few independent scientists proved that certain bacterial had secreted penicillin-inactivating enzyme (Morier, 2021).

2.2 *Ampelocissus* sp. (Vitaceae)

Ampelocissus sp (*Isi Nyaru*) is a genus of Vitaceae that is normally used in several traditional medicines to treat inflammation in damage of tissue, bruises and after childbirth (Rosniyati, 2018). There are more than 90 species found in Asia, tropical Africa, Central America, and Oceania(Ren & Wen, n.d.). Vitaceae is a group of a grape family of woody plants with unisexual apetalous flowers. The parts that can use for medicine are the leaves, fruits, and tubers. Most of the plants in this Vitaceae family possess some similar characteristics, in which their stamens are opposite to its petals, the leaves are opposite tendril and the fruits are in berry types (Karkamkar, Surekha, Patil, & Misra, 2011).

The genus of Vitaceae, *Ampelocissus* is identified by tendril-associated inflorescences, which is a projecting floral disc with 10 linear marks on its side, and for the young part of the plant will normally possess rusty arachnoid hair. It has around 94 species which are habitually distributed in Malaysia, Africa, and southern Asia, and another 5 species scattered in Central America (Chen & Manchester, 2007).

Isi Nyayu is a type of *Ampelocissus* sp. that commonly can be found in mangrove wetland forest, peat wetland forest, limestone hill forest as well as at the lowland, hill dipterocarp forest, and mixed dipterocarp forest. This type of plant can be found in forest gaps, along forest edges, and other environments with enormous light as they are phytophilous plants. The tubers were grown underground and they were formed in purple colour when in dried form. The antibacterial activity of the Philippines *Ampelocissus* contains several phytochemicals such as alkaloids, flavonoids, fixed oils and fat, tannins, saponins, glycosides, and carbohydrates (Chaudhuri & Ray, 2014).

2.3 *Ampellocissus cinnamomea*

Ampellocissus cinnamomea is commonly named as *ubi kertas, ubi nyaru*, and *ubi spring*. The genus of *A. cinnamomea* belongs to the family of Vitaceae.

Table 2.1: Scientific classification of *A. cinnamomea*.

Kingdom	Plantae
Division	Magnoliophyt
Class	Magnoliopsida
Oder	Vitales
Family	Vitaceae
Genus	*Amplelocissus*
Species	*Cinnamomea* Planch

A. cinnamomea is a type of plant with the characteristics of hairy on the stem, leaf veins on upper surfaces, and at petioles. The hair will be getting lesser with its age getting older. When the plant became mature, the hair is visible on the leaf blade surface. Stem from of *A. cinnamomea* is about 1.0 mm across, leaves up to 3-foliolate, rarely more divided, with terminal leaflets 13.5 – 20 cm × 4 – 6.5 cm, and lateral leaflets 11 – 17 cm × 3.5–5 cm; inflorescence 11 – 17 cm long, each flower having red petals and a green disc (Ng, Lim, Ang, Ong, & Yeo, 2014).

Figure 2.1: The whole plant of the *A. cinnamomea.*

Figure 2.2: The tuber of the *A. cinnamomea*.

In Malaysia, the main distribution of the *A. cinnamomea* plant can be found in Pahang, Selangor, Terengganu, and also Federal Territories of Kuala Lumpur. For this experiment, the sample of *A. cinnamomea* was collected from Air Banun, Gerik, Perak. *A. cinnamomea* is a type of wild plant that contains high medicinal properties in the leaves and tuber parts. *A. cinnamomea* is normally used by women after childbirth for womb healing.

2.4 Medicinal Plants Extraction

Extraction is a common technique always used in organic chemistry to isolate a target compound. Extraction is a way that used to separate the desired substance from another substance that had mixed. From the extraction process, a solute is transferred from one phase to another to separate it from unreacted starting materials or impurities.

2.4.1 Types of Extraction Method

There are 4 types of conventional extraction methods and some modern extraction methods that can be used to extract the medicinal plant. Example of conventional extraction methods is maceration, infusion, percolation, and decoction extraction while the modern extraction method included supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), ultrasonic-assisted extraction (UAE), and microwave-assisted extraction (MAE).

Maceration is one of the techniques originated use in winemaking and nowadays had been adopted and widely used in medicinal plants research. This is a very simple method used for the extraction of thermolabile compounds. The step of extraction through maceration is needed to soak the plant sample that had been transformed into powder form with a solvent and leave it a room temperature for a minimum of 3 days onwards with frequent agitation. The aim of this process is used to break down the cell wall of the plant to release the soluble phytochemicals. In these 3 days, the heat is transferred through convection and conduction (Azwanida, 2015). The low temperature is used in this method and the extracted yield collected is similar to the amount extracted by using microwave-assisted extraction (MAE).

Infusion and decoction have the same principle compared with maceration, both are soaking the plant sample in boiled or cold water. The decoction is only suitable to use extracting the plant with can resistance to heat or is a heat-stable compound, such as root or barks of the plant. Besides, using decoction will extract more oil-soluble compounds compare to the maceration and infusion method. Percolation is more efficient compared to the maceration method as percolation is a continuous process in which the saturated

solvent will constantly be replaced and changed by fresh solvent non-stop. The percolation process is usually done at a moderate rate until the extraction is completed before evaporation to get a concentrated extract.

Supercritical fluid extraction (SFE) involved carbon dioxide (CO_2) to compress them into dense liquid at a critical point. This gas is named supercritical fluid (SF) and also name as dense-gas is a gas with solvating characteristics like liquid. This gas will be pumped into a cylinder containing the material to be extracted. Since temperature and pressure is the main factor to push a substance into its critical region, hence a small change in temperature or pressure near the critical point will cause a very significant change in the density of the supercritical fluid. With this characteristic, it is easy to separate solvent and extract. The solvent properties of CO_2 can be controlled by adjusting the temperature and pressure. The temperature and pressure needed to compress the CO_2 into SF are at 31.1^{o}C above and 7380 kPa (Azwanida, 2015). One of the advantages of SFE is CO_2 will evaporate completely and no solvent remain.

Pressurized liquid extraction (PLE) is known as accelerated solvent extraction. High pressure is needed in the PLE method. The high pressure can maintain the boiling point of the solvent and keep it in the liquid state, this restrung in a high solubility and high diffusion rate between the lipid solvent contained in the solvent and high penetration for solvent in the matrix (Zhang, Lin, & Ye, 2018).

Ultrasonic-assisted extraction (UAE) method involved the application of using ultrasound waves ranging from 20 kHz to 2000 kHz, which is a very high frequency to disrupt the plant cell and increase the surface area between solvent and plant sample to penetrate the solvent easily (Abubakar & Haque, 2020). By using this method, the plant sample has to be dried and ground into powder form. Next, the sample will be mixed with the appropriate extraction solvent and packed into the ultrasonic extractor (Altemimi,

Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017). This method has the benefit of reducing time in extraction time and solvent consumption but the ultrasound energy will cause a negative effect on the active phytochemistry through the formation of free radicals (Azwanida, 2015).

Microwave-assisted extraction (MAE) is a method that combined microwave and traditional solvent extraction. The kinetics of the extraction can increase by heating the solvent and tissue through the microwave. The microwave process will cause evaporation to remove the moisture contained in the plant cell and generate tremendous pressure on the cell wall. The active constituents exuding from the broken cell occur hence the yield of phytoconstituents increases (Altemimi et al., 2017).

2.4.2 Solvent Extraction

Solvent extraction is also known as liquid-liquid extraction. The principle of solvent extraction is when the liquid or solid solute is added to a heterogeneous system of two immiscible liquids, the solute distributes between two liquids. This distribution is governed by Nernst distribution law.

The common solvent used in solvent extraction includes ethyl acetate (8.1%), diethyl ether (6.9%), dichloromethane (1.3%), and chloroform (0.8%) dissolved up to 10% in water. Water also dissolves in organic solvents such as ethyl acetate (3%), diethyl ether (1.4%), dichloromethane (0.25%), and chloroform (0.056%). These solvents that contain oxygen can act as hydrogen bond donors and hydrogen bond acceptors causing these solvents more soluble in water. Higher water solubility reduces the solubility of weakly

polar or non-polar compounds in these solvents ("Extraction (Part 1)," 2013). These solvents are purpose to extract the antioxidant from various plants parts such as leave and seeds. All these solvents contain different polarities. A solvent with similar polarity to the solute will generally dissolve the solute. In order to limit the total number of analogous compounds in the desired yield, multiple solvents can be used sequentially. The sequence of solvent is arranged by its polarity from least polar to the most polar.

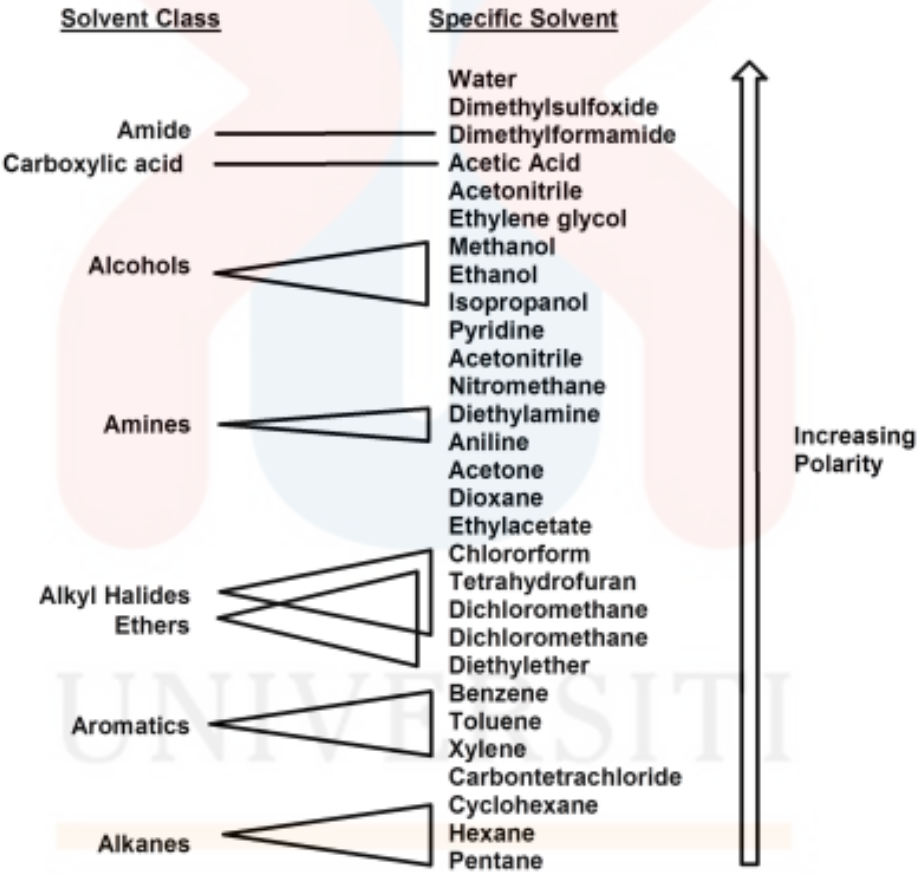


Figure 2.3: Arrangement of solvent polarity.

2.5 Disc Diffusion Assay

Disc diffusion by the Kirby-Bauer method is a standardized technique to test the growth of pathogens. The history of disc diffusion was starting in 1889, agar diffusion method was first used by Martinis Bierock to study the effect of auxins in bacterial growth. In the 1950s, the disc diffusion method was widely used in United States laboratories to determine the susceptibility of bacteria to antimicrobial. But each of the laboratories has its own incubation time, incubation temperature, different media, inoculum concentration, and different concentrations of the antimicrobial agent based on its own needs. Throughout the early 1960s, the lack of standardization to determine bacterial susceptibility become a problem. This method had been improved, refined, and standardized by many scientists' scientific organizations. Kirby and his colleague, A. W. Bauer updated and merge all the descriptions done before by extensively reviewing the susceptibility testing literature using the disk diffusion method and published their research. In 1961, World Health Organization (WHO) publish a report to standardize the procedure for single antimicrobial disc susceptibility testing (Hudzicki, 2009).

2.6 Bioactive Compound

A bioactive compound will affect the live organism, tissue, or cell and it will influence human health. Both plant and animal products possess different bioactive compounds and can be synthesised from both of them. Carotenoids and polyphenols (found in fruits and vegetables), or phytosterols (from oils) are examples of plant bioactive compounds which normally used to be developed. The other example of the bioactive compounds include flavonoids, caffeine, carotenoids, carnitine, choline, phytosterols, polysaccharides, phytoestrogens, polyphenols, anthocyanins prebiotics, and taurine (Golmohamadi, Möller, Powers, & Nindo, 2013; Srivastava & Kulshreshtha, 1989). The table below showed some previous research on the pharmacological study of bioactive extracts from different tuber plants.

Table 2.2: Pharmacological study of bioactive extracts from different tuber plants.

Activities	*Tuber plant*	Part Used	Extract	Compounds	References
Antioxidant	*Ampelocissus* sp.	Tuber	Methanol, n-hexane, chloroform, ethyl acetate	Saponin, alkaloids, terpenoids, and flavonoid	(Rosniyati, 2018)
Antibacterial	*Imperata cylindrica*	Rhizome and roots	Methanol	tocopherol and phenolic	(Savadi, Vazifedoost, Didar, Nematshahi, & Jahed, 2020)
Antibacterial	*Echinops kebericho* Mesfin	Tuber	Ethanol	Phenol, terpenoids, dehydrocostus lactone	(Deyno et al., 2021)
Antibacterial	*Cyclamen persicum*	Tuber	Water, methanol, ethanol, and hexane	Saponin Glycosides and phenolic compound.	(Alkowni, Jodeh, Hussein, & Jaradat, 2018)
Antibacterial	*A. tomentosa*	Roots	Methanol and ethanol	Flavanoids, polyphenols, coumarins	(Dube et al., 2021)
Antibacterial	*Rhoicissus tridentata*	Roots and tubers	Methanol	Phenols, alkaloids, flavonoids, tannins, and saponins	(Dube et al., 2021; Samie, Obi, Bessong, & Namrita, 2005)

2.6.1 Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) is an affinity basic chromatography technique used to separate the compounds of a mixture using a thin stationary phase supported by an aluminium inert backing coated with a thin layer of adsorbent material, silica (SiO_2) or alumina (Al_2O_3) powder. The mobile phase normally consists of a mixture of solvent or volatile organic solvent. The process to separate the volatile component in the extracts is similar to paper chromatography with the advantage of simplicity, better separations, relatively low cost, high sensitivity, running faster, and the choice between different stationary phases. TLC is a highly versatile separation method widely used to monitor chemical reactions of products and is performed on the qualitative and quantitative sample analytical scale of reaction products.

Table 2.3. Chromatographic adsorbents.

Most Strongly Adsorbent	Less Strongly Adsorbent
Alumina (Al_2O_3)	Silica gel (SiO_2)
Charcoal (C)	
Florisil (MgO/SiO_2) (anhydrous)	

The sample will be spotted onto the front line of the TLC plate and placed vertically into a closed chamber with a mobile phase, the best organic solvent have to mix depending on the compound. In the process of separation of organic compounds, one of

the edges of TCL will be immersed in the mobile phase to develop capillary force. The capillary force is used to pull the extracted sample spotted on the front line and travel up to the end line. The speed of traveling depends on the affinities between the stationary and mobile phases (Cheng, Huang, & Shiea, 2011). The degree of polarity of components and mobile phases is critical to understanding and predicting separations. As the time for the solvent travels up to the end of the plate, the components are partitioned into the mobile or stationary phase. If the component is polar, the component will interact with the polar component. Hence, it will move slowly and only move within a very short distance on the TLC plate as the compound is adsorbed strongly. If the component contained in the sample is less polar, the compounds are adsorbed weakly and it will be more soluble in the mobile phase than in the stationary phase, this will cause the traveling time shorter as it moves faster and further on the TLC plate.

The separation of the chemical compounds on a TLC plate is quantified in terms of the value of the retention factor (R_f) (Figure2.4). R_f is defined as the ratio of the distance travelled by the individual components to the total range travelled by the solvent system or mobile phase front. The value of R_f is always between zero and less than one (Sherma, 1996). In general, low polarity compounds have higher R_f values than higher polarity compounds.

$$R_f = \frac{Distance\ travelled\ by\ component}{Distance\ travelled\ by\ solvent\ system}$$

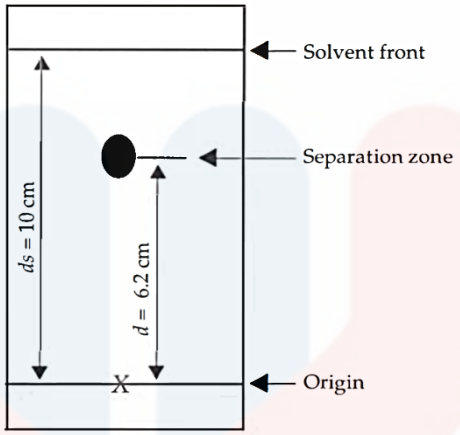


Figure 2.4: TLC plate with the labelled origin and solvent front for calculation of R_f value (Sherma, 1996; Striegel & Hill, 1997).

An illustration of the calculation for the R_f value is shown in Figure 2.4. Comparison of the chromatograms is the way to identify the unknown compound formed on TLC. R_f values facilitate the compound and serve as a guide for the relative migration and sequence of the compound in a mixture. Various factors will affect the value of R_f value. Hence, unknown material is preferred to run with a known material to make a comparison on the same chromatographic plate (Figure 2.5).

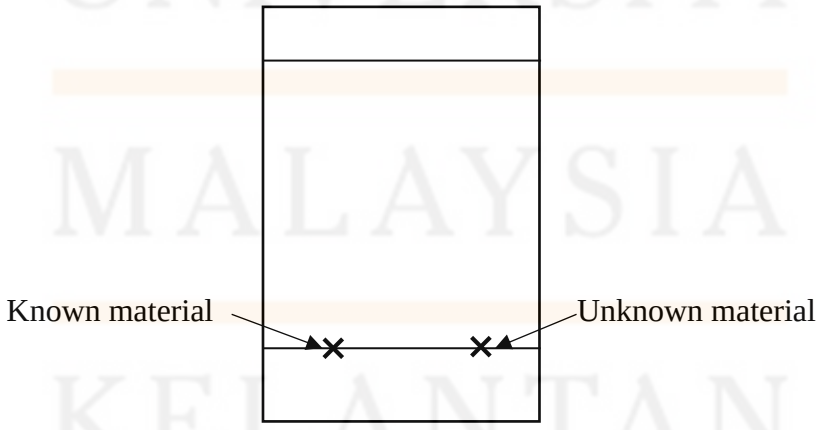


Figure 2.5: Running both known material and unknown material on the same chromatographic plate.

2.6.2 Solvent System

The solvent system is important in the process of separation of the compound in the extract. The solvent system is mixed up with one or more miscible solvents to produce a good solvent mobile phase for TLC. The solvent chosen for the solvent system depends on the equilibrium between the solvent, the solutes, and the sorbent layer of TLC used. Trial and error methods based on similar applications reported in the literature are needed to choose the best solvent system in every experiment (Bruno, Paris, & Svoronos, 1989; Stahly, 1993).

There are several factors needed to consider when selecting the solvent system to get the best separation of the component in the mixture. The selection of mobile phase in solvent system is depended on the nature of the compounds to be separated. The polarity of solvent acts as an important characteristic that affects how fast the compounds travel. A stronger compound needed a solvent that can interact strongly with the sorbent layer of the TLC plate. Cost, volatility, quality, miscibility, availability, and toxicity are the sample for other factors when choosing the solvent system. The functional group of the analyte affects its interaction with the adsorbent layer (Striegel & Hill, 1997). The retention of analytes on silica increases sequentially with the presence of the following functional groups:

RH < ROCH3 < RN-(CH3h < RCOzCH3 < RNHz < ROH < RCONHz < RCOzH.

The technique of rating using solvent strength parameter, E^o can determine the eluting ability reaction between the relative strengths of different solvents on various

adsorbents (Table 2.3). The greater the E^o value, the stronger the reaction between both stationary and mobile phase as the greater E^o value of solvent represents the greater strength ability can displace the original solute. A strong reaction and high E^o value affect the solute's Rf value. Different solvents contain different solvent strengths in each sorbent type. A combination of the elution of solvent series into a binary or ternary mixture of appropriate solvent strengths is needed to prepare a TLC solvent system. In most cases, the power level of a solution from the solvent will be intermediary among the level of power of more than two compounds of the mixture.

The method of using sport test (Bauer, Karin, Gros, & Sauer, 1991) and other simple approaches to the solvent systems are categorized as trial-and-error methods for selecting the solvent system. Hamilton and Hamilton (1987) are the first to start an approach with a low polarity solvent. They mixed with five solvents by adding a more polar solvent in higher proportions accordingly. The compositions volume of the more polar solvents in each mixture were 2%, 4%, 8%, 16%, and 32%. Each increase of the percentage corresponds to an 0.05 unit of the solvent strength parameter in the solvent system. If the R_f value is too high, a solvent system with a lower solvent strength is chosen. If the resulting of R_f value is too low, a solvent system with a higher solvent strength is chosen.

Table 2.4. Solvent strength parameter, E^o (Striegel & Hill, 1997).

Solvent	**E^o**	**Solvent**	**E^o**
Fluoroalkanes	-0.25	Methylene chloride	0.42
n-Pentane	0.00	Ethylene dichloride	0.44
Isooctane	0.01	Methyl ethyl ketone	0.51
Petroleum ether	0.01	1-Nitropropane	0.53
n-Decane	0.04	Triethylamine	0.54
n-Decane	0.04	Acetone	0.56
Cyclopentane	0.05	Dioxane	0.56
1-Pentene	0.08	Tetrahydrofuran	0.57
Carbon disulfide	0.15	Ethyl acetate	0.58
Carbon tetrachloride	0.18	Methyl acetate	0.60
Xylene	0.28	Diethylamine	0.63
i-Propyl ether	0.28	Nitromethane	0.64
i-Propyl chloride	0.29	Acetonitrile	0.65
Toluene	0.29	Pyridine	0.71
n-Propyl chloride	0.30	Dimethyl sulfoxide	0.75
Benzene	0.32	i-Propanol, n-Propanol	0.82
Ethyl bromide	0.35	Ethanol	0.88
Ethyl sulfide	0.38	Methanol	0.95
Chloroform	0.40	Ethylene glycol	1.1

2.6.3 Phytochemical

2.6.3.1 Phenol

The scientific name for *phenol's* simplest number is monohydroxybenzene (C_6H_5OH), or known as benzenol or carbolic acid. Phenol is classified as a hydroxyl (-OH) group in an organic compound attached with a carbon (C) atom to form an aromatic ring. The hydroxyl groups in phenol participate in intermolecular cause it developed stronger hydrogen bonds than alcohols. The ability of phenol enhances the solubility in water and has a higher boiling point compared with alcohol. The colour of phenols is displayed as colourless liquids or white solid at room temperature. High toxicity and causticity will be released from phenols.

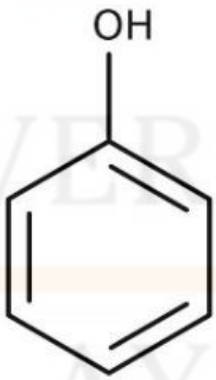


Figure 2.6: The aromatic ring of phenol

Friedlieb Ferdinand Runge was the first person who discovered and extracted phenol in 1834 from impure coal tar. Pure phenol was isolated in 1834 and its structure was proved in 1842 (Hugo, 1978). Phenol is common to be found in nature. Phenolic

compounds are widely distributed in plant foods such as legumes, potatoes, yams, broccoli, and other dark green leafy and brightly-coloured vegetables (Giada, 2013). A plant-based compound containing phenol is defined as an antioxidant. The antioxidant will act as a barrier to stop the reaction of free radicals in our body and prevent affecting health effects.

2.6.3.2 Alkaloid

A naturally occurring organic compound that has nitrogen-containing bases in the structure is classified as an alkaloid. A natural cyclic organic compound containing nitrogen in a negative oxidation state which is of limited distribution among living organisms", defined by Pelletier in 1983 (Verpoorte, 2005). There is one or more than one nitrogen atom with 1°, 2°, or 3° amines, to contribute to the basicity of alkaline. The basicity level of the alkaloid is depend on its functional group location and the structure of the molecule (Nahar & Sarker, 2019). Alkaloids can be divided into different classes, for example, tropanes, pyrrolizidines, indoles, isoquinolines, pyrrolidines, pyridines, quinolines, and terpenoids and steroids. A pure alkaloid is colourless and acts as odourless crystalline solids at room temperature. Alkaloids contain basic properties with a bitter taste. generally insoluble in water but soluble in organic solvents such as alcohol.

Alkaloid is a valuable medical agent that can be found in most plants. There are more than 300 alkaloids are discovered in over different 4000 plant species (Kurek, 2019). Alkaloids are found on the leaves, fruits or seeds, root or even bark of the plant (Grinkevich & Safronich, 1983). The extract from plants containing alkaloids was used

to be medicinally used as drug thousands of years ago. Utilized alkaloids can be used to treat different diseases, for example, cancer, diabetics, cardiac dysfunction, and malaria.

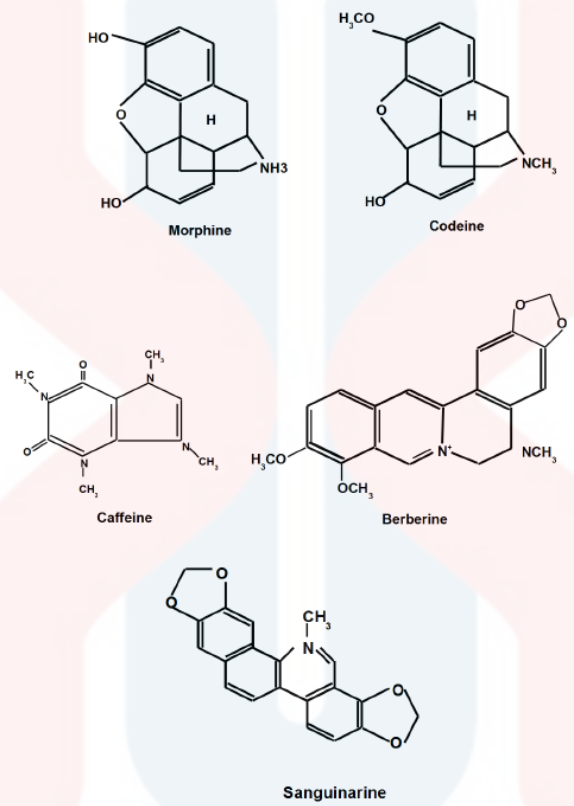


Figure 2.7: The basic structure of alkaloids (Doughari, 2012).

2.6.3.3 Flavonoid

Flavonoids can be found in almost all types of vegetables and fruits as they are the largest diverse group of phytonutrients with more than 6000 types. There is more than one benzene ring on the structure of flavonoids and formed variable phenolic structure. Flavonoids are now widely used in pharmaceutical, medical, nutraceutical, and cosmetic

applications as flavonoids miscellaneous favourable biochemical and antioxidant effects associated with various diseases such as cancer, brain function, Alzheimer's disease (AD), blood sugar, blood pressure regulation, as well as skin protection. The different subgroups of flavonoids are formed by different degrees of unsaturation and oxidation carbon ring, C ring attached on B ring (Fig 2.8) (Panche, Diwan, & Chandra, 2016).

According to Louis Premkumar (2014), the example of natural fruits and vegetables which are rich in flavonoids are strawberries, parsley, onions, citrus fruits, tea, grapes, kale, and Brussels sprouts. Extraction methods are used to collect the flavonoid compound in nature plants. In plants, flavonoids are responsible for the colour and aroma of flowers, attract pollinators in fruits and disperse the fruit consequently assisting in spore germination and the growth and development of seedlings (Griesbach, 2005).

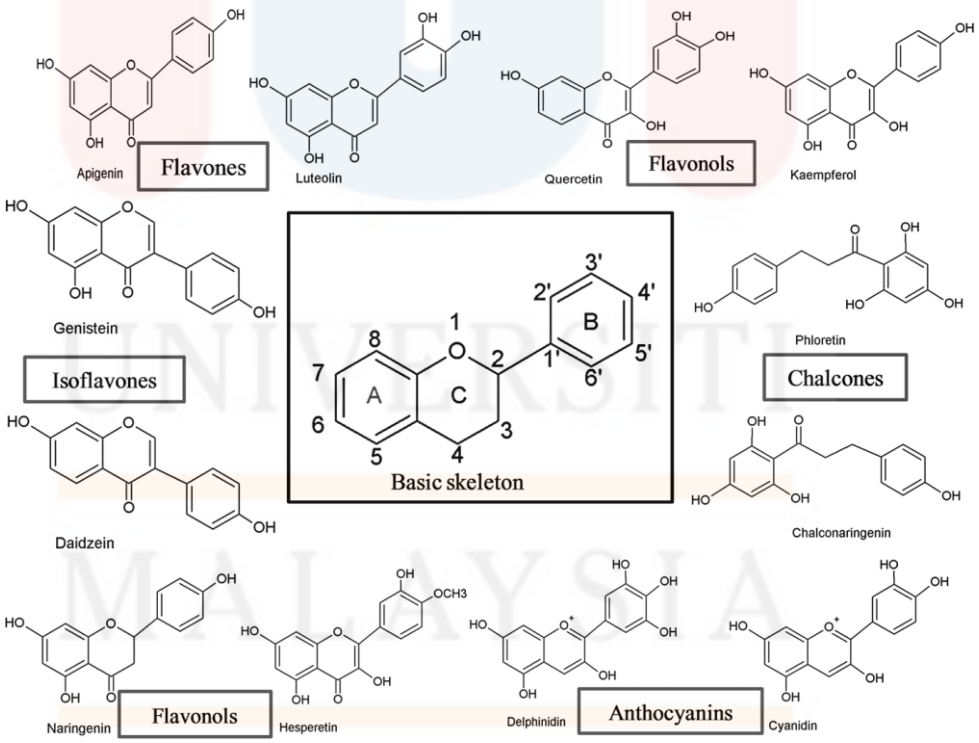
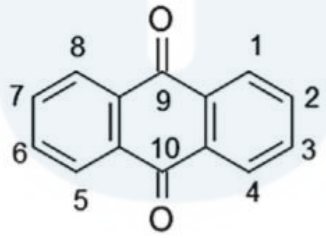


Figure 2.8: Basic skeleton structure of flavonoids and their classes.

2.6.3.4 Anthraquinone

Anthraquinone is a polycyclic aromatic hydrocarbon derived from anthracene or phthalic anhydride (Fig 2.9). Anthraquinone is normally used as a natural dye as these compounds will impart the colours of plants. Derivation of anthraquinone was reported containing anti-viral properties, anti-inflammatory efficacy (Kshirsagar, Panchal, Harle, Nanda, & Shaikh, 2014), and immune booster (Panigrahi, Yadav, Mandal, Tripathi, & Das, 2016). These properties can be demonstrated via the concept of network pharmacology or polypharmacological approach.



Anthraquinone core

Figure 2.9: Anthraquinone core (Simpson & Amos, 2017).

2.6.3.5 Lactone

Lactone is a cyclic carboxylic ester formed by structures attached with 1-oxacycloalkan-2-one structure (−C(=O)−O−), or an unsaturated or heteroatoms replacing one or more carbon atoms on the analogs of the rings, defined by IUPAC (IUPAC, 1997). The major classes of lactones are α-, β-, γ-, δ-, and ω-lactones, which contains 3-, 4-, 5-, 6-, and 7- membrane ring accordingly with different locations attached (Fig 2.10) (Alén, 2018). The biological activity of lactone includes antimicrobial, anti-inflammatory, antitumor, leishmanicidal, and trypanocidal (Sartori, Diaz, & Diaz-Munõz, 2021).

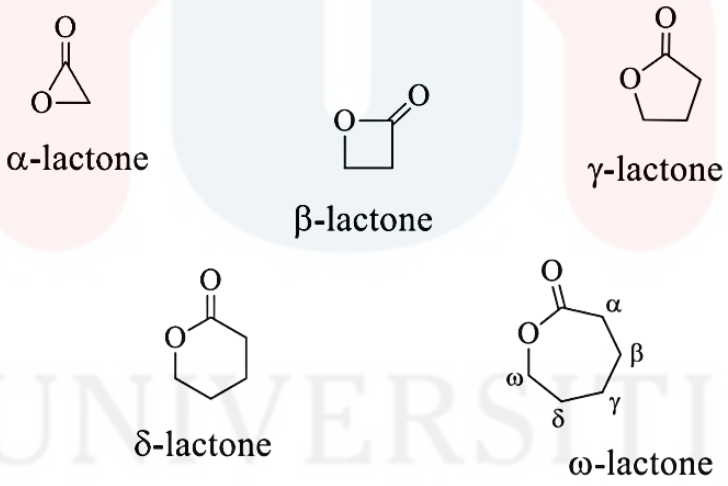


Figure 2.10: Structural representation of α-, β-, γ-, δ-, and ω-lactones (Sartori et al., 2021).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemical and Equipment

3.1.1 Chemical

The chemicals that had been used in this research were hexane (R&M, United Kingdom), chloroform (Fisher Scientific, USA), ethyl acetate (R&M, United Kingdom), acetone (R&M, United Kingdom), methanol (Merck, Germany), and petroleum ether 40 – 60^{o}C (R&M, United Kingdom).

3.1.2 Equipment

The materials that were used in this study included filter paper 240 mm, 20 x 20 cm TLC silica gel 60 F_{254} (Merck, Germany), petri dish, 2 L conical flask, 500 mL media bottle, pipette, universal bottle, forceps, filter funnel, and sprayer. Whereas, the equipment will be used included analytical balance (Mettler Toledo, Switzerland) rotary evaporator (Buchi, Switzerland), grinder, hot plate, oven, desiccator, vortex machine, incubator, and laminar flow.

3.2 Sample

3.2.1 Collection of Plant Sample

The tubers of *A. cinnamomea* sample were collected from Air Banun, Gerik Perak. The plant sample was identified as *Ampelocissus* sp. by FRIM, Kepong in Kuala Lumpur. Besides, the herbarium was further compared to *A. cinnamomea* by Assoc. Prof. Dr. Rahmad Zakaria from University Sains Malaysia Herbarium Collection.

3.2.2 Preparation of Growth Agar Media

The growth media used for the current study were Nutrient Agar (NA) (Oxoid, England) and Mueller Hinton Agar (MHA) (Himedia). 14 g of NA powder and 5 g of agar powder was measured and added into a media bottle containing 500 mL distilled water. For MHA, 17 g of MHA powder was weighed and added into a media bottle containing 500 mL distilled water. Both media were heated and stirred by using a hot plate with 150^{o}C temperature for 10 minutes and continued autoclaving the dissolved mixture for 2 hours to ensure the mixture was fully dissolved and fully sterilized. Once NA and MHA had been autoclaved, allowed it to cool to about 50^{o}C temperature to avoid solidifying. The pouring process of agar media was carried out in the laminar flow to ensure the working environment was sterilized. Poured NA and MHA into each plate and leave plates on the sterile surface until the agar has solidified. Stored all the NA and MHA plates into chiller until the next use.

3.2.3 Preparation of Extracts

An amount of 1 kg of the fresh tuber of *A. cinnamomea* was washed and cleaned under running tap water to remove dirt stuck on the tuber. Next, the skin of the tuber was removed and slid into the thin layer. Dried the slides of tuber in the oven with 40^{o}C of temperature for 3 days until the moisture content in the tuber was dried. The dried tubers were ground into fine powder form. The tuber powder was then kept in a plastic bag and

placed in a desiccator for extraction used to prevent contamination and moisture loss. The tuber powder was extracted using the sequential extraction method following the solvent polarity from low to high, which was started by using hexane and followed by chloroform, ethyl acetate, acetone, and lastly methanol solvent.

An amount of 500 g of the powdered tuber was soaked in 1 L solvent for 72 hours. After that, the mixture was filtered using Whatman filter paper to separate the solvent and biomass. The filtered solvent became concentrated to dryness after using a rotary evaporator and we obtained the extract. The biomass was dried first before proceeding to further extract using successive solvents following similar manners. Extracts obtained from the rotary evaporator were kept in an airtight container at -20°C until further use. The extraction method was conducted according to the method proposed by Chirinos, Rogez, Campos, Pedreschi, and Larondelle (2007)

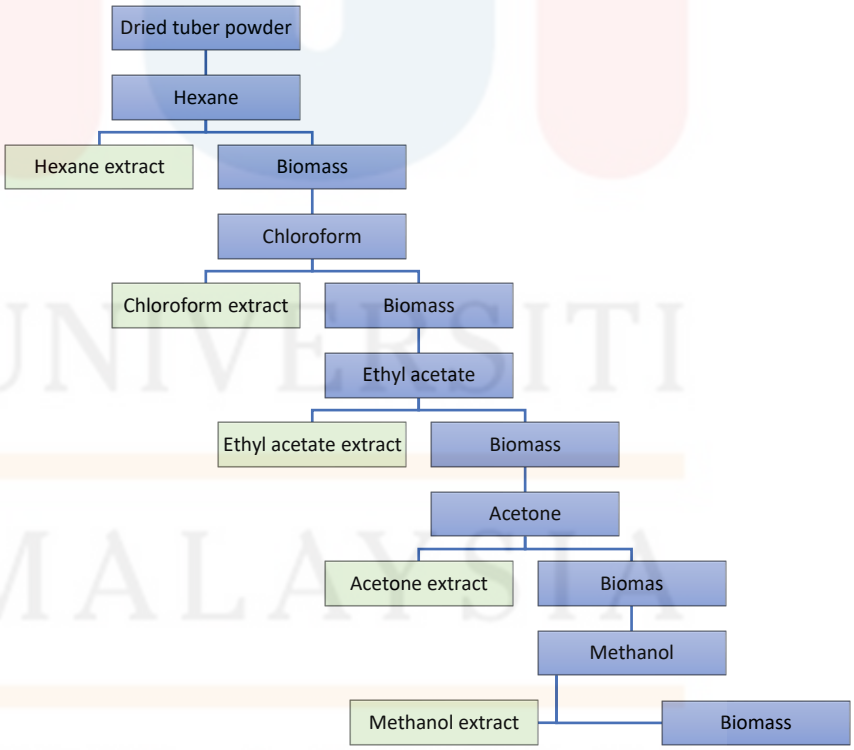


Figure 3.1: Overview of sequential extraction of *A. cinnamomea* tuber by using maceration technique.

3.3 Microorganism and Growth of Bacteria Test

In this research, the antibacterial activity of tuber was investigated using two types of bacteria, viz, four Gram-positive bacterial and three Gram-negative bacterial which provided by Dr. Kharul Azmi Mu'azzam Binti Abdul Rahman in the chemistry lab, University Malaysia Kelantan, Jeli, Malaysia (Table 3.1).

Table 3.1: List of microorganisms used in this study.

Gram-positive bacterial	Gram-negative bacterial
Staphylococcus aureus (S.A.)	*Klebsiella pheumoniae (K.p)*
Methicillin-resistant Staphylococcus aureus (MRSA)	*Escherichia Coli (E. Coli)*
Bacillus cereus (B.C.)	*Yersinia enterocolitica (Y.E.)*
Bacillus Subtilis (B.S.)	

3.4 Preparation of Extraction Solution

A total of 20 mg of crude extracts was dissolved in 0.5 mL of dimethyl sulfoxide (DMSO). Next, a total of 0.5 mL of sterile distilled water was added to the extract after the extract was completely dissolved to yield stock with 20 mg/ mL concentration (the concentration of DMSO in the extract stock solution was 50%). After that, a 0.2 µm pore size of sterile nylon membrane was used to filter the extract solution.

3.5 Test Inoculum and Seeded Agar Plate

Bacterial inoculum was prepared and seeded on agar plates according to the method of Clinical and Laboratory Standards Institute (CLSI) standard M2-A9 (CLSI, 2010). A loopful of pure bacterial colonies were picked from a fresh bacterial culture 24 hours old on Nutrient Agar (NA) (Oxoid, England). Next, suspended in a 5 mL sterile physiological saline (0.85% sodium chloride) solution contained in a universal bottle. The resultant suspension was stirred uniformly by using a vortex machine and the turbidity of the suspension was adjusted visually to meet the 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL). A sterile cotton swab was dipped into the bacterial suspension. The cotton swab was pressed firmly on the inner wall of the universal bottle to ensure all the excess inoculum had been removed from the swab. The surface of Mueller Hinton Agar (MHA) surface was then streaked over by the cotton swab thrice, rotated the MHA with 60^o angle to ensure the bacterial inoculum had distributed uniformly on the surface. After mixing well, the MHA was incubated at 37^oC for 24 hours.

3.6 Preparation of Susceptibility Test

According to the Bauer-Kirby procedure, Whatman No. 1 filter paper with 0.14 mm of thickness has been punched into a 6 mm diameter disc size. The discs were autoclaved for 15 min with 121^oC to ensure the discs were fully sterilized. An amount of 10 µL extract solution (20 mg/mL) was pipetted onto a sterile disc and left to air-dry for

a moment before impregnating the disc with another 10 µL of extract to produce the disc with 0.4 mg of extract and 10% DMSO. The discs were needed to be air dried before placing the disc onto the agar plate which seeded with test microorganisms later.

3.6.1 Disc Diffusion Susceptibility Test (Antibacterial Activity)

The antibacterial activity of several crude extracts was determined by disc diffusion according to Bauer *et al*., (1966) with modifications. The sterile Whatman antibiotic disc was placed on the surface of inoculated medium carefully. The negative control of 10% DMSO was included for solvent effect detection whilst 30 µg per disc (20 µL of 1.5 mg/ mL) chloramphenicol served as a positive control for bacteria. The agar plates were incubated at 37^{o}C for 16 to 18 hours. The diameter of inhibition zones formed around the discs was measured and recorded. The research was carried out in triplicates on separate occasions starting from the initial step until the diameter measurement of the clear inhibition zone.

3.7 Interpretation of Result

The formation zone of inhibition around the agar plugs by bacterial was observed and measured after 24 hours of incubation time. All the measurements were

independently repeated three times. Chloramphenicol (30 μg/mL) was used for bacteria for positive control. The result was recorded as zone inhibition -, +, ++ and +++ .

Table 3.2: Meaning of symbol for inhibition zone.

Symbol	Meaning
-	No inhibition zone
+	Small inhibition zone (≤ 10 mm)
++	Medium inhibition zone (11 to ≤ 20 mm)
+++	Large inhibition zone (≥ 21 mm)

3.8 Preliminary Screening of Bioactive Compounds in the Extract

3.8.1 Thin-Layer Chromatography (TLC)

The extracts of *A. cinnamomea* were subjected to a silica gel plate (20 x 20 cm Silica gel 60G F254 Merck) of thin-layer chromatography (TLC). The plate was cut into 2 cm x 12 cm and lines were drawn across the TLC plate 1.5 cm from the bottom and 0.5 cm from the top using pencil (Fig 3.2). The crude extract contains compounds with different polarities. To achieve the best separation, three solvents were mixed and adjusted to form the best solvent system. The solvent system previously optimized by Farouq (2017) was used which is 1:2:7 of petroleum ether: ethyl acetate: methanol. In this

experiment, a TLC plate was run through a solvent system of petroleum ether: ethyl acetate: methanol with ration 1:1:1 (v/v/v).

TLC plates were activated in the oven at temperature 80ºC for about 30 min. They were ready to be used after totally cooling to room temperature. An amount of 1 mg/mL of the extracts was dissolved using their respective solvent and loaded on the front line of the TLC silica gel plate to form a tiny round sport. The TLC silica gel plate was inserted in a beaker containing the particular solvent systems and covered with a glass lid to avoid evaporating. The solvent system acted as mobile phase whereas silica gel on TLC plate acted as stationary phase, the mobile phase of extract spotted on silica gel was left to run up on TLC plate. TLC plate was taken out immediately when the solvent reached to the end line (0.5 cm from the top). The developed TLC plate was swung for speed drying purposes to further detection.

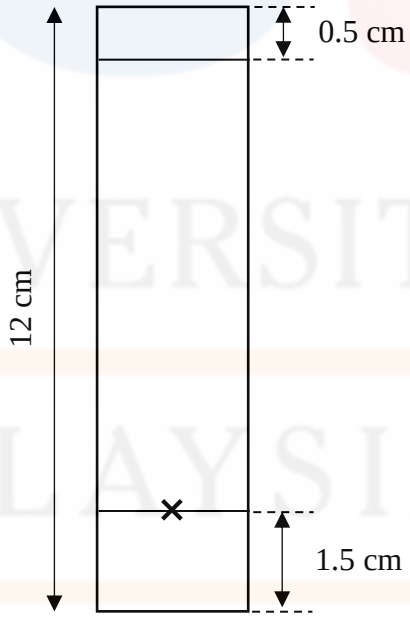


Figure 3.2: TLC plate with the labelled origin and solvent front.

3.8.2 Preliminary Screen of Antibacterial Compound

TLC plats developed through a solvent system of ethyl acetate: methanol: petroleum ether with ratio 1:1:1 (v/v/v) were used in the detection of various structure groups of the compound such as phenol, alkaloid, flavonoid, anthraquinone, and lactone (Table 3.3).

Table 3.3: List of spray reagents used to stain developed TLC plates.

Compound	Reagent
Phenol	1% aqueous ferric chloride ($FeCl_3$)
Alkaloid	Wagner's reagent (iodine and potassium iodide)
Flavonoid	1 M Sodium hydroxide (NaOH) and 1 M hydrochloric acid (HCl)
Anthraquinone	10% methanolic potassium hydroxide (KOH)
Lactone	Iodine vapour

3.8.2.1 Detection of Phenol

Phenol compound was detected by using freshly prepared 1% aqueous ferric chloride ($FeCl_3$). The reagent was prepared by dissolving 1 g of $FeCl_3$ in distilled water.

The developed TLC plate was sprayed with the reagent. An appearance of intense green, purple, blue, or black colours indicated the presence of phenol (Tepal, 2016).

3.8.2.2 Detection of Alkaloid

TLC plate was sprayed with Wagner's reagent, a solution prepared by dissolving 1.27 g of iodine and 2 g of potassium iodide in 5 mL distilled water, afterward top up the reagent with distilled water until 100 mL. The appearance of reddish-brown colour indicated the presence of alkaloid.

3.8.2.3 Detection of Flavonoid

1 M of sodium hydroxide (NaOH) was sprayed on a TLC plate to determine the presence of flavonoids. Flavonoids appear of yellow colour indicating the presence of flavonoids. The yellow colour of the indicator on the TLC plate can be decolorised after the addition of 1 M hydrochloride acid (HCl) (Onwukaeme, Ikuegbvweha, & Asonye, 2007).

3.8.2.4 Detection of Anthraquinone

Anthraquinone compound can be detected using 10% methanolic potassium hydroxide (KOH), a solution of 100 mL methanol, and 10 g KOH. The change of original colour to red, violet, green, or purple showed the presence of anthraquinone.

3.8.2.5 Detection of Lactone

To detect lactone, the developed plate was placed in a closing chamber containing iodine crystals. The appearance of brown sport indicates a positive reaction to lactose compounds.

3.9 Statistic Analysis

All the experiments were independently repeated thrice and the data represent the mean of three replicates ± standard deviation (SD). One-way ANOVA and Post Hoc-Tukey Test statistical analysis available from Statistical Package for the Social Sciences, SPSS (version 22) software packages were used in the data analysis. One-way ANOVA was used to analyze the significant differences between the mean of the inhibitory activity of the extracts whereas the Post Hoc-Tukey test was used as a comparison of the means with the significant different level at 5% ($p < 0.05$).

CHAPTER 4

RESULT AND DISCUSSION

4.1 Sample Extraction

The extraction yield of *A. cinnamomea* powdered tuber was collected through the sequential extraction method. The total extracted weight of yield for sequential solvent hexane, chloroform, ethyl acetate, acetone, and methanol was 0.15 g, 0.19 g, 20.90 g, 39.90 g, and 25.34 g, respectively. Table 4.1 showed the extraction from 500 g *A. cinnamomea* powdered tuber using acetone solvent collected the highest percentage of extracted yield (7.95%) compared to other solvents and the extraction using hexane as solvent collected the least percentage of extracted yield, which is only 0.03% of extract yield from 500 g *A. cinnamomea* powdered tuber.

The colour of the extract for hexane and chloroform is yellow, ethyl acetate extract is in orangish-red colour, while red colour formed for the acetone and methanol solvent extract. The colour of the extracts was changed from lighter to darker colour depending on their increasing polarity (Fig 4.1).

Table 4.1: Extraction yield of *A. cinnamomea* powdered tuber using sequential extraction method.

Solvent	Extract weight (g)	Extract yield (%)	Colour
Hexane	0.15	0.03	Yellow
Chloroform	0.19	0.04	Yellow
Ethyl acetate	20.90	4.18	Orangish red
Acetone	39.90	7.97	Red
Methanol	25.34	5.07	Red

Note: Initial weight of powdered tuber used was 500 g and extracted in 1 L of respective solvent (1:2 ratio).

Figure 4.1: Colour of sequential extraction of *A. cinnamomea* arranged ascendingly according to polarity.

Note: From left hexane, chloroform, ethyl acetate, acetone, and methanol.

4.2 Screening of Antibacterial Activity Determination

In this current research, various extracts of tuber of *A. cinnamomea* were tested against Gram-positive bacteria *viz.*, *S. aureus*, *Methicillin-resistant Staphylococcus aureus*, *B. cereus*, *B. subtilis*, Gram-negative bacteria *viz.*, *K. pheumoniae*, *E. Coli*, and *Y. enterocolitica*. Table 4.2 shows the primary screening result of 7 selected bacterial against agar plug diffusion assay. The screening was conducted by placing 0.6 mm diameter of sterile Whatman antibiotic disc on the surface of inoculated medium carefully. The finding of the present study is in line with the study conducted by Kusuma, Arung, and Kim (2014) who used hexane, ethyl acetate, and ethanol to extract the yield from twelve different plant samples, one of the plant sample, the leaves of *A. cinnamomea* showed inhibitory activity against *Propionibacterium acnes*. While another same finding from the study by Khan et al. (2013) using the extracts from *B. ciliata* (roots), *J. officinale* (leaves), and *S. album* (wood) also demonstrated the potential of antimicrobial activity against human pathogens.

Table 4.2. Primary screening of 7 bacteria reactions against agar plug diffusion assay.

Extract (Primary screening)	Mean of inhibition zone (diameter) against test microorganism						
	Gram-positive				Gram-negative		
	[1]S.A.	[2]MRSA	[3]B.C.	[4]B.S.	[5]K.P.	[6]E.C.	[7]Y.E.
+Ve control	+++	+++	+++	+++	+++	+++	+++
-Ve control	-	-	-	-	-	-	-
Hexane	-	+	+	-	-	+	+
Chloroform	+	-	-	+	+	-	+
Ethyl Acetone	+	++	++	+	++	+	++
Acetone	+	+	++	++	++	+	+
Methanol	+	+	++	++	++	+	++

Notes: [1]***Staphylococcus aureus,*** [2]***Methicillin-resistant Staphylococcus aureus,*** [3]***Bacillus cereus,*** [4]***Bacillus subtilis,*** [5]***Klebsiella pheumoniae,*** [6]***Escherichia Coli,*** [7]***Yersinia enterocolitica***

Indicator: (-) = No inhibition zone, (+) = Inhibition zone ≤ 10 mm, (++) = Inhibition zone 11 to ≤ 20 mm, (+++) = Inhibition zone ≥ 21 mm

Table 4.3: Antibacterial activities of extracts against test bacterial.

Extracts	Mean of inhibition zone diameter against test microorganism (mm ± SD)						
	S. aureus	**MRSA**	***B. cereus***	***B. subtilis***	***K. pneumoniae***	***E. coli***	***Y. enterocolitica***
AC-HEX	-	6.8 ± 0.3	7.2 ± 0.3	-	-	8.7 ± 0.6	6.8 ± 0.3
AC-CLF	6.8 ± 0.3	-	-	9.3 ± 0.6	8.3 ± 0.3	-	7.5 ± 0.5
AC-EAT	7.7 ± 0.6	11.2 ± 0.3	14.3 ± 0.6	9.2 ± 0.3	18.0 ± 1.0	9.7 ± 2.0	12.2 ± 0.3
AC-ACT	10.0 ± 1.0	9.2 ± 0.3	11.5 ± 0.5	12.3 ± 0.6	12.8 ± 0.6	9.7 ± 1.2	9.8 ± 0.3
AC-MEOH	9.3 ± 0.6	9.8 ± 0.3	10.7 ± 0.6	10.7 ± 0.6	10.2 ± 0.3	10.0 ± 1.0	10.7 ± 0.6
Positive control (Chloramphenicol)	32.6 ± 2.5	23.3 ± 3.2	26.7 ± 0.4	27.4 ± 5.8	25.1 ± 0.8	26.0 ± 0.9	25.1 ± 3.3
Negative control	-	-	-	-	-	-	-

Key: - = No inhibition zone, AC-HEX = *A. cinnamomea* hexane extract, AC-CLF = *A. cinnamomea chloroform* extract, AC-EAT = *A. cinnamomea* ethyl acetate extract, AC-ACT = *A. cinnamomea* acetone extract, AC-MEOH = *A. cinnamomea* methanol extract

The result of antimicrobial activity of extracts against several bacteria is presented in Table 4.3. The mean zone of inhibition for bacteria from the extracts of tuber of *A. cinnamomea* ranged between 6.8 ± 0.3 and 18.0 ± 1.0. The highest inhibition zone was formed by the ethyl acetate extract against *K. pneumoniae* with measurement 18.0 ± 1.0 mm. Hexane extract revealed the minimal inhibition activity against MRSA, *B. cereus, E. coli, and Y. enterocolitica* bacterial, followed by 6.8 ± 0.3, 7.2 ± 0.3, 8.7 ± 0.6, and 6.8 ± 0.3 respectively. The extract of hexane, chloroform, ethyl acetate, and acetone from the tuber of *A. cinnamomea* revealed a significant difference ($p < 0.05$) but surprisingly, methanol extract revealed a non-significant difference ($p > 0.05$). A similar situation has happened in the past study from Hashim, Shaari, Mamat, and Ahmad (2016), the 100% methanol extract has not significantly different at 4 and 8 hours extraction in their study. They stated that the reason for the result was due to the long-time extended for extraction which would prime decreasing of phenolic compounds cause of the occurred extending and exposing to environmental factors. For the current study, methanol extract showed a non-significant value might be due to the uncontrollable environmental effect.

The result obtained from primary screening indicated that Gram-negative bacteria were more susceptible compared to Gram-positive bacteria. This result was affected by the different structures of Gram-negative and Gram-positive bacteria. Gram-negative bacteria consist of a three-layer envelope (Fig 4.2). Gram-negative bacteria consist of a thin cell wall enclosed by an outer membrane that has the protective and unique feature formed only in Gram-negative bacteria. Lipopolysaccharides are bounded on the outer leaflet of the membrane and porins are attached to the inner leaflet of the membrane. The second layer of Gram-negative bacteria is the peptidoglycan cell wall. The concentration of peptidoglycan for Gram-negative bacteria is composed of repeating units of the disaccharide N-acetyl glucosamine-N-acetylmuramic acid (Murray, Rosenthal, & Pfaller,

2020) is very thin with aid to act as the rigid exoskeleton to maintain the cell shape. The thin layer of peptidoglycan can't protect the cell break down easily and penetrated inside the cell. The inner layer is the third layer of Gram-negative bacteria which is a multifunctional phospholipid bilayer that plays the role of transport, structure, and biosynthetic function.

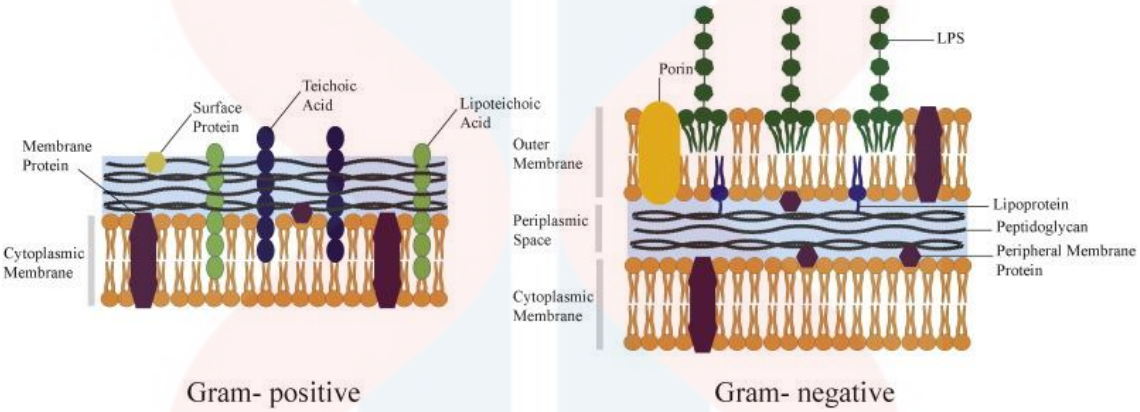


Figure 4.2: Comparison of Gram-positive and Gram-negative bacterial cell membranes.

Gram-positive bacteria lack the outer membrane and have a thicker layer of peptidoglycan surrounded by the plasma membrane to protect the Gram-positive bacteria from the harsh environment (Silhavy, Kahne, & Walker, 2010). The comparison of peptidoglycan thickness between Gram-positive and Gram-negative bacterial become the reason why Gram-positive bacteria are more resistant against the antibacterial and the extracts from *A. cinnamomea* tuber.

The other factors that affected antimicrobial susceptibility included the time for incubating the bacteria, different temperatures, pH value of the medium, and as well as the size of the bacterial inoculum. According to Khaliq, Ghauri, and Akhtar (2013), the best antimicrobial susceptibility was shown on the 5^{th} day of the incubation period, while the antifungal needed to incubate 8 days to get the maximum inhibition zone. The result for the study mentioned has to maintain the constant 5% inoculum size, initial pH of the

medium 7.2, and the temperature-controlled at 30°C. This is because the changes of any other factor will affect the antimicrobial activity. Different inoculum sizes and incubation temperatures can be achieved the maximum antimicrobial activity (Laure, Dawan, & Ahn, 2021; Mu, Chen, Li, Zhang, & Jiang, 2009). Temperature is an essential requirement for bacterial growth. Most of the significant microorganisms grow optimally at temperatures between 25°C to 40°C, these bacteria are named as mesophilic bacteria (Hajdu et al., 2010). If the incubating temperature is lower than 25°C, some of the bacterial might not grow and the result will be affected. Microorganisms have pH growth limits. Most of the microorganisms prefer to live and grow optimally in a natural pH value but most of them will stop growing at a pH of 5.0. Some of the bacteria can grow in an acidic pH value condition. Based on the past research, the lowest growth limit was considered at pH 4.6.

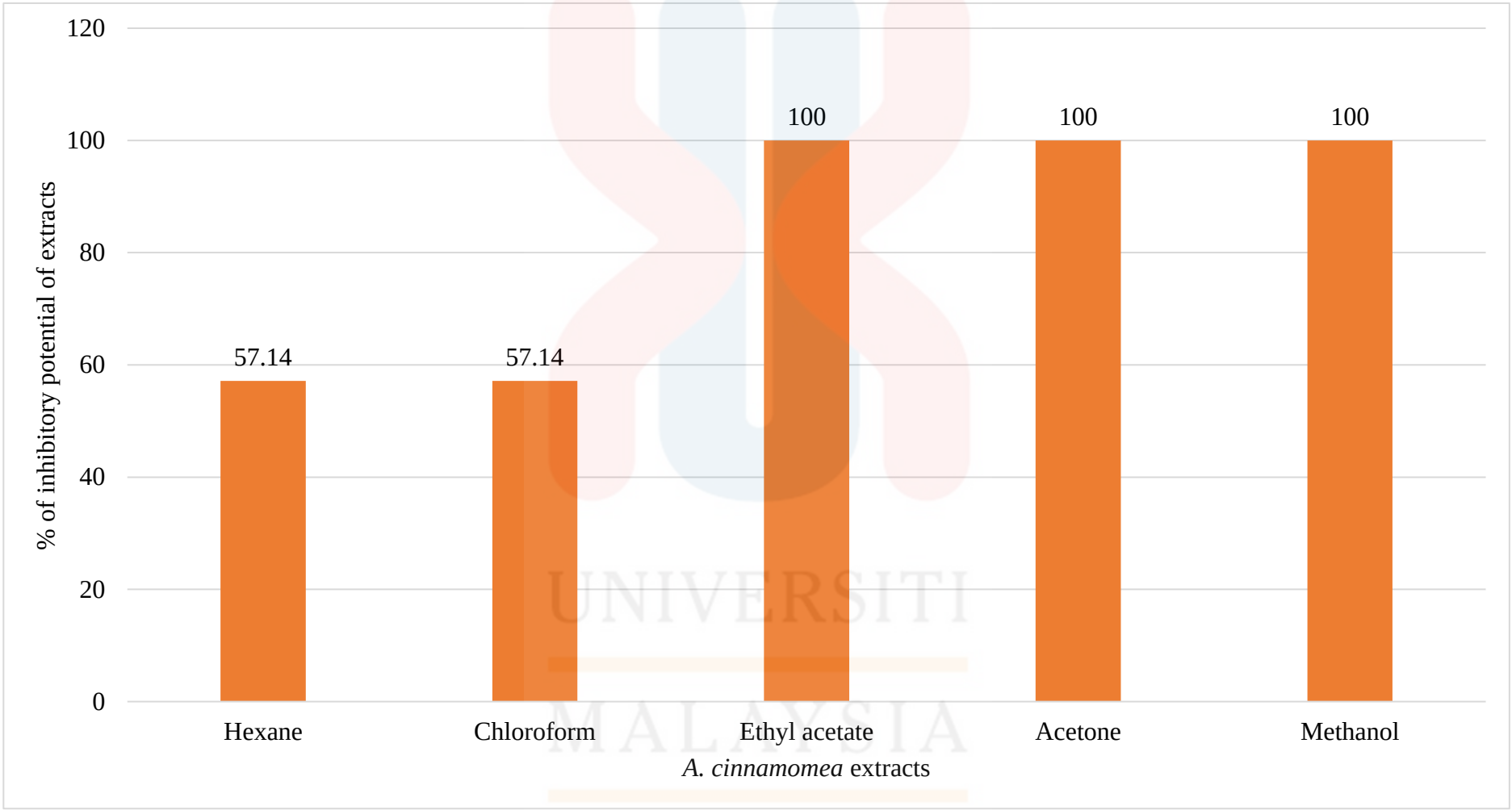


Figure 4.3: The percentage of inhibitory against several *A. cinnamomea* extract**.**

The present research work showed the percentage of inhibitory against a different extract from *A. cinnamomea* in Figure 4.3. Based on the result, we can notice that hexane and chloroform contained the minimum percentage of the inhibitory potential of extract, both of the extracts formed the inhibition zone in 4 out of 7 different bacteria in the current research, which only 57.14% of inhibitory on the bacterial against the extracts. There was a 100% inhibitory for the other three extracts, ethyl acetate, acetone, and methanol. These three extracts successfully get the inhibition zone in all the tested bacterial provided in this research.

Figure 4.4 until 4.22 displayed the inhibition region formed by the *A. cinnamomea* extracts against selected bacteria of interest. The result showed that the mean inhibition zone for Gram-negative bacterial was mostly bigger compared to Gram-positive bacteria. Gram-positive bacteria contain a more solid structure consisting of peptidoglycan. Hence, this directly affected the Gram-negative with a lower percentage of peptidoglycan less resistant to the antibiotics compared to Gram-positive bacterial with a greater percentage of peptidoglycan. Antibacterial activity of the extracts from the dried tuber of *A. cinnamomea* was higher and can easily penetrate through Gram-negative bacteria's cell wall.

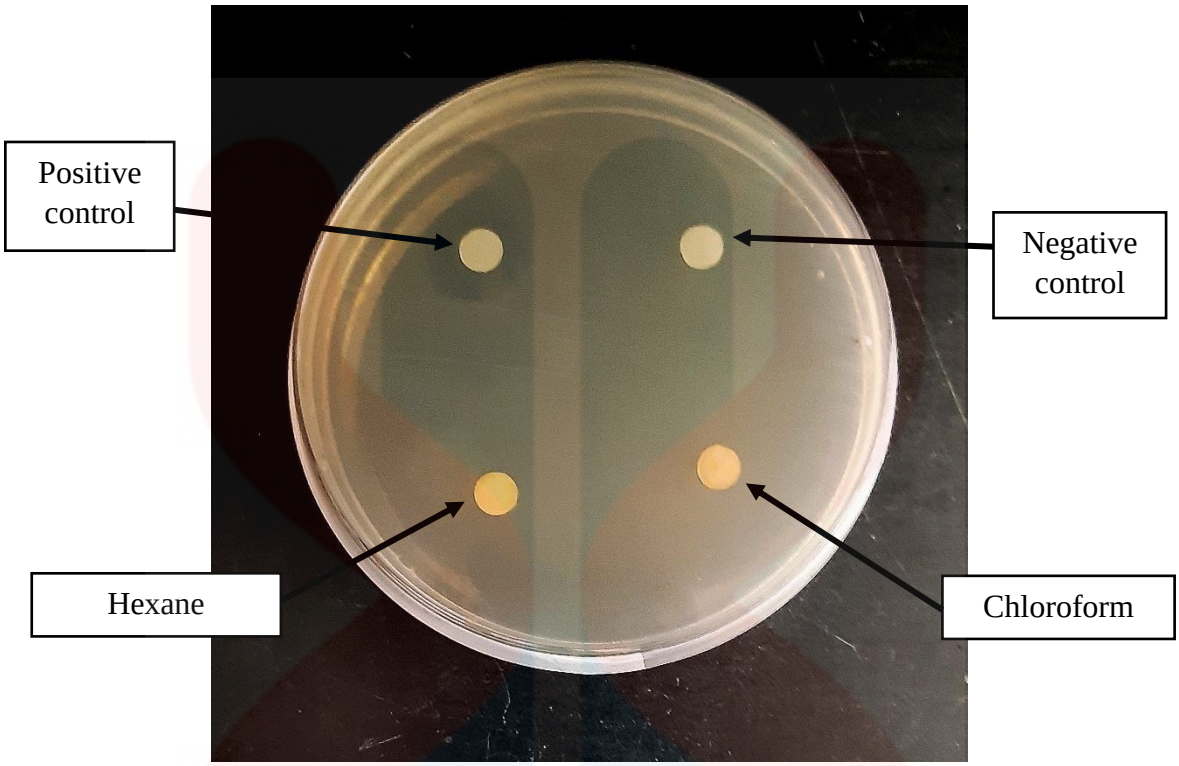


Figure 4.4: Inhibitory zone of the different extracts against *S. aureus*.

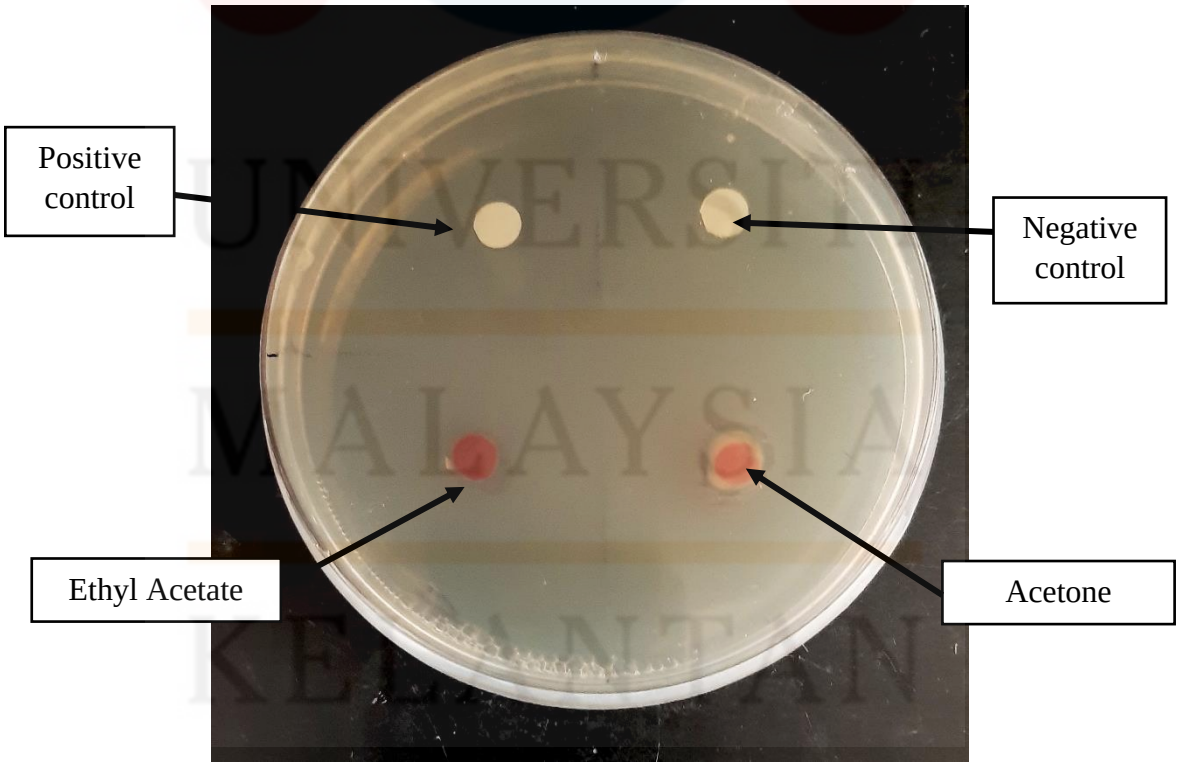


Figure 4.5: Inhibitory zone of the different extracts against *S. aureus*.

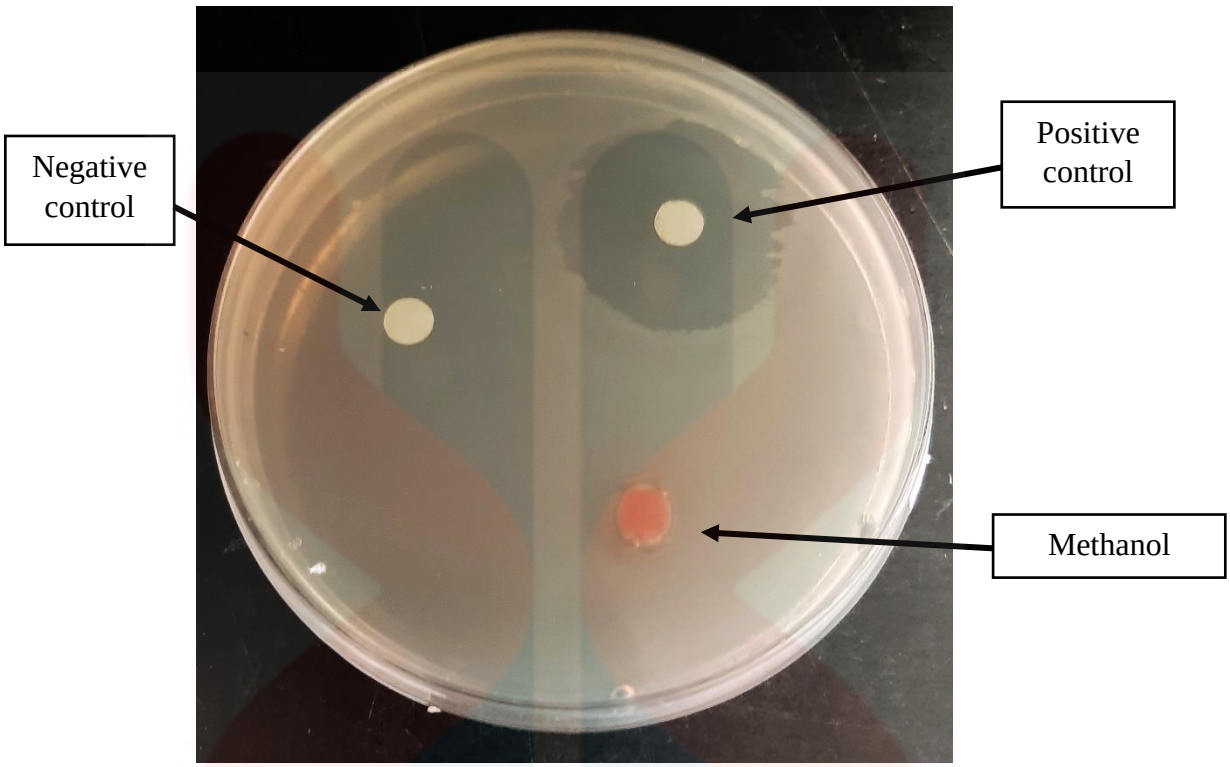


Figure 4.6: Inhibitory zone of the different extracts against *S. aureus*.

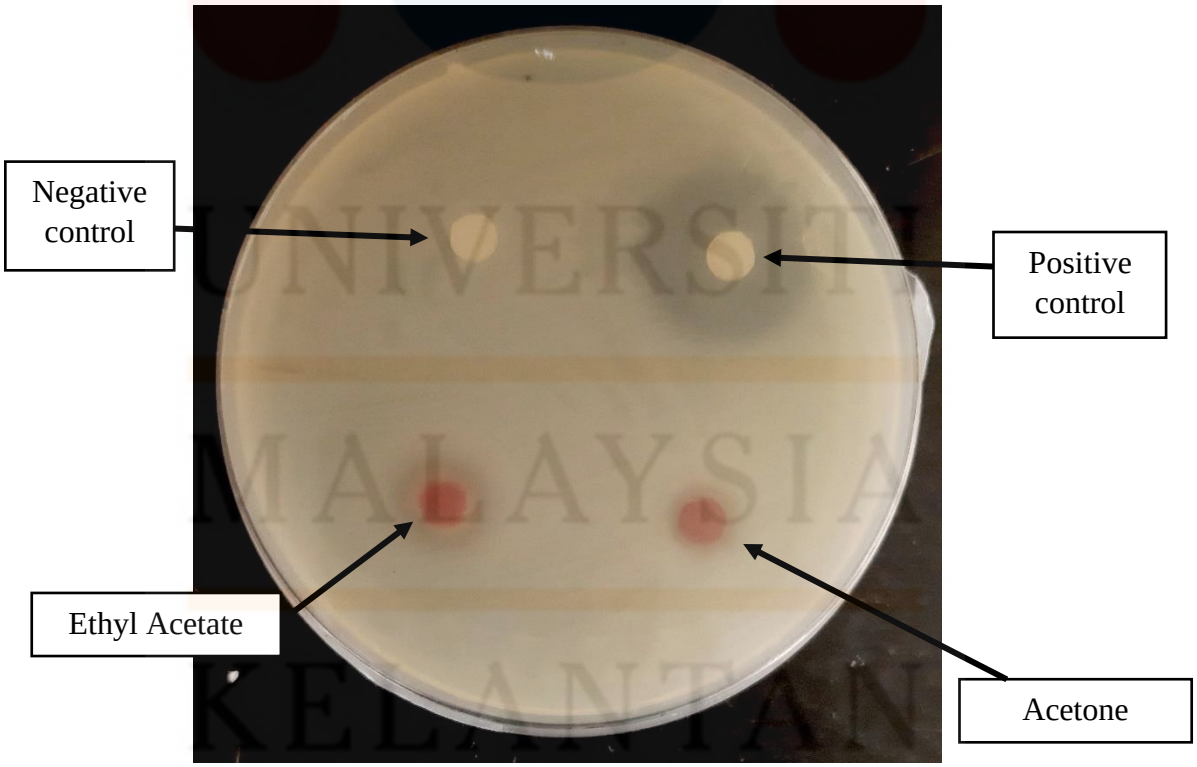


Figure 4.7: Inhibitory zone of the different extracts against MRSA.

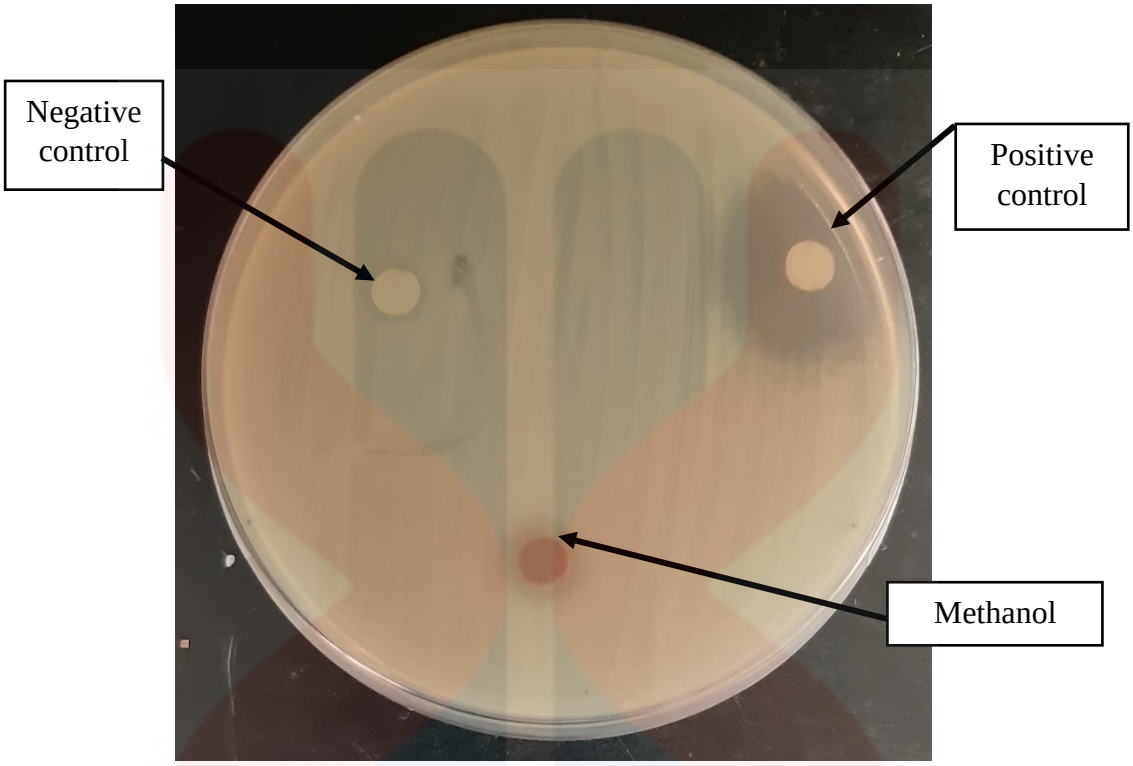


Figure 4.8: Inhibitory zone of the different extracts against MRSA.

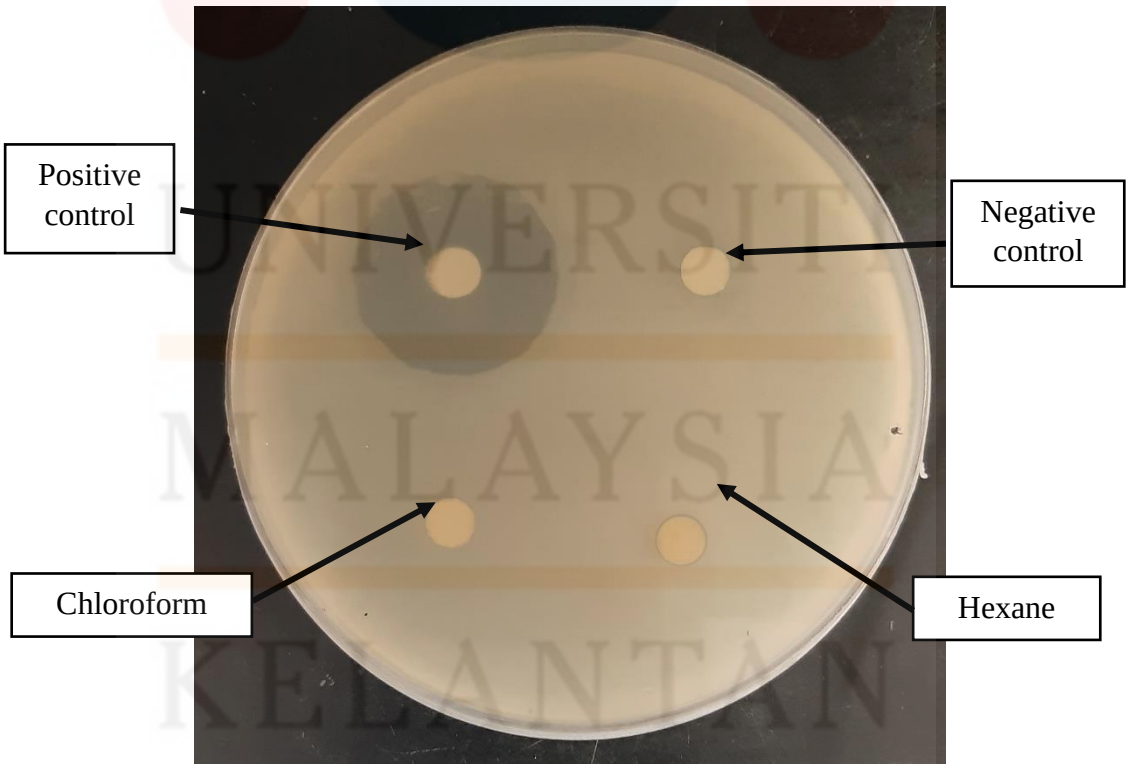


Figure 4.9: Inhibitory zone of the different extracts against *B. cereus (B.C.).*

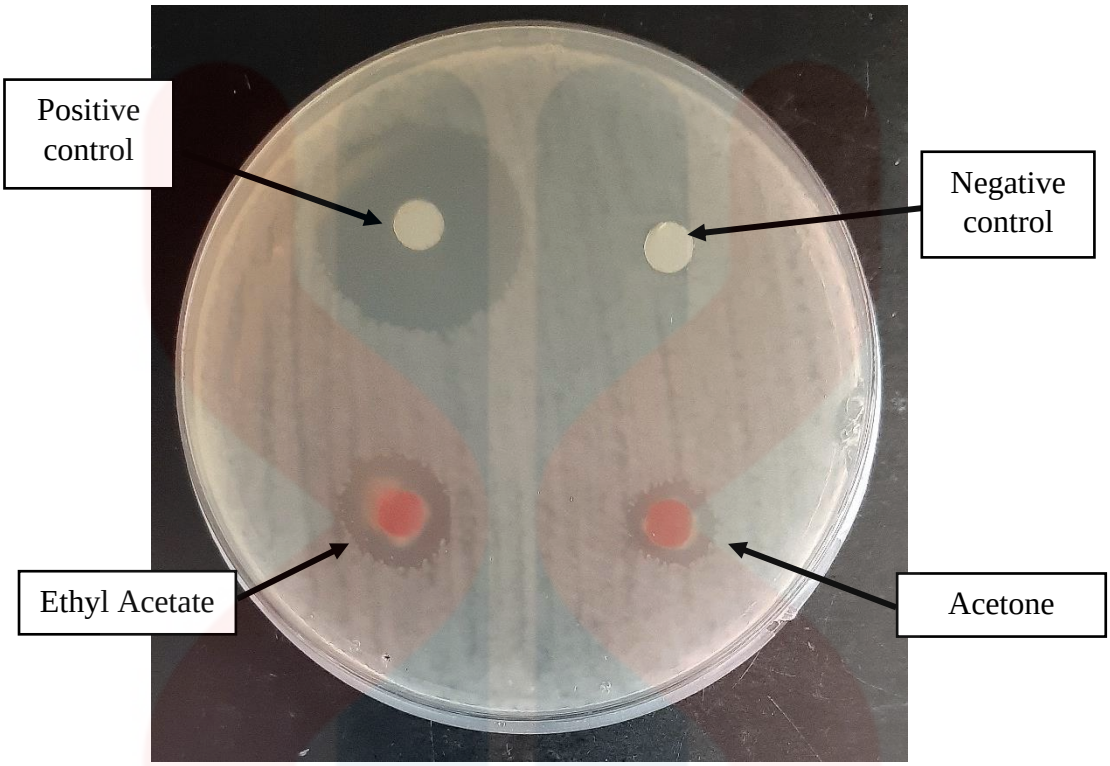


Figure 4.10: Inhibitory zone of the different extracts against *B. cereus (B.C.)*.

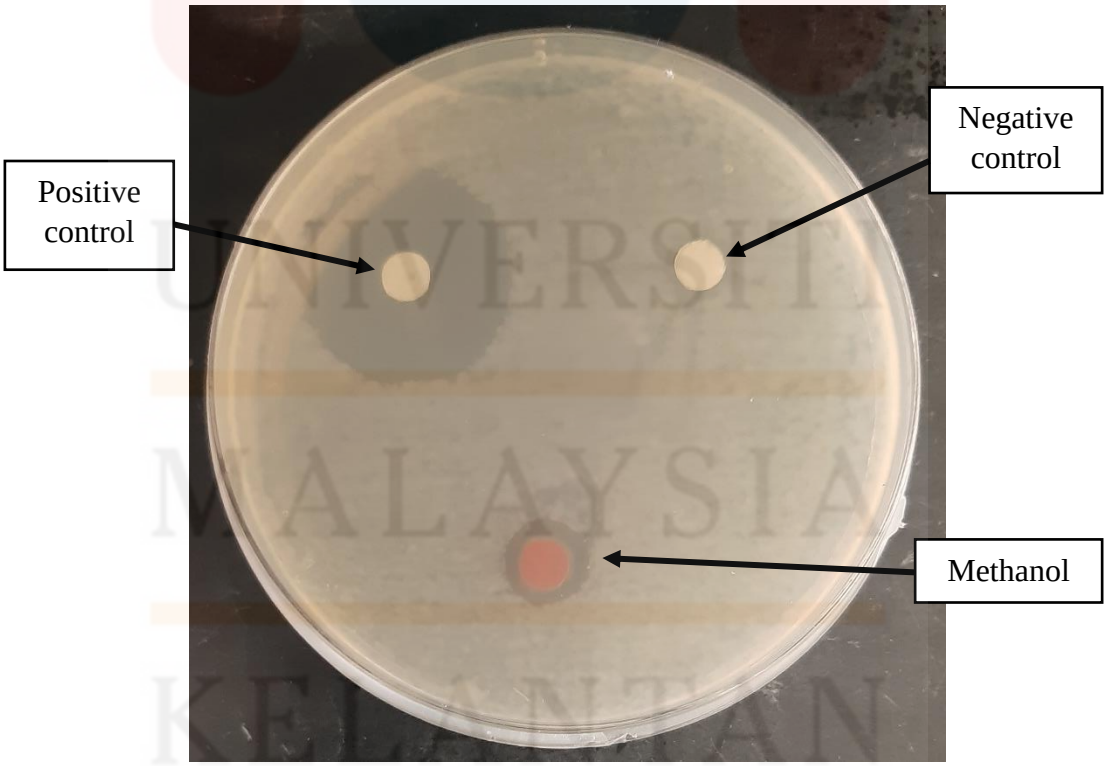


Figure 4.11: Inhibitory zone of the different extracts against *B. cereus (B.C.)*.

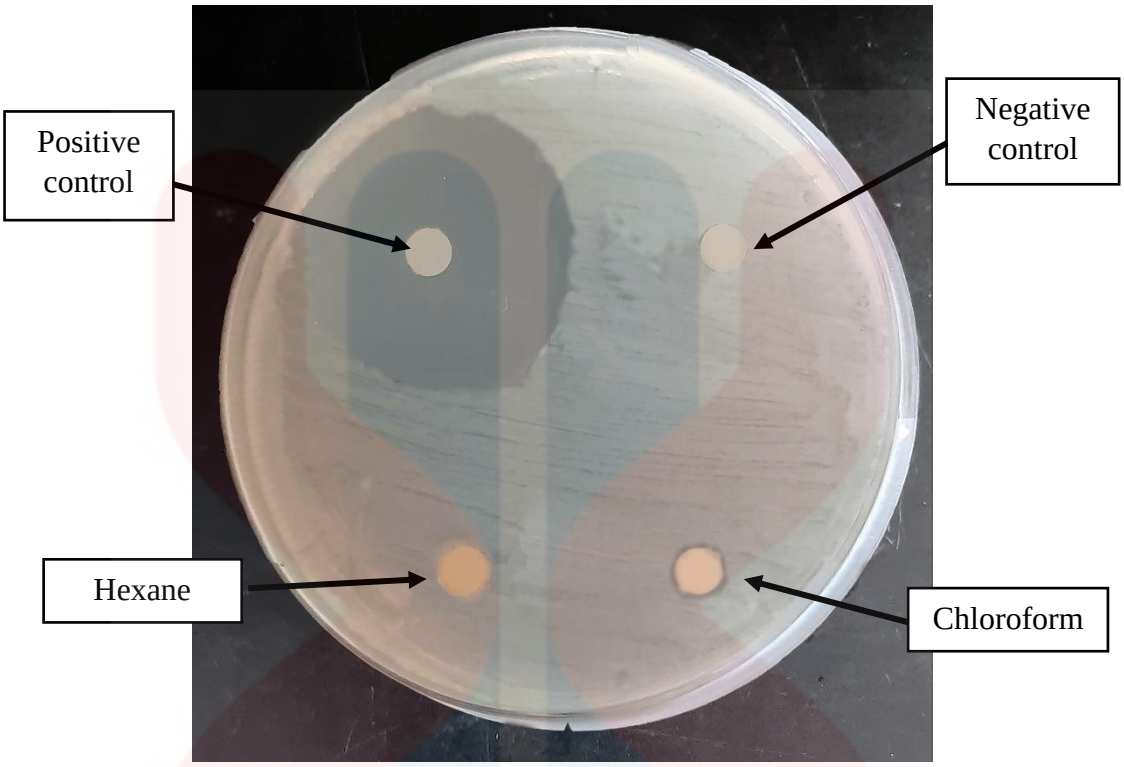


Figure 4.12: Inhibitory zone of the different extracts against *B. Subtilis (B.S.)*.

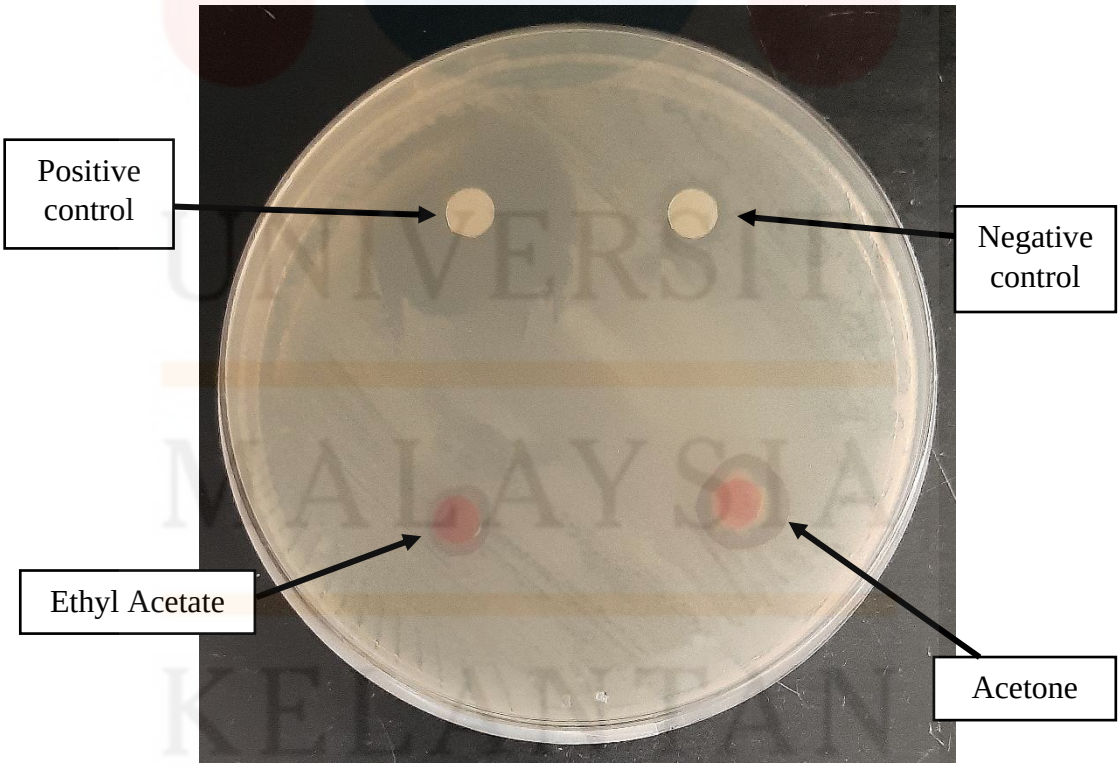


Figure 4.13: Inhibitory zone of the different extracts against *B. Subtilis (B.S.)*.

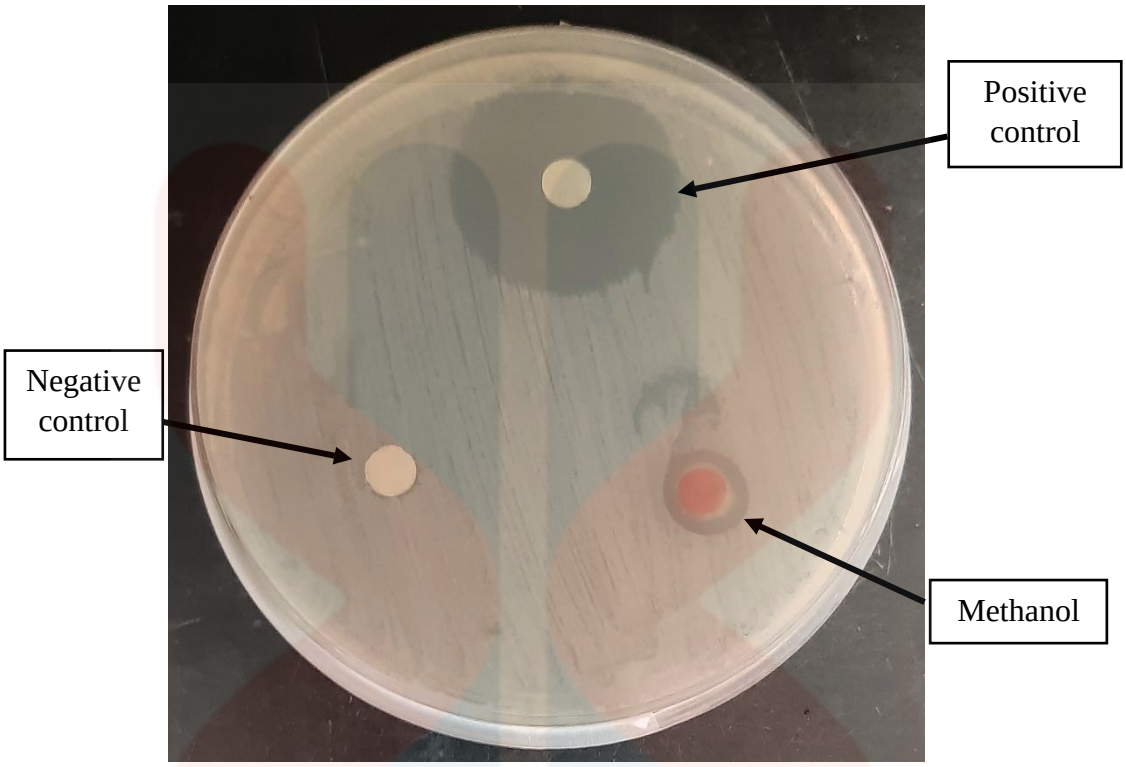


Figure 4.14: Inhibitory zone of the different extracts against *B. Subtilis (B.S.)*.

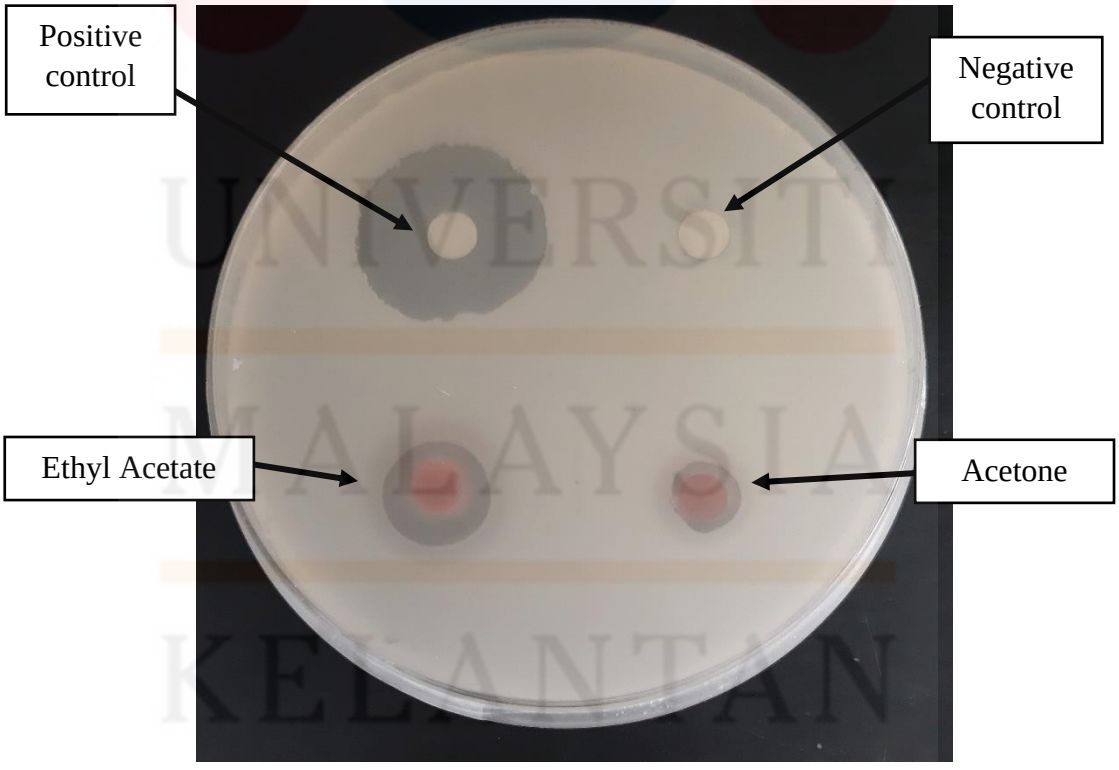


Figure 4.15: Inhibitory zone of the different extracts against *K. pneumonia*.

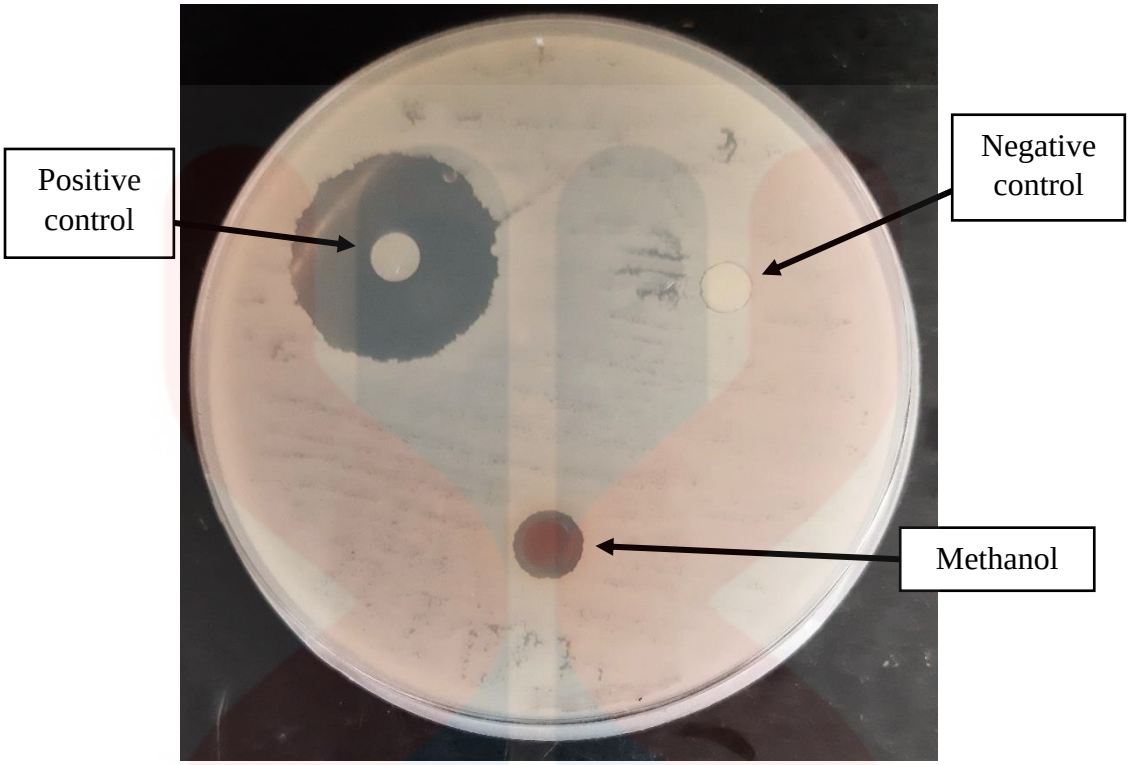


Figure 4.16: Inhibitory zone of the different extracts against *K. pneumonia*.

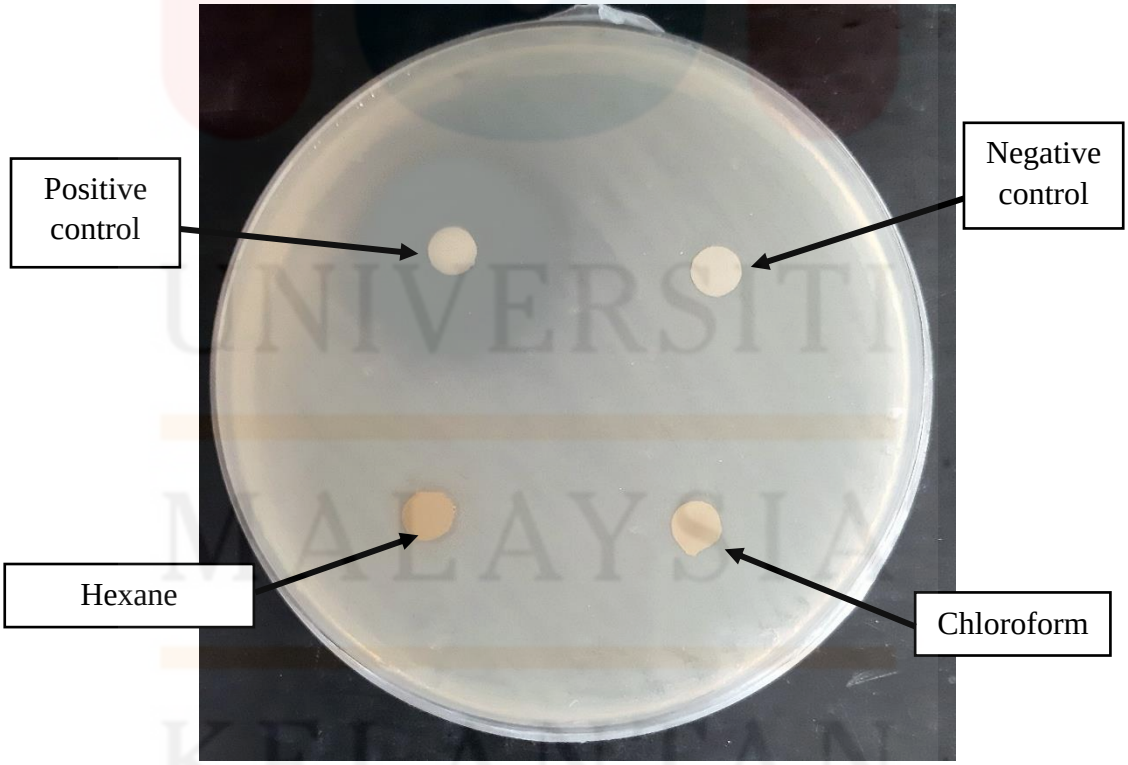


Figure 4.17: Inhibitory zone of the different extracts against *E. Coli*.

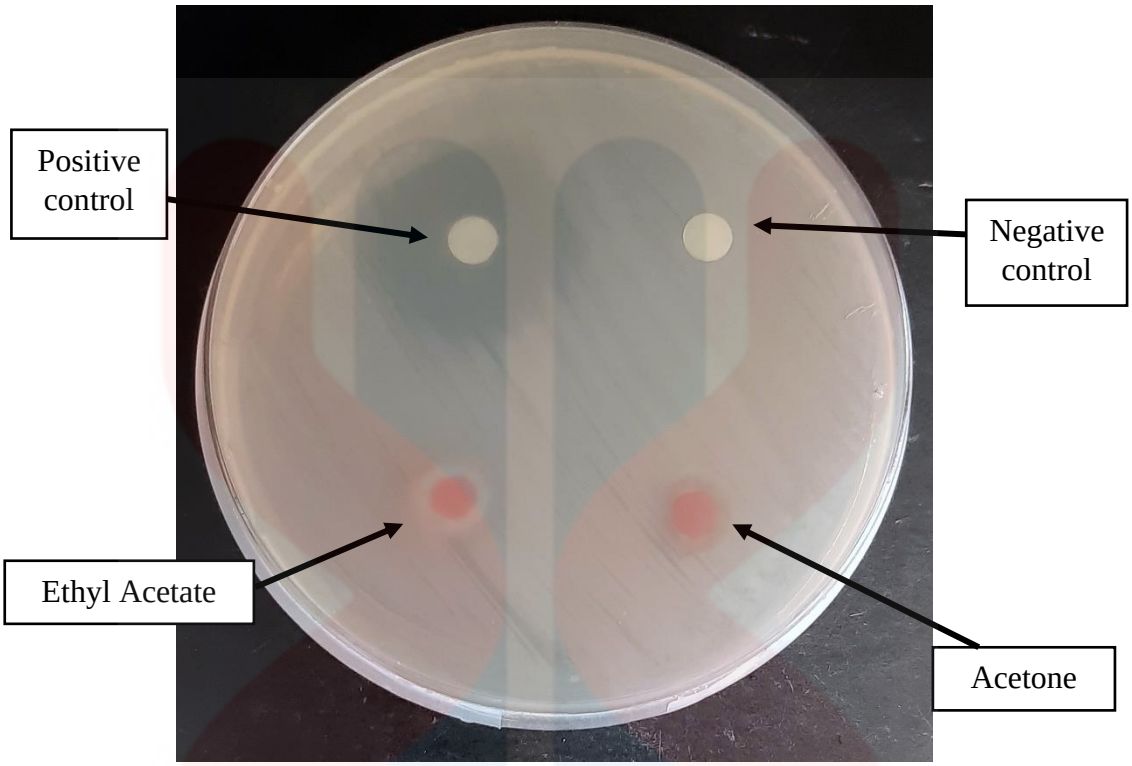


Figure 4.18: Inhibitory zone of the different extracts against *E. Coli*.

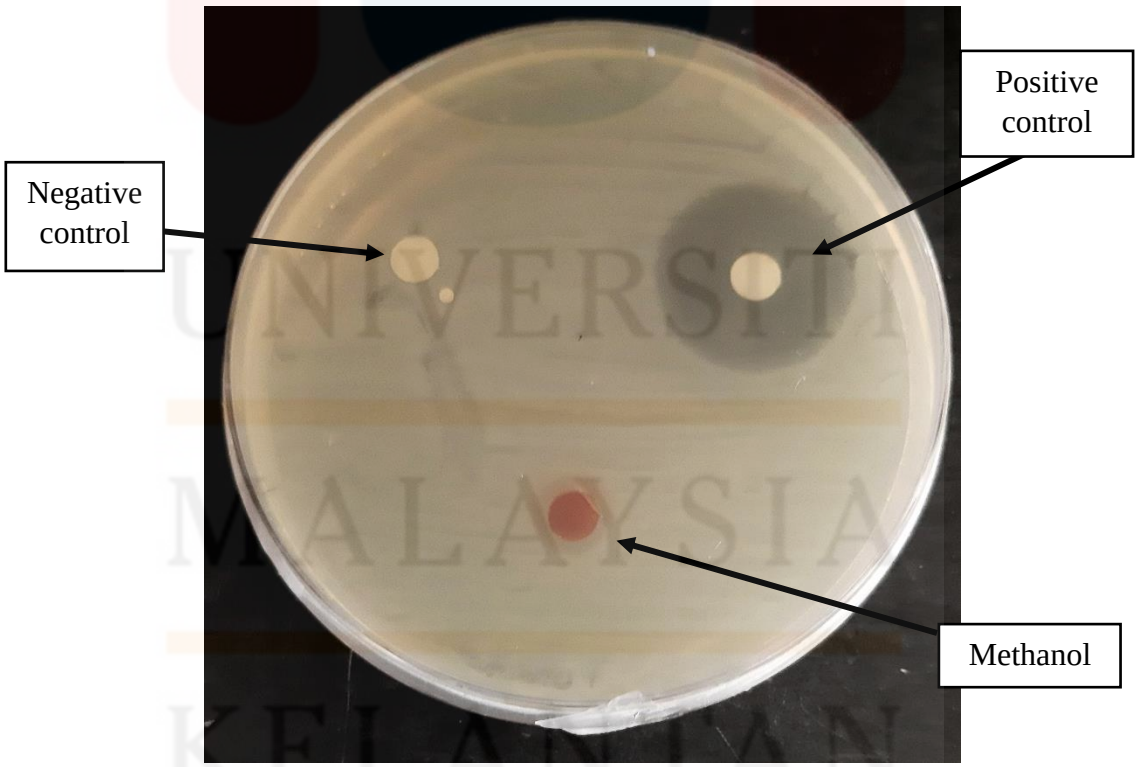


Figure 4.19: Inhibitory zone of the different extracts against *E. Coli*.

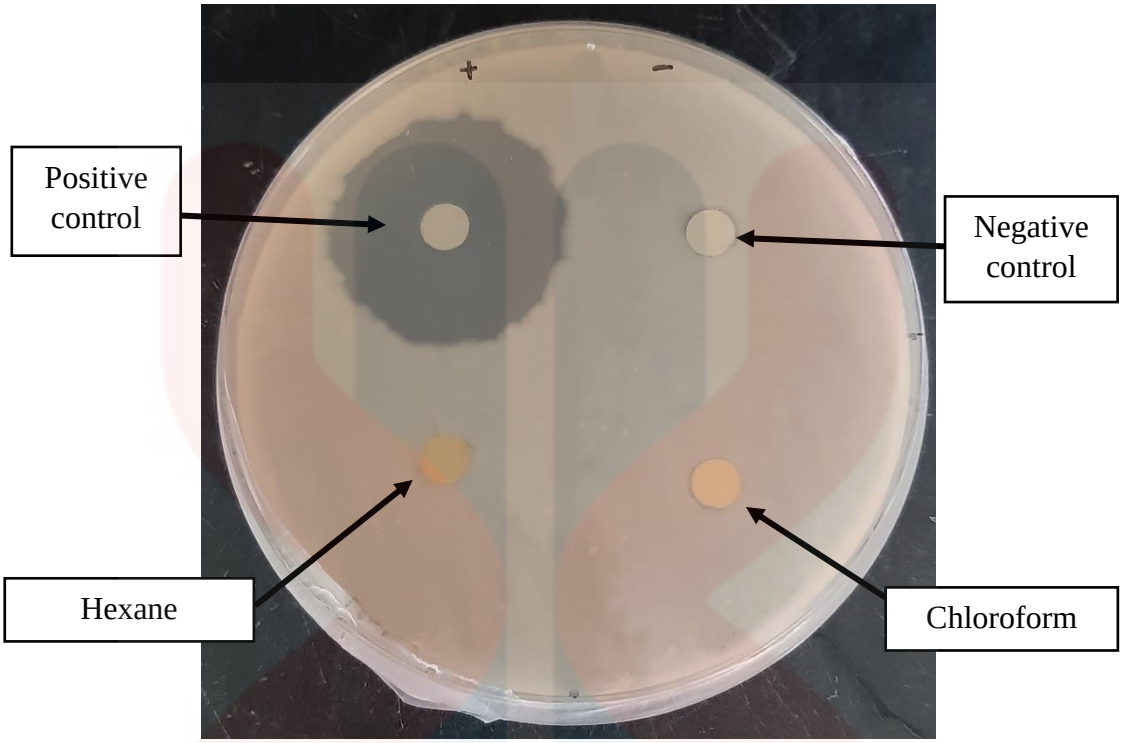


Figure 4.20: Inhibitory zone of the different extracts against *Y. enterocolitica (Y.E.)*.

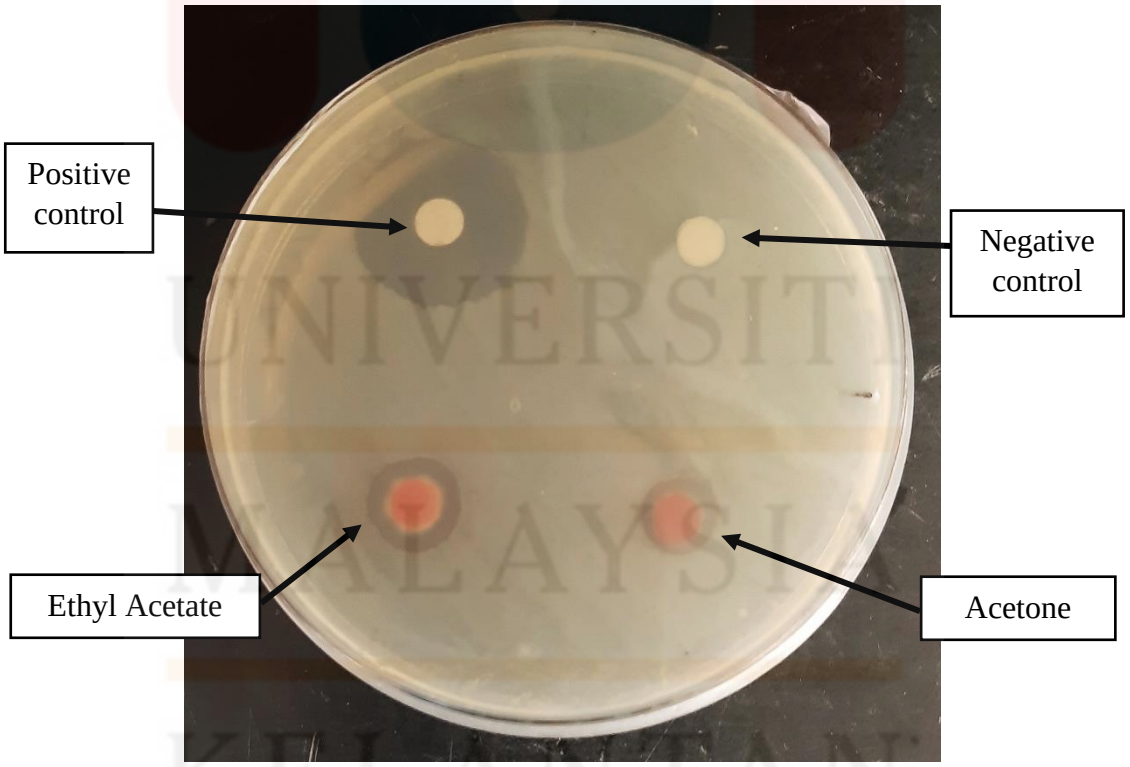


Figure 4.21: Inhibitory zone of the different extracts against *Y. enterocolitica (Y.E.)*.

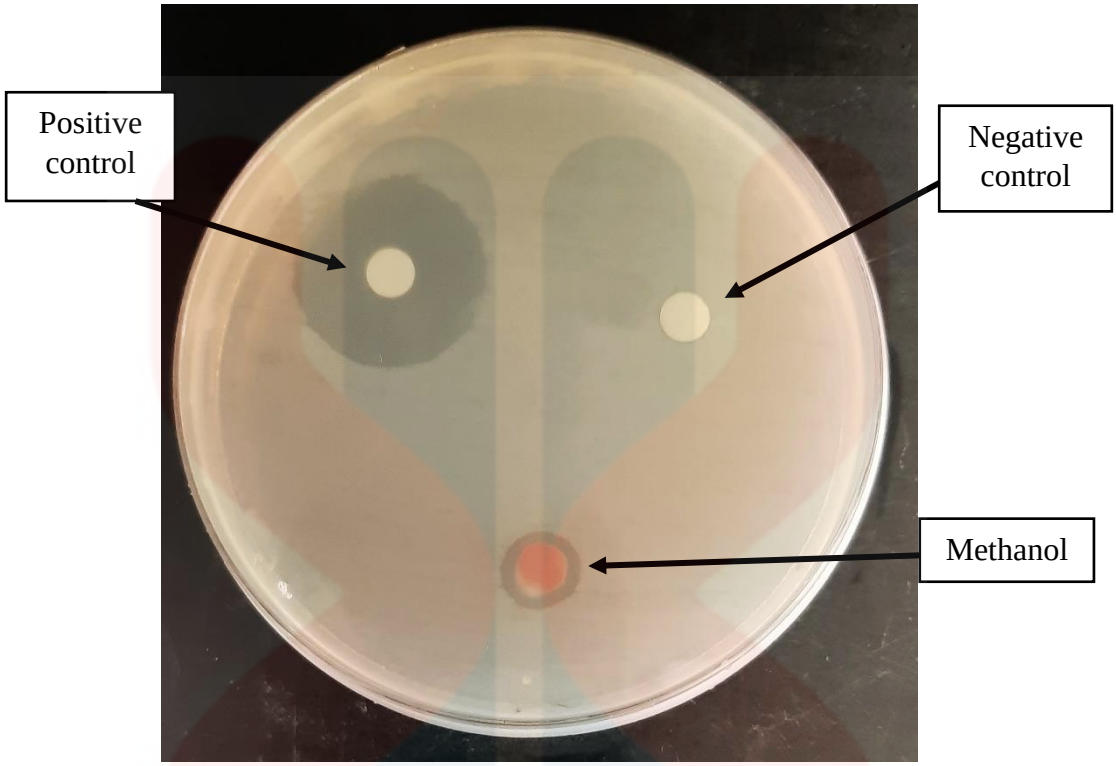


Figure 4.22: Inhibitory zone of the different extracts against *Y. enterocolitica (Y.E.)*.

4.3 Preliminary Screening of Antibacterial Compounds

Extraction of *A. cinnamomea* is a process of separating active plant materials or secondary metabolites from inert or inactive material using an appropriate solvent and standard extraction procedure. To investigate what active compounds contained in the different extracts of *A. cinnamomea*, several chemical tests were accomplished. The compounds were classified based on several chemical reactions by using silica gel 60 F254 plate (Merck, Germany) through the solvent system of petroleum ether, ethyl acetate, and methanol with ratio 1:1:1 (v/v/v). The testing reagent was sprayed on the developed TLC plate, except for the analysis of lactone compound, placed the developed TLC plates in the closing chamber containing iodine crystal. The result of different chemical reactions against several extracts was reported in Table 4.4.

In the detection of phenol, 1% aqueous $FeCl_3$ spraying reagent was used while Wagner's reagent was sprayed to detect alkaloid. Flavonoid was detected by spraying with 1 M NaOH and decolourised with 1 M of HCl. 10% of methanolic KOH spraying reagent was used to detect the presence of anthraquinone while for the detection of lactone compound in the extract, iodine vapour was used in a close chamber. In the solvent system of petroleum, ethyl acetate, and methanol with ratio 1:1:1 (v/v/v), the presence of greenish-yellow in of hexane and black colour sprit confirmed the presence of phenols in ethyl acetate, acetone, and methanol extract. When sprayed with Wagner's reagent, reddish-brown indicate the presence of alkaloid while red colour represents the presence of anthraquinone in the extracts. Appeared yellow colour indicated the presence of flavonoids and the yellow colour of the indicator can be decolorised after being sprayed again with 1 M HCl. To detect lactone, the developed plate was placed in a closing

chamber containing iodine crystals. The appearance of brown sport indicates a positive reaction to lactose compounds.

Based on the result of the experiment (Table 4.4), the *A. cinnamomea* extracts of ethyl acetate, acetone, and methanol showed a positive reaction towards all the chemical classes. In another word, ethyl acetate, acetone, and methanol extract of *A. cinnamomea* tuber contained phenol, alkaloid, flavonoid, anthraquinone, and lactone compounds. The extract of hexane only showed a positive reaction to phenol, alkaloid, flavonoid, and lactone compound. A negative reaction was shown on the anthraquinone compound for hexane extract. Chloroform extract of *A. cinnamomea* tuber contains the least compound, which included alkaloid, flavonoid, and lactone only. Phenol and anthraquinone compounds showed a negative reaction, which does not appear any intense colour in the phytochemical screening analysis test. The same finding from the study by Rosniyati (2018) detected saponin, alkaloids, terpenoids, and flavonoids in the *Ampelocissus* sp. extract and the findings concluded that the research showed the extracts of *Ampelocissus* sp. possessed good antioxidant activity.

Table 4.4: Preliminary screening of antibacterial compounds.

Chemical classes	Method	Reactions				
		Hex	**CF**	**EA**	**AC**	**MeOH**
Phenol	1% aqueous ferric chloride ($FeCl_3$)	Black spot at the bottom line, greenish-yellow on top (+)	Remain yellow (-)	Black (+)	Black (+)	Black (+)
Alkaloid	Wagner's reagent test	Redish brown (+)	Redish brown (+)	Redish brown (+)	Redish brown (+)	Redish brown (+)
Flavonoid	1 M Sodium hydroxide (NaOH)	Yellow (+)	Dark yellow (+)	Brownish-yellow (+)	Brownish-yellow (+)	Brownish-yellow (+)
	1 M hydrochloric acid (HCl)	Light yellow (+)	Light yellow (+)	Light red (+)	Light red (+)	Light red (+)
Anthraquinone	10% methanolic (KOH)	Remain yellow (-)	Remain yellow (-)	Red (+)	Red (+)	Red (+)
Lactone	Iodine vapour	Brown (+)	Brown (+)	Brown (+)	Brown (+)	Brown (+)

Key: + = positive reaction; - = negative reaction, Hex = hexane, CF = chloroform, EA = ethyl acetate, AC = Acetone, MeOH = methanol

The method of extraction for this research was sequential extraction by using the maceration technique. Depending on the different solvent systems used in the extraction method, different biologically active compounds in plants will be extracted. The polarity of *A. cinnamomea* extract was increasing from the least polar solvent, hexane, chloroform, ethyl acetate, acetone, and methanol is the most polar solvent. Polar compounds in the plants were dissolved by polar solvents while non-polar solvents commonly will dissolve the non-polar compounds. The polarity can be measured as the dielectric constant (Elmaa et al., 2018). The greater the constant, the more polar the molecule is, the higher the polarity of the solvent, the more component contain in the extract. Organic solvents' polarity determines their water solubility and miscibility (Chormey, Büyükpınar, Turak, Komesli, & Bakırdere, 2017). Polar solvents were able to improve cell permeability and penetrate into the cells, extracting more intracellular secondary metabolites, both polar and less polar chemicals, when compared to non-polar solvents like hexane. Nonpolar solvents with zero polarity index, such as n-hexane, only dissolved lipophilic substances such as alkanes, waxes, colour pigments, sterols, many terpenoids, and alkaloids, extracting fewer secondary metabolites (Yusnawan & Inayati, 2017). The most popular solvents used to extract the polar bioactive compounds from the plant materials are methanol, ethanol, and their mixtures with water in maceration, percolation, reflux, and Soxhlet method (Krishnan, Chandran, Vadivel, & Rajan, 2016; Oniszczuk et al., 2014) while polar solvent used in MAE and SPE method can be developed in the separation of flavonoids from various plant materials (Kryževičiūtė, Kraujalis, & Venskutonis, 2016).

In the test for detection of various structure groups of antibacterial chemical compounds by different spraying reagents, several extract yields from the tuber of *A. cinnamomea* by using sequential solvent extract appearance different colours on the TLC plate shown as a proof to indicate the presence for each of the compounds. Solvent

polarity plays the role to increase the active compound contained in the extract. The research showed that the colour changes became more obvious and darker when tested with the spraying agent from the least polar to the most polar extract of *A. cinnamomea* tuber. This result showed the higher polarity of the extracts will contain more active compounds and hence cause the colour to change darker and more obvious.

Phenol has different functions in different fields as an antioxidant, antimicrobial, anti-inflammatory, antitumor, antiviral, analgesic, and antipyretic (Jahromi, 2019). The phytochemical screening of the several extracts in this research showed a positive result. Hence we can say that the phenol compound contains in the extract of the tuber of *A. cinnamomea* has the ability to improve human health. Flavonoids are one of the main groups that can be found in the natural plant which contained polyphenols compounds. Flavonoids contain antioxidants compound which can use to protect plants from harsh natural environmental conditions (Oniszczuk & Podgórski, 2015). The aromatic ring of flavonoids attached to the hydroxyl groups at curtained position enhances the antibacterial effect (Xie, Yang, Tang, Chen, & Ren, 2015). Table 4.10 below shows some of the previous research which stated that the extracts for those different tuber plants contained different types of compounds and had proved that the extracts possessed antibacterial properties and different functions contained. According to the screening studies from Basile, Giordano, López-Sáez, and Cobianchi (1999) and Özçelik, Kartal, and Orhan (2011), their results both showed that alkaloids, flavonoids, and phenolic acids possessed antibacterial activity against some of the bacterial such as *P. aeruginosa*, and also *E. coli* than *S. aureus*. So we can conclude that the bioactive compound contained in the tuber of *A. cinnamomea* can be further developed as antibacterial to cure or maintain human health.

The chloroform extract of the leaves of *Leptadenia hastata* has concluded that the phytochemical components such as tannins, saponins, terpenoids, alkaloids, flavonoids, phenols, and steroids compounds performed different levels of antimicrobial activity on different tested bacterial and the research (Umaru, Badruddin, Assim, & Umaru, 2018). Furthermore, Rosniyati (2018) concluded that *Ampelocissus* sp. possessed acetylcholinesterase inhibition potential to be used for Alzheimer's disease treatment. Hence, the active compounds found in the current study containing phenol, flavonoid, alkaloid, anthraquinone, and lactone showed the relationship of the antibacterial activity for several extract yields from the tuber of *A. cinnamomea* by using sequential solvent extract.

CHAPTER 5

CONCLUSION

5.1 Conclusion

The research revealed a total of 5 extracts from the tuber of *A. cinnamomea* showed the inhibitory activity against Gram-positive bacteria *viz*., *S. aureus*, *Methicillin-resistant Staphylococcus aureus*, *B. cereus*, *B. subtilis*, and Gram-negative bacteria *viz.*, *K. pheumoniae*, *E. Coli*, and *Y. enterocolitica*. Extracts from the tuber of *A. cinnamomea* by using solvent hexane, chloroform, ethyl acetate, and acetone revealed a significant difference ($p < 0.05$) in this current study. The result also revealed that the extracts exhibit more inhibitory activity towards Gram-negative bacteria compared to Gram-positive bacteria. Among all the extracts, ethyl acetate showed the maximum inhibition zone (18.0 ± 0.8) against *K. pheumonia*. The extract of solvent hexane showed the minimum inhibition zone diameter against MRSA, *B. cereus*, *E. Coli*, and *Y. enterocolitica* in 6.8 ± 0.2 mm,7.2 ± 0.2 mm, 8.7 ± 0.5 mm, and 6.8 ± 0.2 mm respectively. The highest percentage of the inhibitory potential of extract which showed the most promising antibacterial activity are ethyl acetate, acetone, and methanol as they can inhibit all the test bacteria used in this study.

Furthermore, the preliminary chemical profiling of antibacterial compounds on TLC plats developed through a solvent system of ethyl acetate: methanol: petroleum ether with ratio 1:1:1 (v/v/v) sprayed with respective reagent showed the positive reaction to the phenol, flavonoid, alkaloid, anthraquinone, and lactone compounds possessed in the tuber of *A. cinnamomea*. Ethyl acetate, acetone, and methanol extract showed the most promising reaction as these three solvent extracts showed all positive results to all tested compounds. The presence of the compounds in the extract support the more inhibitory activities formed on the Gram-positive and Gram-negative bacterial.

In conclusion, the tuber powder of *A. cinnamomea* has great potential and can be a great possible candidate as an antimicrobial agent in the future due to its antibacterial activity and the bioactive compounds contained in the extracts. In the future, the active compounds are needed to be isolated and purified for further identification. The active compound might be extracted to be developed and commercialized as an antibiotic.

5.2 Recommendation

Other parts of the *A. cinnamomea* plant such as bark, flower, and leaves can be used for further study as different plants parts might possess different bioactive compounds. Each chemical solvent used to extract the yield from *A. cinnamomea* tuber can be placed as the negative control to determine that the inhibitory activity formed on the bacterial is due to the extract of the plant sample.

Further study on the bioactive compound of the tuber of *Ampelocissus cinnamomea* by using TLC also can be improved by optimizing a different ratio of the solvent system to obtain better separation of the compound on the TLC plates. Moreover, both high performance liquid chromatography (HPLC) and gas chromatography-mass spectroscopy (GCMS) methods can be used to analyse the purified extracts from the plant sample by determining the bioactive compound contained and also can be used to analyse the concentration of the compound on the sample more accurately.

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APPENDIX

Table A.1: Mean inhibition zone of extracts against test bacterial developed from SPSS.

Report

Bacteria		Hexane	Chloroform	Ethyl Acetate	Acetone	Methanol	Positive Control
SA	Mean	.0000	6.8333	7.6667	10.0000	9.3333	32.5556
	Std. Deviation	.00000	.28868	.57735	1.00000	.57735	2.49305
MRSA	Mean	6.8333	.0000	11.1667	9.1667	9.8333	23.3333
	Std. Deviation	.28868	.00000	.28868	.28868	.28868	3.16228
BC	Mean	7.1667	.0000	14.3333	11.5000	10.6667	26.7222
	Std. Deviation	.28868	.00000	.57735	.50000	.57735	.36324
BS	Mean	.0000	9.3333	9.1667	12.3333	10.6667	27.4444
	Std. Deviation	.00000	.57735	.28868	.57735	.57735	5.83333
KP	Mean	.0000	8.3333	18.0000	12.8333	10.1667	25.0556
	Std. Deviation	.00000	.28868	1.00000	.76376	.28868	.76830
EC	Mean	8.6667	.0000	9.6667	9.6667	10.0000	26.0000
	Std. Deviation	.57735	.00000	2.08167	1.15470	1.00000	.86603
YE	Mean	6.8333	7.5000	12.1667	9.8333	10.6667	25.0556
	Std. Deviation	.28868	.50000	.28868	.28868	.57735	3.32081
Total	Mean	4.2143	4.5714	11.7381	10.7619	10.1905	26.5952
	Std. Deviation	3.79332	4.13003	3.42279	1.49682	.69779	3.95525

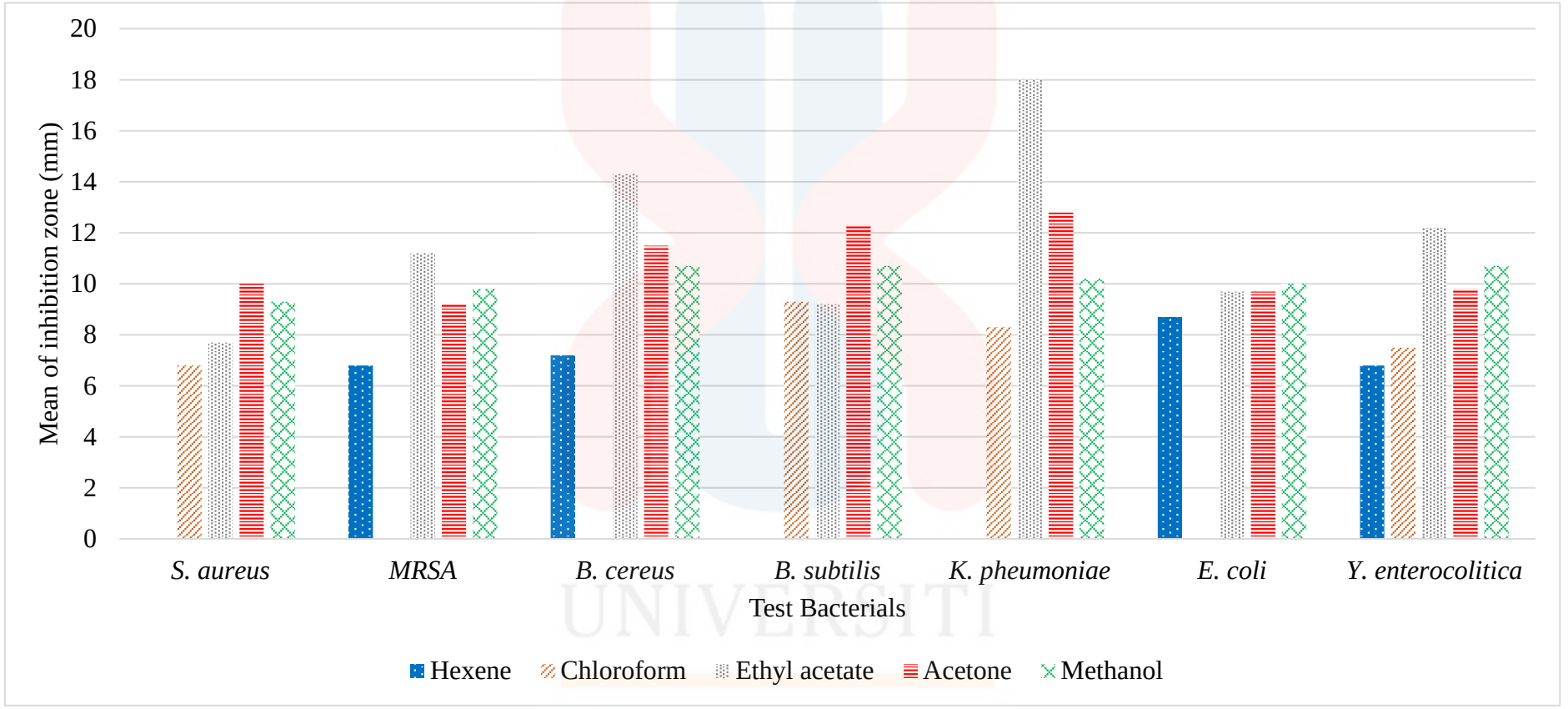

Figure A.1: The comparison of the antimicrobial activity between the mean inhibition zone of 7 types of bacterial.

Table A.2: The comparison of significant different of the mean inhibition zone for 7 types of bacterial in One-way ANOVA test.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Positive_Control	Between Groups	467.984	6	77.997	8.702	.000
	Within Groups	501.944	56	8.963		
	Total	969.929	62			
Hexane	Between Groups	286.619	6	47.770	573.238	.000
	Within Groups	1.167	14	.083		
	Total	287.786	20			
Chloroform	Between Groups	339.643	6	56.607	528.333	.000
	Within Groups	1.500	14	.107		
	Total	341.143	20			
Ethyl Acetate	Between Groups	221.810	6	36.968	41.404	.000
	Within Groups	12.500	14	.893		
	Total	234.310	20			
Acetone	Between Groups	37.476	6	6.246	11.924	.000
	Within Groups	7.333	14	.524		
	Total	44.810	20			
Methanol	Between Groups	4.738	6	.790	2.211	.104
	Within Groups	5.000	14	.357		
	Total	9.738	20			

Table A.3: Post Hoc Tests of the antimicrobial activity between the mean inhibition zone of 7 types of bacterial.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Bacteria	(J) Bacteria	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Positive Control	SA	MRSA	9.22222*	1.41133	.000	4.9064	13.5381
		BC	5.83333*	1.41133	.002	1.5175	10.1492
		BS	5.11111*	1.41133	.011	.7953	9.4270
		KP	7.50000*	1.41133	.000	3.1842	11.8158
		EC	6.55556*	1.41133	.000	2.2397	10.8714
		YE	7.50000*	1.41133	.000	3.1842	11.8158
	MRSA	SA	-9.22222*	1.41133	.000	-13.5381	-4.9064
		BC	-3.38889	1.41133	.217	-7.7047	.9270
		BS	-4.11111	1.41133	.072	-8.4270	.2047
		KP	-1.72222	1.41133	.883	-6.0381	2.5936
		EC	-2.66667	1.41133	.496	-6.9825	1.6492
		YE	-1.72222	1.41133	.883	-6.0381	2.5936
	BC	SA	-5.83333*	1.41133	.002	-10.1492	-1.5175
		MRSA	3.38889	1.41133	.217	-.9270	7.7047
		BS	-.72222	1.41133	.999	-5.0381	3.5936
		KP	1.66667	1.41133	.898	-2.6492	5.9825
		EC	.72222	1.41133	.999	-3.5936	5.0381
		YE	1.66667	1.41133	.898	-2.6492	5.9825
	BS	SA	-5.11111*	1.41133	.011	-9.4270	-.7953
		MRSA	4.11111	1.41133	.072	-.2047	8.4270
		BC	.72222	1.41133	.999	-3.5936	5.0381
		KP	2.38889	1.41133	.624	-1.9270	6.7047
		EC	1.44444	1.41133	.946	-2.8714	5.7603
		YE	2.38889	1.41133	.624	-1.9270	6.7047
	KP	SA	-7.50000*	1.41133	.000	-11.8158	-3.1842
		MRSA	1.72222	1.41133	.883	-2.5936	6.0381
		BC	-1.66667	1.41133	.898	-5.9825	2.6492
		BS	-2.38889	1.41133	.624	-6.7047	1.9270
		EC	-.94444	1.41133	.994	-5.2603	3.3714

		YE	.00000	1.41133	1.000	-4.3158	4.3158
	EC	SA	-6.55556*	1.41133	.000	-10.8714	-2.2397
		MRSA	2.66667	1.41133	.496	-1.6492	6.9825
		BC	-.72222	1.41133	.999	-5.0381	3.5936
		BS	-1.44444	1.41133	.946	-5.7603	2.8714
		KP	.94444	1.41133	.994	-3.3714	5.2603
		YE	.94444	1.41133	.994	-3.3714	5.2603
	YE	SA	-7.50000*	1.41133	.000	-11.8158	-3.1842
		MRSA	1.72222	1.41133	.883	-2.5936	6.0381
		BC	-1.66667	1.41133	.898	-5.9825	2.6492
		BS	-2.38889	1.41133	.624	-6.7047	1.9270
		KP	.00000	1.41133	1.000	-4.3158	4.3158
		EC	-.94444	1.41133	.994	-5.2603	3.3714
Hexane	SA	MRSA	-6.83333*	.23570	.000	-7.6382	-6.0285
		BC	-7.16667*	.23570	.000	-7.9715	-6.3618
		BS	.00000	.23570	1.000	-.8048	.8048
		KP	.00000	.23570	1.000	-.8048	.8048
		EC	-8.66667*	.23570	.000	-9.4715	-7.8618
		YE	-6.83333*	.23570	.000	-7.6382	-6.0285
	MRSA	SA	6.83333*	.23570	.000	6.0285	7.6382
		BC	-.33333	.23570	.786	-1.1382	.4715
		BS	6.83333*	.23570	.000	6.0285	7.6382
		KP	6.83333*	.23570	.000	6.0285	7.6382
		EC	-1.83333*	.23570	.000	-2.6382	-1.0285
		YE	.00000	.23570	1.000	-.8048	.8048
	BC	SA	7.16667*	.23570	.000	6.3618	7.9715
		MRSA	.33333	.23570	.786	-.4715	1.1382
		BS	7.16667*	.23570	.000	6.3618	7.9715
		KP	7.16667*	.23570	.000	6.3618	7.9715
		EC	-1.50000*	.23570	.000	-2.3048	-.6952
		YE	.33333	.23570	.786	-.4715	1.1382
	BS	SA	.00000	.23570	1.000	-.8048	.8048
		MRSA	-6.83333*	.23570	.000	-7.6382	-6.0285
		BC	-7.16667*	.23570	.000	-7.9715	-6.3618
		KP	.00000	.23570	1.000	-.8048	.8048
		EC	-8.66667*	.23570	.000	-9.4715	-7.8618
		YE	-6.83333*	.23570	.000	-7.6382	-6.0285
	KP	SA	.00000	.23570	1.000	-.8048	.8048
		MRSA	-6.83333*	.23570	.000	-7.6382	-6.0285
		BC	-7.16667*	.23570	.000	-7.9715	-6.3618

		BS	.00000	.23570	1.000	-.8048	.8048
		EC	-8.66667*	.23570	.000	-9.4715	-7.8618
		YE	-6.83333*	.23570	.000	-7.6382	-6.0285
	EC	SA	8.66667*	.23570	.000	7.8618	9.4715
		MRSA	1.83333*	.23570	.000	1.0285	2.6382
		BC	1.50000*	.23570	.000	.6952	2.3048
		BS	8.66667*	.23570	.000	7.8618	9.4715
		KP	8.66667*	.23570	.000	7.8618	9.4715
		YE	1.83333*	.23570	.000	1.0285	2.6382
	YE	SA	6.83333*	.23570	.000	6.0285	7.6382
		MRSA	.00000	.23570	1.000	-.8048	.8048
		BC	-.33333	.23570	.786	-1.1382	.4715
		BS	6.83333*	.23570	.000	6.0285	7.6382
		KP	6.83333*	.23570	.000	6.0285	7.6382
		EC	-1.83333*	.23570	.000	-2.6382	-1.0285
Chloroform	SA	MRSA	6.83333*	.26726	.000	5.9207	7.7459
		BC	6.83333*	.26726	.000	5.9207	7.7459
		BS	-2.50000*	.26726	.000	-3.4126	-1.5874
		KP	-1.50000*	.26726	.001	-2.4126	-.5874
		EC	6.83333*	.26726	.000	5.9207	7.7459
		YE	-.66667	.26726	.232	-1.5793	.2459
	MRSA	SA	-6.83333*	.26726	.000	-7.7459	-5.9207
		BC	.00000	.26726	1.000	-.9126	.9126
		BS	-9.33333*	.26726	.000	-10.2459	-8.4207
		KP	-8.33333*	.26726	.000	-9.2459	-7.4207
		EC	.00000	.26726	1.000	-.9126	.9126
		YE	-7.50000*	.26726	.000	-8.4126	-6.5874
	BC	SA	-6.83333*	.26726	.000	-7.7459	-5.9207
		MRSA	.00000	.26726	1.000	-.9126	.9126
		BS	-9.33333*	.26726	.000	-10.2459	-8.4207
		KP	-8.33333*	.26726	.000	-9.2459	-7.4207
		EC	.00000	.26726	1.000	-.9126	.9126
		YE	-7.50000*	.26726	.000	-8.4126	-6.5874
	BS	SA	2.50000*	.26726	.000	1.5874	3.4126
		MRSA	9.33333*	.26726	.000	8.4207	10.2459
		BC	9.33333*	.26726	.000	8.4207	10.2459
		KP	1.00000*	.26726	.028	.0874	1.9126
		EC	9.33333*	.26726	.000	8.4207	10.2459
		YE	1.83333*	.26726	.000	.9207	2.7459
	KP	SA	1.50000*	.26726	.001	.5874	2.4126
		MRSA	8.33333*	.26726	.000	7.4207	9.2459

		BC	8.33333*	.26726	.000	7.4207	9.2459
		BS	-1.00000*	.26726	.028	-1.9126	-.0874
		EC	8.33333*	.26726	.000	7.4207	9.2459
		YE	.83333	.26726	.084	-.0793	1.7459
	EC	SA	-6.83333*	.26726	.000	-7.7459	-5.9207
		MRSA	.00000	.26726	1.000	-.9126	.9126
		BC	.00000	.26726	1.000	-.9126	.9126
		BS	-9.33333*	.26726	.000	-10.2459	-8.4207
		KP	-8.33333*	.26726	.000	-9.2459	-7.4207
		YE	-7.50000*	.26726	.000	-8.4126	-6.5874
	YE	SA	.66667	.26726	.232	-.2459	1.5793
		MRSA	7.50000*	.26726	.000	6.5874	8.4126
		BC	7.50000*	.26726	.000	6.5874	8.4126
		BS	-1.83333*	.26726	.000	-2.7459	-.9207
		KP	-.83333	.26726	.084	-1.7459	.0793
		EC	7.50000*	.26726	.000	6.5874	8.4126
EthylAcetate	SA	MRSA	-3.50000*	.77152	.007	-6.1344	-.8656
		BC	-6.66667*	.77152	.000	-9.3011	-4.0323
		BS	-1.50000	.77152	.486	-4.1344	1.1344
		KP	-10.33333*	.77152	.000	-12.9677	-7.6989
		EC	-2.00000	.77152	.200	-4.6344	.6344
		YE	-4.50000*	.77152	.001	-7.1344	-1.8656
	MRSA	SA	3.50000*	.77152	.007	.8656	6.1344
		BC	-3.16667*	.77152	.014	-5.8011	-.5323
		BS	2.00000	.77152	.200	-.6344	4.6344
		KP	-6.83333*	.77152	.000	-9.4677	-4.1989
		EC	1.50000	.77152	.486	-1.1344	4.1344
		YE	-1.00000	.77152	.843	-3.6344	1.6344
	BC	SA	6.66667*	.77152	.000	4.0323	9.3011
		MRSA	3.16667*	.77152	.014	.5323	5.8011
		BS	5.16667*	.77152	.000	2.5323	7.8011
		KP	-3.66667*	.77152	.004	-6.3011	-1.0323
		EC	4.66667*	.77152	.000	2.0323	7.3011
		YE	2.16667	.77152	.142	-.4677	4.8011
	BS	SA	1.50000	.77152	.486	-1.1344	4.1344
		MRSA	-2.00000	.77152	.200	-4.6344	.6344
		BC	-5.16667*	.77152	.000	-7.8011	-2.5323
		KP	-8.83333*	.77152	.000	-11.4677	-6.1989
		EC	-.50000	.77152	.994	-3.1344	2.1344
		YE	-3.00000*	.77152	.021	-5.6344	-.3656

	KP	SA	10.33333*	.77152	.000	7.6989	12.9677
		MRSA	6.83333*	.77152	.000	4.1989	9.4677
		BC	3.66667*	.77152	.004	1.0323	6.3011
		BS	8.83333*	.77152	.000	6.1989	11.4677
		EC	8.33333*	.77152	.000	5.6989	10.9677
		YE	5.83333*	.77152	.000	3.1989	8.4677
	EC	SA	2.00000	.77152	.200	-.6344	4.6344
		MRSA	-1.50000	.77152	.486	-4.1344	1.1344
		BC	-4.66667*	.77152	.000	-7.3011	-2.0323
		BS	.50000	.77152	.994	-2.1344	3.1344
		KP	-8.33333*	.77152	.000	-10.9677	-5.6989
		YE	-2.50000	.77152	.068	-5.1344	.1344
	YE	SA	4.50000*	.77152	.001	1.8656	7.1344
		MRSA	1.00000	.77152	.843	-1.6344	3.6344
		BC	-2.16667	.77152	.142	-4.8011	.4677
		BS	3.00000*	.77152	.021	.3656	5.6344
		KP	-5.83333*	.77152	.000	-8.4677	-3.1989
		EC	2.50000	.77152	.068	-.1344	5.1344
Acetone	SA	MRSA	.83333	.59094	.788	-1.1845	2.8511
		BC	-1.50000	.59094	.217	-3.5178	.5178
		BS	-2.33333*	.59094	.019	-4.3511	-.3155
		KP	-2.83333*	.59094	.004	-4.8511	-.8155
		EC	.33333	.59094	.997	-1.6845	2.3511
		YE	.16667	.59094	1.000	-1.8511	2.1845
	MRSA	SA	-.83333	.59094	.788	-2.8511	1.1845
		BC	-2.33333*	.59094	.019	-4.3511	-.3155
		BS	-3.16667*	.59094	.002	-5.1845	-1.1489
		KP	-3.66667*	.59094	.000	-5.6845	-1.6489
		EC	-.50000	.59094	.975	-2.5178	1.5178
		YE	-.66667	.59094	.909	-2.6845	1.3511
	BC	SA	1.50000	.59094	.217	-.5178	3.5178
		MRSA	2.33333*	.59094	.019	.3155	4.3511
		BS	-.83333	.59094	.788	-2.8511	1.1845
		KP	-1.33333	.59094	.328	-3.3511	.6845
		EC	1.83333	.59094	.086	-.1845	3.8511
		YE	1.66667	.59094	.139	-.3511	3.6845
	BS	SA	2.33333*	.59094	.019	.3155	4.3511
		MRSA	3.16667*	.59094	.002	1.1489	5.1845
		BC	.83333	.59094	.788	-1.1845	2.8511
		KP	-.50000	.59094	.975	-2.5178	1.5178
		EC	2.66667*	.59094	.007	.6489	4.6845

		YE	2.50000*	.59094	.011	.4822	4.5178
	KP	SA	2.83333*	.59094	.004	.8155	4.8511
		MRSA	3.66667*	.59094	.000	1.6489	5.6845
		BC	1.33333	.59094	.328	-.6845	3.3511
		BS	.50000	.59094	.975	-1.5178	2.5178
		EC	3.16667*	.59094	.002	1.1489	5.1845
		YE	3.00000*	.59094	.002	.9822	5.0178
	EC	SA	-.33333	.59094	.997	-2.3511	1.6845
		MRSA	.50000	.59094	.975	-1.5178	2.5178
		BC	-1.83333	.59094	.086	-3.8511	.1845
		BS	-2.66667*	.59094	.007	-4.6845	-.6489
		KP	-3.16667*	.59094	.002	-5.1845	-1.1489
		YE	-.16667	.59094	1.000	-2.1845	1.8511
	YE	SA	-.16667	.59094	1.000	-2.1845	1.8511
		MRSA	.66667	.59094	.909	-1.3511	2.6845
		BC	-1.66667	.59094	.139	-3.6845	.3511
		BS	-2.50000*	.59094	.011	-4.5178	-.4822
		KP	-3.00000*	.59094	.002	-5.0178	-.9822
		EC	.16667	.59094	1.000	-1.8511	2.1845
Methanol	SA	MRSA	-.50000	.48795	.940	-2.1661	1.1661
		BC	-1.33333	.48795	.160	-2.9995	.3328
		BS	-1.33333	.48795	.160	-2.9995	.3328
		KP	-.83333	.48795	.622	-2.4995	.8328
		EC	-.66667	.48795	.810	-2.3328	.9995
		YE	-1.33333	.48795	.160	-2.9995	.3328
	MRSA	SA	.50000	.48795	.940	-1.1661	2.1661
		BC	-.83333	.48795	.622	-2.4995	.8328
		BS	-.83333	.48795	.622	-2.4995	.8328
		KP	-.33333	.48795	.992	-1.9995	1.3328
		EC	-.16667	.48795	1.000	-1.8328	1.4995
		YE	-.83333	.48795	.622	-2.4995	.8328
	BC	SA	1.33333	.48795	.160	-.3328	2.9995
		MRSA	.83333	.48795	.622	-.8328	2.4995
		BS	.00000	.48795	1.000	-1.6661	1.6661
		KP	.50000	.48795	.940	-1.1661	2.1661
		EC	.66667	.48795	.810	-.9995	2.3328
		YE	.00000	.48795	1.000	-1.6661	1.6661
	BS	SA	1.33333	.48795	.160	-.3328	2.9995
		MRSA	.83333	.48795	.622	-.8328	2.4995

		BC	.00000	.48795	1.000	-1.6661	1.6661
		KP	.50000	.48795	.940	-1.1661	2.1661
		EC	.66667	.48795	.810	-.9995	2.3328
		YE	.00000	.48795	1.000	-1.6661	1.6661
	KP	SA	.83333	.48795	.622	-.8328	2.4995
		MRSA	.33333	.48795	.992	-1.3328	1.9995
		BC	-.50000	.48795	.940	-2.1661	1.1661
		BS	-.50000	.48795	.940	-2.1661	1.1661
		EC	.16667	.48795	1.000	-1.4995	1.8328
		YE	-.50000	.48795	.940	-2.1661	1.1661
	EC	SA	.66667	.48795	.810	-.9995	2.3328
		MRSA	.16667	.48795	1.000	-1.4995	1.8328
		BC	-.66667	.48795	.810	-2.3328	.9995
		BS	-.66667	.48795	.810	-2.3328	.9995
		KP	-.16667	.48795	1.000	-1.8328	1.4995
		YE	-.66667	.48795	.810	-2.3328	.9995
	YE	SA	1.33333	.48795	.160	-.3328	2.9995
		MRSA	.83333	.48795	.622	-.8328	2.4995
		BC	.00000	.48795	1.000	-1.6661	1.6661
		BS	.00000	.48795	1.000	-1.6661	1.6661
		KP	.50000	.48795	.940	-1.1661	2.1661
		EC	.66667	.48795	.810	-.9995	2.3328

*. The mean difference is significant at the 0.05 level.

Table A.4: Homogeneous subsets of the means group in positive control.

Positive_Control

Tukey HSD[a]

Bacteria	N	Subset for alpha = 0.05	
		1	2
MRSA	9	23.3333	
KP	9	25.0556	
YE	9	25.0556	
EC	9	26.0000	
BC	9	26.7222	
BS	9	27.4444	
SA	9		32.5556
Sig.		.072	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

Table A.5: Homogeneous subsets of the means group in hexane extract.

Hexane

Tukey HSD[a]

Bacteria	N	Subset for alpha = 0.05		
		1	2	3
SA	3	.0000		
BS	3	.0000		
KP	3	.0000		
MRSA	3		6.8333	
YE	3		6.8333	
BC	3		7.1667	
EC	3			8.6667
Sig.		1.000	.786	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table A.6: Homogeneous subsets of the means group in chloroform extract.

Chloroform

Tukey HSD[a]

Bacteria	N	Subset for alpha = 0.05			
		1	2	3	4
MRSA	3	.0000			
BC	3	.0000			
EC	3	.0000			
SA	3		6.8333		
YE	3		7.5000	7.5000	
KP	3			8.3333	
BS	3				9.3333
Sig.		1.000	.232	.084	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table A.7: Homogeneous subsets of the means group in ethyl acetate extract.

Ethyl Acetate

Tukey HSD[a]

Bacteria	N	Subset for alpha = 0.05				
		1	2	3	4	5
SA	3	7.6667				
BS	3	9.1667	9.1667			
EC	3	9.6667	9.6667	9.6667		
MRSA	3		11.1667	11.1667		
YE	3			12.1667	12.1667	
BC	3				14.3333	
KP	3					18.0000
Sig.		.200	.200	.068	.142	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table A.8: Homogeneous subsets of the means group in acetone extract.

Acetone

Tukey HSD[a]

Bacteria	N	Subset for alpha = 0.05		
		1	2	3
MRSA	3	9.1667		
EC	3	9.6667	9.6667	
YE	3	9.8333	9.8333	
SA	3	10.0000	10.0000	
BC	3		11.5000	11.5000
BS	3			12.3333
KP	3			12.8333
Sig.		.788	.086	.328

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table A.9: Homogeneous subsets of the means group in methanol extract.

Methanol

Tukey HSD[a]

Bacteria	N	Subset for alpha = 0.05
		1
SA	3	9.3333
MRSA	3	9.8333
EC	3	10.0000
KP	3	10.1667
BC	3	10.6667
BS	3	10.6667
YE	3	10.6667
Sig.		.160

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.