ANTIBACTERIAL ACTIVITY DETERMINATION OF EXTRACT FROM LEAF OF *Terminalia catappa*

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DECLARATION

I hereby 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 Determination of Extract from Leaf of *Terminalia catappa* by Fatin Nurilyana Binti Muhd Hisyam with the matric number F18B0033 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Food Security), Faculty of Agro-Based Industry, University Malaysia Kelantan.

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Table of Contents

LIST OF TABLE

LIST OF FIGURE

LIST OF ABBREVIATION

Number	Abbreviation	Meaning
1	AMR	Antimicrobial Resistance
2	TCST	Two component signal transduction
3	WHO	World Health Organization
4	SFE	Supercritical fluid extractions
5	CO_2	Carbon dioxide
6	S-CO_2	Supercritical carbon dioxide
7	PLE	Pressurized liquid extraction
8	MRSA	*Methilin-resistant Staphylococcus aureus*
9	NA	Nutrient agar
10	MHA	Mueller Hinton agar
11	DMSO	Dimethyl sulfoxide
12	TLC	Thin layer chromatography
13	NaOH	Sodium hydroxide
14	HCl	Hydrochloric acid
15	$FeCl_3$	Ferric chloride
16	KOH	Potassium hydroxide

LIST OF SYMBOLS

Number	Symbols	Meaning
1	ml	Milliliter
2	mm	Millimeter
3	g	Gram
4	mg	Milligram
5	SD	Standard deviation
6	°C	Degree celsius
7	%	Percentage
8	v	Volume

Antibacterial Activity Determination Of Extract From Leaf Of *Terminalia catappa*

ABSTRACT

Since the beginning of antibiotic era in 1928, numerous antibiotics have been found or synthesized from natural resources. Nowadays, many studies have been conducted intensively To tackle the antibiotic resistance dilemma that becoming increasing lately. Therefore, the purposes of this research were to study the antibacterial potential of five different extracts (hexane, chloroform, acetone, ethyl acetate, and methanol) of *T. catappa* leaves on selected Gram-positive and Gram-negative bacteria using disc diffusion assay and to preliminary screen the group of compound present in leaves extract of *T. catappa*. Result of the antibacterial activity showed that all of the extracts were able to inhibit Gram-positive and Gram-negative bacteria with zone inhibition ranging from 5.33 until 24.00 mm. However, acetone extracts of *T. catappa* showed better zone of inhibition, 24.00 mm against *Bacillus cereus* and *Staphylococcus aureus* compared to other extracts. Furthermore, chloroform, acetone, ethyl acetate, and methanol extracts of *T. catappa* revealed that the presence of secondary metabolites such as flavonoids, alkaloids, phenols, lactones, and anthraquinone through chemical profiling of antibacterial compound using TLC plates. In conclusion, acetone, ethyl acetate, and methanol extracts of *T. catappa* have the potential as a new source of antibacterial agent and can become a valuable source of novel medications due to their promising potential in antibacterial testing.

Keywords: antibacterial, antibacterial agents, secondary metabolites, thin layer chromatography, Terminalia catappa

Aktiviti Antibakteria Penentuan Ekstrak Dari Daun *Terminalia catappa*

ABSTRAK

Sejak permulaan era antibiotik pada tahun 1928, banyak antibiotik telah ditemui atau disintesis daripada sumber semula jadi. Pada masa kini, banyak kajian telah dijalankan secara intensif untuk menangani dilema rintangan antibiotik yang semakin meningkat akhir-akhir ini. Oleh itu, tujuan penyelidikan ini adalah untuk mengkaji potensi antibakteria lima ekstrak berbeza (heksana, kloroform, aseton, etil asetat, dan metanol) daun T. catappa ke atas bakteria Gram-positif dan Gram-negatif terpilih menggunakan ujian resapan cakera dan untuk menyaring awal kumpulan sebatian yang terdapat dalam ekstrak daun T. catappa. Keputusan aktiviti antibakteria menunjukkan bahawa semua ekstrak mampu menghalang bakteria Gram-positif dan Gram-negatif dengan perencatan zon antara 5.33 hingga 24.00 mm. Walau bagaimanapun, ekstrak aseton T. catappa menunjukkan zon perencatan yang lebih baik, 24.00 mm terhadap Bacillus cereus dan Staphylococcus aureus berbanding ekstrak lain. Tambahan pula, ekstrak kloroform, aseton, etil asetat, dan metanol T. catappa mendedahkan bahawa kehadiran metabolit sekunder seperti flavonoid, alkaloid, fenol, lakton, dan antrakuinon melalui pemprofilan kimia sebatian antibakteria menggunakan plat TLC. Kesimpulannya, ekstrak aseton, etil asetat, dan metanol T. catappa mempunyai potensi sebagai sumber baru agen antibakteria dan boleh menjadi sumber berharga ubat baru kerana potensinya yang menjanjikan dalam ujian antibakteria.

Kata kunci: antibakteria, agen antibakteria, metabolit sekunder, kromatografi lapisan nipis, Terminalia catappa

CHAPTER 1

INTRODUCTION

1.1 Research background

Asia is one of the largest biodiversity regions in the world, having most of the richest countries in the term of plant resources. Malaysia is a home of great diversify of forests and is in the 17th place for mega biodiversity in the country for the exceptional numbers of unique plant and animal species found along the forest (Safavi *et al*., 2015). Malaysia is located in the equatorial region and has a tropical rainforest climate which helps the medicinal plants to maintain their importance value. These plants are widely known since thousands of years for their medicinal value that can cure any sickness. Due to the research findings about their useful of biological compounds, Malaysian herbal medicine market had experienced an extraordinary growth.

The species of *Terminalia catappa* which also known as Indian almond, comes from *Combretaceae* family is a large tropical tree found in biodiversity of Malaysia and constantly growing in other tropical country. Some of the bioactive compounds, as well as their molecular interactions, have been established in previous studies (Venkatalakshmi *et al*., 2014).

The leaves of *T. catappa* carries medicinal properties such as flavonoids and tannins that has been used by local people from long time ago as they believed with use of medicinal plants can help to treat any wound, skin infection as well as possess for antimicrobial, anticancer and antioxidant properties (MN, 2017). The aquarists placed the leaves of the *T. catappa* in an aquarium as research proved that the releasing of tannins from the leaves gradually into water can lower the pH of water (devi *et al*., 2019).

1.2 Problem statement

People have had to adapt a variety of challenges for thousands of years, including hunger caused by diseases in plants, sickness in humans such as diabetes and virus infections. Since Alexander Fleming's discovery of penicillin in 1928 (Landecker, 2016), it has been shaped the field of antibiotics and has been used to treat a variety of diseases all around the world. Antimicrobial Resistance (AMR) is widely known as a global health and development threat. AMR occurs when bacteria, viruses, fungi or parasites continue to evolve and lose their ability to react towards

antibiotics. These problems that makes infections more difficult to handle and maximize the risk of disease transmission, serious illness and death.

Antibiotic resistance raises treatment costs, extends hospital stays and increases mortality rates. The way antibiotics are prescribed and used in the world needs to change immediately. Even if new drugs are created, antibiotic resistance will continue to be a major problem unless people change their habits of taking all those synthetic antibiotics. Besides, antibiotics resistance is accelerated due to the misuse and overuse of antibiotics, as well as poor prevention and control.

To address these issues, scientist had found novel bioactive compound with the application of antimicrobial possess from the medicinal plant's leaf extract. Herbal remedies' active compounds have the advantage of being mixed with a variety of other chemicals that tend to be inactive. These complementary components, on the way round, give the plants as a whole a degree of protection and efficiency well beyond that of its isolated and pure active components (Hejniak *et al*., 2019).

1.3 Objectives of study

i. To prepare the leaves extract of *T. catappa* using sequential solvent extraction method.

ii. To investigate the antimicrobial potential of five different extracts of *T. catappa* leaves (hexane, chloroform, acetone, ethyl acetate, and methanol) on the selected Gram-positive and Gram-negative bacteria using disc diffusion assay.

iii. To preliminary screen the group of compound present in the leaves extract of *T. catappa.*

1.4 Hypothesis

The leaves of *T.catappa* are thought to have antibacterial activity against both Gram-positive and Gram-negative bacteria. This extract is hypothesised to have antibacterial potential and could be developed as an antimicrobial agent.

1.5 Scope of study

The present study was focusing on the antibacterial activity of the leaves of local medicinal plant, *T. catappa*. The preparation of *T. catappa* leaf extract was performed using sequential extraction method using the increasing polarity of solvent from hexane, chloroform, acetone, ethyl acetate, and methanol. The antibacterial potential of this leaf extract will be evaluated by using a few selected pathogenic Gram-negative and Gram-positive bacteria using disc diffusion assay method. The preliminary detection of bioactive compounds in the extracts was performed using developed TLC plates and sprayed with respective reagents to detect the presence of group of organic compounds.

1.6 Significance of study

Many studies on natural compounds with significant pharmacological activity have been conducted recently. Local medicinal plants have been shown to have

antibacterial properties due to their ability to synthesize a variety of bioactive compounds (Mickymaray, 2019). Antibacterial substances generated from medicinal plants can be used to develop of novel antimicrobial agents to counteract the recent rise in bacterial resistance to existing antibiotics (Abuga *et al*., 2021).

Medicinal plants are fascinating sources that can be used to isolate many potential antimicrobial agents. Therefore, the current study is carried out to investigate the potential of antibacterial activity from local medicinal herb *T. catappa* which is believed to possess antibacterial properties. The finding from this study may give an insight to pharmaceutical companies in developing natural antimicrobial agent

CHAPTER 2

LITERATURE REVIEW

2.1 The history of antibiotic resistant

Antibiotics are one type of medicines that helps to stop any infections that comes from bacteria by killing the bacteria or just keeping them with copying themselves or just reproducing. The majority of bacteria in humans' body are harmless but some of them are also beneficial. However, bacteria can also damage nearly every organ, fortunately antibiotics will normally help (Durand *et al*., 2019). Penicillin was the first commercialized antibiotic that had been discovered in 1928 by Alexander Fleming (Mohr, 2016). During 1940s, antibiotic was first prescribed to treat serious infections. Along with the discovery of a new antibiotic, resistance to that antibiotic has been detected and identified. During World War II, penicillin was administered to the general population for surgical purposes and to the Allied Forces to prevent any wound infections (Ragheb *et al*., 2019).

Antibiotic resistance is escalating around the world. New resistance mechanisms are arising and spreading across the country, posing a challenge to humanity's ability to handle common infectious diseases (Jacoby, 1999). As antibiotic become less effective, an increasing list of infections like pneumonia, tuberculosis, blood poisoning, gonorrhoea and foodborne diseases are becoming more difficult to treat as antibiotics become less effective (Zaman *et al*., 2017). Nevertheless, antibiotic resistance is a matter of interest to epidemiologists, microbiologists, doctors, health economists and public health officials who are concerned with the practical issue of what to do in the event of crisis (Ragheb *et al*., 2019).

2.2 Development of antibacterial agent

Antibacterial agent or antibiotic is a substance that prevents bacteria from growing and reproducing. Antibiotics are now more widely known as agents that are used to disinfects surfaces and kill bacteria that may be harmful (Theuretzbacher *et al*., 2020). Antibacterial agents are a class of materials that can combat bacteria that cause disease. Thus, bacteria's pathogenic impact in biological settings can be reduced by destroying or reducing their metabolic activity (Belete, 2019).

Thinking back on the evolution of human disease, infectious disease has witnessed a significant increase portion of all diseases. Microorganisms were discovered to be responsible for a number of infectious disease that had ruined

humans since ancient times until the latter half of the 19th century (Durand *et al*., 2019). Salvarsan, a syphilis treatment developed by Ehrlich in 1910, was the world's first antimicrobial agent then followed by Domagk and other researchers invented sulphonamides in 1935 (Bahar & Ren, 2013).

Generally, the development of antibacterial agents from the past years till now have been classified into three main groups which are inhibition of cell wall synthesis, inhibition of protein synthesis, and inhibition of bacterial nucleic acid synthesis (Vila *et al*., 2020). Continuing advances have been made for antimicrobial agents in different ways in addition to the antimicrobial continuum and operation (Don *et al*., 2016). The drugs have been designed to achieve improved pharmacodynamics including the absorption of oral drugs, concentration in the blood and distribution to the inflammatory emphasis (Lam *et al*., 2018).

Antibacterial agent has been steadily increasing in many common bacterial pathogens such as *Staphylococcus aureus, Streptococcus pneumoniae* and *Enterococcus faecalis* in recent years (Tesfaye *et al*., 2020). Table 2.1 shows the examples of intrinsic resistance and its mechanism. The type of organism were classified according to the antibiotic class that believed can be the antibacterial agents in order to kill all of those type of bacteria. So, there is a significant need for the discovery and creation of new antibiotic groups to supplement our existing inventory. Only till today, the pharmaceutical industry's primary strategy was to pursue gradual changes in existing products.

Table 2.2: The examples of intrinsic resistance and its mechanism (Sun *et al*., 2020) (Yang *et al*., 2014)

Organism	Antibiotic class	Mechanism of resistance
Anaerobic bacteria	Aminoglycosides	Lack of oxidative mechanisms which reduces the uptake of antibiotics
Aerobic bacteria	Metronidazole	Inability to reduce the drug anaerobically
Gram-positive bacteria	Aztreaonam	Lack of penicillin binding site
Gram-negative bacteria	Vancomycin	Existence of outer membrane layer around cell membrane blocks the vancomycin uptake
Klebsiella sp.	Ampicillin	Production of beta-lactamases that degrades the antibiotic before reaching penicillin binding site
Lactobacilli	Vancomycin	Lack of binding site on cell wall which not allow the antibiotic to bind and interrupt cell wall

Bacteria have several two component signal transduction (TCST) systems which usually include a membrane-bound histidine kinase and a cytoplasmic response regulator (Motamedi *et al*., 2015). Bacteria use these mechanisms to track and adapt to their external environment in order to survive. Consequently, finding a large spectrum selective inhibitor of TCST systems will jeopardise the bacteria's ability to adapt and function in any climate, making this a promising antibacterial strategy (Pushkareva *et al*., 2017).

2.3 Medicinal plant as a source of antibacterial agent

Microorganisms have developed resistance to antibiotics as a result of their widespread use. In addition to this problem, antibiotics have been linked to a variety of side effects in humans, including hypersensitivity, depletion of beneficial gut and mucosal microorganisms, immunosuppression and allergic reactions (Shaik *et al*., 2014). This has resulted in a significant clinical issue in the treatment of infections, in which the alternative antimicrobial drugs for the treatment of infectious diseases are required and in this case, local medicinal plants are depicted as the potential source of natural antimicrobial drugs (Elnaggar *et al*., 2016). Based on World Health Organization (WHO), herbal medicine has been used by quarter of our world's population and also a group of traditional therapies actively includes the beneficial crude extracts from the plant to treat people (Adnan *et al*., 2014).

The development and pathogenicity variety of microbes have been shown to be inhibited by plants used in conventional medical practices against contaminants. As an adaptions for self-defence and contact with other species in their environments, plants synthesize a wide range of chemicals known as secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids (Tungmunnithum *et al*., 2018). Furthermore, these secondary metabolites have a number of benefits for the production of anti-infective drugs includes being typically bioactive, drug-like and

metabolite-like also having the potential for synergy with other secondary metabolites as part of a plant's multicomponent protection mechanism (Puspita *et al*., 2019).

The antimicrobial function of herbs plants in particular, has been used in a variety of applications like raw and modified food preservations, pharmaceuticals, herbal medicine, and natural therapies (Motamedi *et al*., 2015). Medicinal plants have been found to be a possible source for new antimicrobial agents. Presently, plant derivatives have been used as antimicrobials almost exclusively by the pharmaceuticals in United States since they found healing points in these plants (Egamberdieva *et al*., 2017). Medicinal plant having an almost infinite number of aromatic compounds, the majority of which are phenols or their oxygen-substituted derivatives. These substances are also used by plants to protect themselves from microorganisms, insects and herbivores (Chauhan *et al*., 2017). There are many types of antimicrobial phytochemicals that can be used from these medicinal plants.

Next is the flavones, flavonoids and flavanols. Flavones have phenolic structures that contains one carbonyl group, but the addition of 3-hydroxyl group yields a flavanol. However, flavonoids are also having the hydroxylated phenolic substances but it occur as a C_6-C_3 unit linked in aromatic rings. It is not shocking that they have been found to be successful antimicrobial substances in vitro against a broad range of microbes. Their ability to complex with extracellular and soluble proteins, also bacterial cell walls contributes for their activity. Microbial membranes can be disrupted by more lipophilic flavonoids (Ghasemzadeh & Ghasemzadeh, 2011).

Other than that, cancer is a disease that affects populations worldwide. New treatments to cure and prevent this life-threatening disease are still in demand (Bussa

& Belayneh, 2019). Natural-derived compounds are attracting scientific and academic attention because they are thought to have less toxic sides effects than conventional therapies like chemotherapy (Kadima *et al*., 2016). Multiple researchers have described plant species with anticancer properties with a particular emphasis on those used in herbal medicine in developing countries (Bussa & Belayneh, 2019). Polyphenols' cytotoxicity on a variety of cancer cells has been shown and their antioxidant properties have been determined (Kuruppu *et al*., 2019). Besides, polyphenols are thought to induce apoptosis and therefore have anticancer properties that can be used (Rao *et al*., 2016). Plant polyphenols also have the ability to inhibit the growth of cancer cells by interfering with proteins that found in those cells (Ling *et al*., 2019). As mentioned by (Ozkan *et al*., 2016) by directly bonding with cancer agents, those polyphenols can regulate acetylation, methylation or phosphorylation.

2.4 Medicinal plant extract as a source of antibacterial agent

Medicinal plant extracts as well as other alternative medical therapies, have become increasingly common in recent years all over the world. Antimicrobial plant extracts catch the attention of clinical microbiologists. Historically, crude extracts of various sections of medicinal plants such as the root, stem, flower, fruits, leaves and twigs were commonly used to treat a variety of human diseases naturally (Alsammarraie *et al*., 2018). Despite the fact that oregano essential oils and its component carvacrol increased the incidence of apoptotic cell death by a small

amount, they demonstrated substantial antimicrobial activity even at low concentrations (Tesfaye *et al*., 2020).

Natural antiseptic properties of plants have long been used in medicine. As a result, research has focused on the properties and applications of these plants extracts in the preparation of potential nanomaterial-based drugs for diseases such as cancer (Siew *et al*., 2019). Several plant species are now being used to cure or prevent cancer. Anticancer medicines extracted from plants are preferred because they are safe and readily available. They are simple to prescribe orally as part of a patient's diet. Since they are derived from plants, they are usually more tolerable and non-toxic to normal human cells (Tesfaye *et al*., 2020).

Apart from that, food spoilage which is caused by microorganisms also affects all forms of food and results in food waste and loss, particularly in developed countries (Xu *et al*., 2017). The consequence, new environmentally friendly methodologies are needed to reduce pathogenic bacteria growth and extend the shelf life of food products without the adding of chemical preservatives. Many studies have recently looked into the possibility of using plant extract as natural preservatives (Aziman *et al*., 2014). The Dorigo pepper, *Tasmania stipitate* is an endemic Australian plant that have similar taxonomically with *Tasmania lanceolata*, which has been used for food flavouring and shown to have medicinal properties too (Yadav *et al*., 2019).

2.5 *Terminalia catappa*

Table 2.5: Scientific classification of *T. catappa*

Kingdom	Plantae
Order	Myrtales
Family	Combretaceae
Genus	*Terminalia*
species	*T. catappa*

Terminalia catappa is also commonly called as tropical almond or Indian almond is a medium to large deciduous tropical tree with a spreading crown and tier of horizontal branching that grows to 75'-90' tall but often lower in cultivation. It is native to Asia's coastal regions, Polynesia and northern Australia but it is now cultivated in a variety of tropical and subtropical locations around the world. The genus of Terminalia to the family of *Combretaceae* is a type of monoecious tree. This ornamental tree is grown for the deep shade it provides with its huge leaves. The fruit is edible and has a mildly acidic flavour while the wood is reddish-brown in colour and has a high water resistance.

The leaves having several flavonoids like kaempferol or quercetin, several tannins such as punicalin, punicalagin or tercatin, also contains saponines and phytosterols. The leaves and the bark are used in various purposes due to their

chemical richness (Oyeleye *et al.*, 2018). Addition to that, by keeping the leaves in an aquarium will cause the water's pH and heavy metal content to drop. For several years, fish breeders have used it in this way and it is effective against parasites and bacterial pathogens (Claudiano *et al.*, 2012). It is even intended to help keep fungi from growing on the fish's eggs (Webster *et al*., 2017).

In addition, punicalin and punicalagin the anti-AIDS compounds that found in water extract of *T. catappa* leaves, have been reported (Ben *et al*., 2019). Tannin and flavonoid glycosides from *T. catappa* leaves displayed free radical effect thus inhibit Cu^{2+} induced LDL oxidation (Kankia, 2014). The high contents of tannins in *T. catappa* leaves that they may provide as a source of essential antioxidants. Depending on the dosage, water extract of *T. catappa* dried leaves prevented lipid peroxidation in vitro and TPA-induced hydrogen peroxide production in human mononuclear leukocytes (Tenpe *et al*., 2008). The antioxidants potential of *T. catappa* leaves also has been suggested as a possible explanation for their anticlastogenic impact (Chyau *et al*., 2002).

A wound occurs when the living tissues' cellular and functional ability is lost or broken. The development of synthetic antibacterial medicines to heal wounds was hampered by antibiotic resistance and toxicity. According to the findings (Cock, 2015), applying *T. catappa* ointment to a wound reduces the wound are by 97% when compared to a control, 81% and betadine ointment as the conventional treatment. *T. catappa* ointment accelerates epithelization, implying that the bark extracts have significant wound-healing properties (Nugroho *et al.*, 2019).

Moreover, diabetes is becoming more prevalent in both emerging and developed countries (Ceriello *et al*., 2020). Carbohydrate, protein, and lipid

metabolism are all disrupted by diabetes. Natural medicines derived from plants may represent culturally appropriate complementary or alternative treatments, as well as provide strong clinical chances and aid in the hunt for new antidiabetic drugs (Chaachouay *et al*., 2019). In alloxan-induced animal models, the aqueous and cold extract of fresh leaves of *T. catappa* has the ability to lower elevated blood glucose levels and lipids (Behl & Kotwani, 2017). Rats were also shown to exhibit hypocholesterolaemia effects from *T. catappa* fruit extract and *T. catappa* fallen dry leaves extract (Ratnasooriya *et al*., 2002).

2.6 Basic extraction for medicinal plant extract

Antioxidant compounds are usually extracted from plant materials using various extraction techniques (Ranilla *et al*., 2010). The procedure of extraction will be the key to determine how much of the desired bioactive components that have been extracted from the parts of the plants. However, this will be different in every sample that being tested as the solvent used is one of the most contributing factors in extraction methods (Kaur, 2018). The separation of medicinally active parts of plant or animal tissues from inactive or inert materials using selective solvents is known as standard extraction procedure (Yadav *et al*., 2019). Plants produce comparatively impure liquids, semisolids, or powders that are only intended for oral external use. So, standardization of extraction procedures has a significant impact on the herbal drug's final consistency (Dhiman *et al*., 2020).

The first elements in separating the desired natural products from the raw materials is extraction. Distillation, sublimation, extraction of solvent and pressing are prior to extraction principle for getting the active portions from the natural products (Azmir *et al*., 2013). The most common approach is solvent extraction. The following steps are involved in the extraction of natural products. Firstly, the penetration between solvent and solid matrix. Next, solutes will dissolve into the solvents. Thirdly, solute will be diffused out from solid matrix and final steps are the desired extract is produced (Mandal *et al*., 2015).

When it comes to solvent extraction, it is important to choose wisely for the solvent that intended to be used. When choosing a solvent, consider the effects of selectivity, solubility, cost and safety (Swami Handa *et al*., 2008). Solvents that have high polarity value tends to work better such as ethanol and methanol. These two types of alcohol are used in wide range for solvent extraction in phytochemical investigations (Saad *et al*., 2015). In general, the crude extraction will produce in good form when it is in small particles as this will increase the extraction efficiency. Thus, extraction period also increases. Long time ago, percolation and maceration are the most used extraction methods to obtain the desired active components from the plant extract (Giacometti *et al*., 2018). As for now in modern era, extraction can be done with shorter extraction time also giving benefits of lowering the intake of organic solvent.

2.6.1 Maceration extraction

Maceration is a straightforward extraction method that while it is efficient, it has the flaws of a long extraction time and low performance. This technique entails soaking the raw material, either coarse or powdered in a chosen solvent for at least 3 days at room temperature with regular agitation (Chen *et al*., 2016). As to concentrate the substance, the solvent is extracted from the mixture after extraction usually by vacuum evaporation. The choice of solvent is critical in this process since it defines the types of compounds retrieved from the samples and also allows for the extraction of thermolabile components using maceration technique (Deng *et al*., 2017).

2.6.2 Percolation extraction

Percolation is a method of removing all of the soluble constituents from a comminute plant substance by extracting the crude drug with a new solvents. The percolate is reintroduced as the solvent in re-percolation, which reduces solvent consumption. Besides, it is the preparation of tinctures and fluid extracts which this is the most popular method for extracting active ingredients (Chanda & Kaneria, 2012). A percolator is a small, cone-shaped vessel that is open on both ends. The percolator's outlet is then opened and the liquid inside is allowed to drip slowly.

The solvent's selectivity is important not only for the yield of one or more principal substances, but also for the qualitative and quantitative composition of the substance that surround them (Zhong *et al*., 2018). Moreover, the mixed falling rate determine the solvent's flow rate. As a result, the contact time between the solvent and the drug is determined. The drug or solvent ratio is the amount of drug used divided by the total amount of solvent use (Ravanfar *et al.*, 2018). Despite the importance of temperature in percolation, it is seldom used as controlling factor.

It plays an important role since some phytoconstituents need a warm environment to extract more effectively, while others may be lost at higher temperatures. As a result, when preparing the percolation process, temperature must always be taken into account. However, the concentration of extracts containing thermolabile substances and the concentration of hydro-alcoholic mixtures are numerous risks of the traditional percolation process (Mizuki & Katoh, 2021).

2.6.3 Supercritical Fluids Extraction

Hannay and Hogarth were the first to report supercritical fluids extraction (SFE) and their ability to dissolve low vapour-pressure solid materials in 1879 (Herrero *et al*., 2010). Since then, the use of these fluids as extraction solvents has increased at a distinctive rate. Since SFE associated with natural or organic methods, it helps to improve the appeal of certain goods. Carbon dioxide (CO_2) is the most common supercritical solvent, particularly in the food, pharmaceutical and

nutraceutical industries (da Silva *et al*., 2016). As it is well suited for extracting low volatility compounds like essential oils, lipophilic compounds and slightly polar compounds. Supercritical carbon dioxide (S-CO_2) also may have modifier applied to it to greatly improve its solvating properties (Yousefi *et al*., 2019).

2.6.4 Pressurized Liquid Extraction

Different research groups have referred to pressurized liquid extraction (PLE) as accelerated solvent extraction, enhanced solvent extraction, pressurized fluid extraction and also high pressure solvent extraction (Mustafa & Turner, 2011). When it comes to extraction, PLE uses a lot of pressure. As compared to other approaches, PLE significantly reduced extraction time and solvent usage while also providing improved repeatability (Andreu & Picó, 2019). When compared to conventional low-pressure extraction methods such as maceration, the solvent consumption and extraction time are drastically reduced. However, in order to concentrate the finished product, the solvents must be separated from the extract as much as they must in the conventional method (Liang *et al*., 2020).

2.7 Organic solvent used as extraction compound to dissolve *T. catappa* leaves

Organic compounds are any chemical molecules that have the carbon-hydrogen bonds in chemistry (Majuste *et al.*, 2018). Thus, there are many of other organic compounds that have been identified due to the carbon's potential to form chains with the other carbon atoms. The studies of characteristics, reactions and the syntheses of organic substances are known as organic chemistry. The naming of the organic compounds are varies based on the number and the chain of the carbon-hydrogen bonds they have. The effects of several solvents, including hexane, ethyl alcohol, and methanol on antioxidant extraction from diverse plant parts such as leaves and seed have been investigated and assessed by (Kartika Sari *et al.*, 2018). Diverse solvents with varying polarity must be employed to extract various phenolic compounds from plants with a high degree of precision (Delange & Rico, 2016). (Tzanova *et al.*, 2020) also emphasized that varying solvents resulted in different extraction yields which is due to the fact that changes in the polarity of the extraction solvents could result in a wide range of bioactive compounds levels in the extract. Methanolic extract and ethanolic extract had higher extraction yields than chloroform, dichloromethane, and acetone extracts, showed that highly polar solvents had a better extraction efficiency (Suryavanshi & Saxena, 2019).

CHAPTER 3

MATERIALS AND METHOD

3.1 Preparation of plant material

T. catappa plant sample were collected from Bandar Seri Alam, Masai, Johor by hand picking method. All of the healthy plant leaves sample was washed thoroughly under running tap water in order to remove any remaining dust or debris then they were dried under the shade for 10-12 days. The leaves will be then cut into small pieces followed by grounding in an electric grinder to produce a powder. Then, the powdered leaves will be then kept in a zip lock plastic bag in a desiccator to prevent moisture loss and contamination.

Figure 3.1: Freshly picked leaves of *T. catappa*

3.2 Preparation of extract

The powdered material was extracted using the sequential extraction method following the solvent polarity which was started by using hexane and followed by chloroform, acetone, ethyl acetate, lastly methanol solvent. The powdered leaves were soaked in each solvent for 24 hour. 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 the extracts were obtained. The biomass were dried first before proceeded to further extract using successive solvents following similar manners. Extracts obtained from rotary evaporator was kept in an airtight container at 20°C until further use.

3.3 Test microorganism

In this study, the antibacterial activities for the leaves extract of *T. catappa* will be tested on two different types of bacteria as listed below:

i. Gram-positive bacteria

- *Bacillus subtilis*
- *Bacillus cereus*
- *Staphylococcus aureus*
- *Methilin-resistant Staphylococcus aureus* (MRSA)

ii. Gram-negative bacteria

- *Escherichia coli*
- *Klebsiella pneumonia*
- *Yersinia enterocolitica*

3.4 Preparation of test inoculum and seeded agar plate

A loopful of a pure bacterial colony that will be picked from a 24 hours old bacterial culture on nutrient agar (NA) (Oxoid, England) followed by suspended in a 5 ml of sterile physiological saline (0.85% sodium chloride) solution. The resulting suspension will be vortex for uniform mixing and the suspension turbidity will be

adjusted visually to match 0.5 McFarland standards (approximately 1.5 x 10^8 CFU/mL). After that, a sterile cotton swab will be dipped into the bacterial suspension and pressed hardly on the inside wall of universal bottle to ensure the removal of any excessive inoculum from the cotton swab. The cotton swab will be then streaking over the whole Mueller Hinton Agar (MHA) surface thrice with the rotation angle of 60^0 to ensure the uniform distribution of bacterial inoculum (CLSI, 2006).

3.5 Preparation of extract solution

A total of 20 mg extracts in 0.5 mL dimethyl sulfoxide (DMSO). After the extract was completely dissolved, a total of 0.5 mL of sterile distilled water will be added into the extract to yield a stock with 20 mg/ mL concentration (the concentration of DMSO in the extract stock solution was 50%). Then the extract solution will be filtered using 0.2 µm pore size of sterile nylon membrane.

3.6 Preparation of susceptibility test

Whatman no. 1 filter papers (0.14mm of thickness) that will be punched to become a 6 mm of diameter disc will be autoclaved at 1210°C for 15 min for the sterilization purpose. After that, 10 µL of extract solution (20 mg/mL) will be pipetted onto sterile disc and left it to air dry for a moment prior to impregnated the disc with another 10 µL of extract to produce the disc with 0.4 mg of extract and 1% DMSO.

This disc then will be left air dried prior to placing onto the agar plate that will be seeded with test microorganisms.

3.6.1 Disc diffusion susceptibility test

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

3.7 Statistical analysis

All the experiments will be independently repeated three times, and average zone of inhibition of test extracts relative to negative control was calculated using Microsoft Excel 2007 software.

3.8 Interpretation of results

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

- = No inhibition zones

\+ = Small inhibition zone (≤10 mm)

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

+++ = Large inhibition zone (≥ 21 mm)

3.9 Preliminary chemical profiling of extract

3.9.1 Thin Layer Chromatography (TLC)

The extracts of the *T. catappa* were subjected to a silica gel plate (20x20 cm Silica gel 60G F254 Merck) of thin layer chromatography (TLC). It was sliced into 2cm x 12cm strip and pencil lines were drawn across TLC plate 1.5cm from bottom

and 0.5cm from the top. 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 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).

For around 30 minutes, the TLC plate was activated in an oven at 80°C. They were ready to use once they had completely cooled. An amount of 1mg/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 spot. The TLC silica gel plate was inserted in a beaker containing the particular solvent system and covered with a glass lid to avoid evaporating. The mobile phase was the solvent system, while the stationary phase was silica gel on the TLC plate. The mobile phase was left to run up the TLC plate. The TLC plate was taken out immediately when the solvent reached the end line (0.5cm from the top). The developed TLC plate was a swing for speed drying purpose to further detection.

3.9.2 Preliminary chemical profiling of antibacterial compounds

Bioactive spots detected in section 3.9.1 were proceeded to characterize the group of compounds. The TLC plates that developed in solvent system were used in

detection of various structural groups of compounds such as flavonoids, alkaloids, phenols, lactones, and anthraquinone.

3.9.2.1 Detection of flavonoids

The presence of flavonoid was also tested by spraying with increasing amount of 1M sodium hydroxide (NaOH). Appearance of yellow colour indicated the presence of flavonoid; it discolours after addition of 1M hydrochloric acid (HCl) (Das *et al*., 2019).

3.9.2.2 Detection of alkaloids

Alkaloids were detected using Wagner's reagent (Nuraskin *et al*., 2019). The reagent was prepared by dissolving 1.27g iodine and 2g potassium iodide in 5ml distilled water, afterwards top up with distilled water until 100ml. the developed TLC plate was sprayed with Wagner's reagent. Appearance of reddish brown colour indicated the presence of alkaloid.

3.9.2.3 Detection of phenols

Phenolic compounds can be detected using freshly prepared 1% aqueous ferric chloride ($FeCl_3$). The reagent was prepared by dissolving 1g $FeCl_3$ in distilled water. The developed TLC plate was sprayed with the reagent (Kustrin David W, 2015). Appearance of intense green, purple, blue or black colours indicated the presence of phenols.

3.9.2.4 Detection of lactones

Lactones were detected by placing the developed plates in a chamber containing iodine crystals. The appearance of brown spot indicates a positive reaction (Buchholtz *et al*., 2006).

3.9.2.5 Detection of anthraquinone

TLC plate was sprayed with 10% methanolic potassium hydroxide (KOH), a solution of 100ml methanol and 10g KOH. The change of original colour to red, violet, green or purple showed the presence of anthraquinone (Wahyuni *et al*., 2019).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction of *T. catappa* using different type of solvents

Filter paper and nylon membrane were used to filter all of the milled *T. catappa* leaves with several types of solvents. The leaves were filtered according to the increasing polarity of solvents, which are hexane, chloroform, acetone, ethyl acetate, and methanol. The rotary evaporator extraction method was used to extract the yield of *T. catappa* leaves samples.

Table 4.1.1: Percentage of yield extract and colour of the extract from each type of solvents

SOLVENTS	YIELD EXTRACT (%)	COLOUR
Hexane	0.064	Yellowish green
Chloroform	0.14	Black
Acetone	0.235	Dark green
Ethyl Acetate	0.018	Green
Methanol	1.008	Dark brown

Based on table 4.11, each solvent gave a different amount of yield percentage and appeared in distinct colours due to the biological compounds included in each extract. Ethyl acetate extract appeared to be the lowest yield (0.018%) followed by hexane (0.064%), chloroform (0.140%), acetone (0.235%), and lastly methanol which produce the highest percentage of yield extract (1.008%). The amount of substance that can be extracted by the solvent, as well as the polarity of the solvent, have an impact on the amount of yield collected. Due to the presence of antioxidants in each extract, the colour of the extract differed for each solvent.

Regardless of the fact that all of the oven-dried powdered leaves were immersed in all of the solvents for the same period which was overnight, the crude of each solvent produced was differed due to the polarity of the compounds that appeared in each type of solvents. The solvent polarity, which influences both qualitatively and quantitatively the extracted antioxidant components, has a big influence on the extraction yield and antimicrobial properties of the plant extracts

(Sowmya & Raveesha, 2021). Methanol has the highest yield percentage of all the solvents because of its high polarity, which allows it to absorb more components from the plant extracts. Hence, the higher the polarity of the solvents, the higher the bioactive compounds are absorbed (Dhana Rangesh Kumar *et al*., 2021).

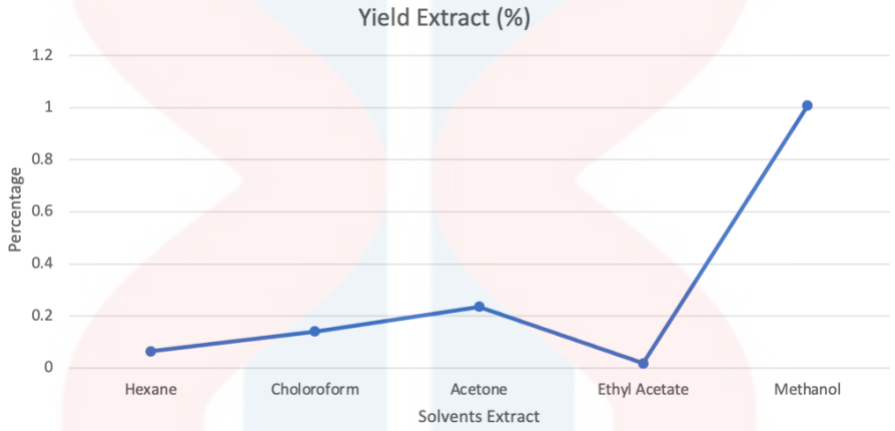

Figure 4.1: The percentage of yield extract for each type of solvents according to the polarity increment

The percentage of yield extract for each type of solvent is shown in Figure 4.1. As can be observed in Figure 4.1, the percentage of yield extract for each type of solvent was increasing from hexane to acetone and dropped significantly for ethyl acetate extract to become the lowest yield (0.018%). This was affected when the extraction is done in a continuous process, mass transfer becomes crucial to the operation's efficiency (Dhanani *et al.*, 2017). Hence, due to the weight of *T. catappa* leaves affected the volume of the solvents to be dissolved with them, the volume of the solvents required to completely dissolved the sample was varied.

Table 4.1.2 shows the weight of *T. catappa* leaves needed to be soaked with desired amount of each type of solvents. The dried *T. catappa* leaves needed to be weighed first before being soaked with each solvents with the ratio of 1g : 20ml as being used in study of (Chirinos *et al*., 2007) that used one gram of *Tropaeolum tuberosum* to be dissolved in 20ml methanol. Solvent extraction is a method for separating soluble phenolic compounds from a solid matrix (plant tissue) by diffusion using a liquid matrix (solvent). This method is widely used to extract phenolic compounds from a variety of plant materials (Jones & Kinghorn, 2012). The different type of solvents, pH temperature, number of extraction steps, ratio solvent/solid, and particle size of the solid matrix are all the elements that influence the solvent extraction process' effectiveness (Dabetić *et al*., 2020). In terms of phenolic extraction, each plant material has its own characteristics. Thus, developing an optimal extraction procedure for quantification and identification is vital (Bentley *et al*., 2020).

Table 4.1.2: The weight of *T. catappa* leaves needed to be soaked with desired amount of each type of solvents

SOLVENTS	**WEIGHT OF *T. catappa* LEAVES (g)**	**AMOUNT OF SOLVENTS NEEDED (ml)**	**BOILING POINT (°C)**
Hexane	105.02	2100.4	69
Chloroform	99.42	1988.4	61.2
Acetone	93.76	1875.2	56
Ethyl Acetate	89.50	1790.0	77.1
Methanol	87.96	1759.2	64

4.2 Antibacterial activity of *T. catappa* towards different extract

The existence of antibacterial activity in plant extracts can be examined using the disc diffusion method (Kader *et al.*, 2012). The inhibition zone formations of the tested extracts are shown on Figure 4.2. The size of the zone of inhibition represent the activity of the extract. The wider the inhibition zone, the stronger the antibacterial potential of the extracts. The positive control, chloramphenicol, will have the biggest inhibitory zone because it is already a pure compound that has been used as an antibiotics. So, if the five extracts can produce a wider inhibition zone than

chloramphenicol, it means that extracts are capable of becoming a potential antibacterial agent.

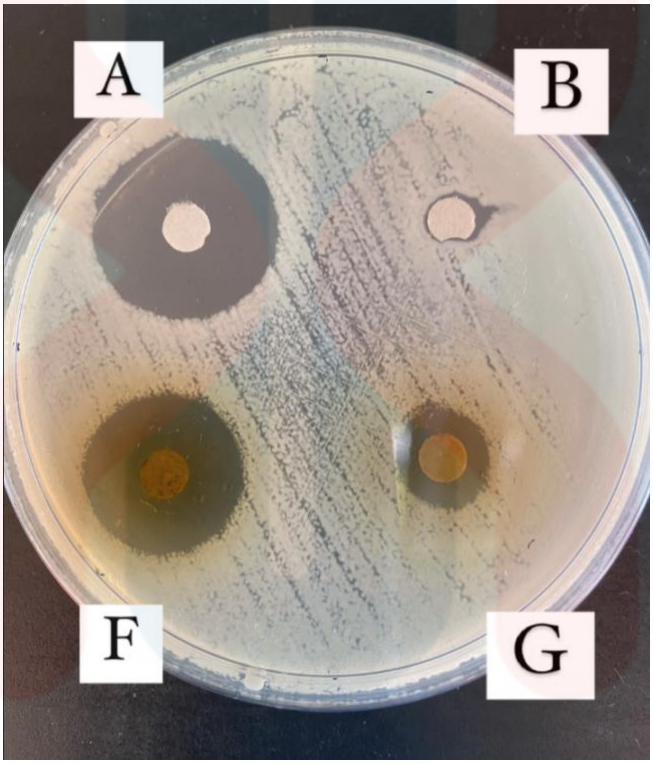

Figure 4.2.1: Agar disc diffusion method for antibacterial activity testing on *B. subtilis* .

[A= Positive Control containing of 30 µg/disc of chloramphenicol, B= Negative Control containing 1% DMSO, F= Acetone extracts and G= Ethyl Acetate extracts.]

Table 4.2.1: Mean of inhibition zone diameter of various extract against test microorganism

	Diameter of inhibition zone (mm ± SD)						
Bacteria	**Hexane**	**Chloroform**	**Acetone**	**Ethyl acetate**	**Methanol**	**Positive control**	**Negative control**
B. subtilis	9.00±1.00	5.33±0.58	20.33±0.58	12.00±0.00	21.33±1.53	23.67±0.58	-
B. cereus	12.67±1.15	13.67±0.58	24.00±1.00	23.67±1.53	21.00±1.00	28.67±1.15	-
MRSA	8.00±1.00	7.00±1.00	22.67±0.58	11.00±1.00	13.33±1.15	19.00±1.00	-
S. aureus	10.00±1.00	6.67±1.53	24.00±1.00	16.67±1.53	17.33±1.53	25.67±1.53	-
Y. enterocolitica	11.00±1.00	11.33±0.58	17.00±1.00	11.00±1.00	18.33±0.58	18.00±0.00	-
K. pneumoniae	7.67±1.53	7.00±0.00	18.00±1.00	10.67±0.58	18.33±1.15	24.67±0.58	-
E. coli	6.67±1.15	6.33±0.58	15.00±1.00	7.00±1.00	17.00±1.00	24.33±0.58	-

Table 4.2.1 shows the mean inhibition zone diameter of various extract against test microorganisms. From the results obtained in Table 4.2.1, all of the extracts were able to inhibit Gram-positive and Gram-negative bacteria with zone inhibition ranging from 5.33 ± 0.58 (mm ± SD) until 24.00 ± 1.00 (mm ± SD). Hexane demonstrated the weakest antibacterial potential reflecting from its lowest value of diameter of inhibition zone. The diameter of the zone of inhibition reflected the potency of the crude extract's ability to retard the pathogen growth. *B. cereus* bacteria

was the most susceptible Gram-positive bacteria followed by *B. subtilis* while *Y. enterocolitica* was most susceptible bacteria compared to the other two Gram-negative bacteria. Each strain of bacteria had varying levels of susceptibility to chemical compounds, which was related to their resistance level.

Based on the polarity of the solvents, all of the extracts were subjected to solvent-to-solvent extraction in this study. Due to metabolites having a high attraction for their polarity equivalents, it is expected that varied polarities of solvents will be able to extract all secondary metabolites formed (Allyn *et al.*, 2018). Figure 4.2.2 show the 21.00 mm zone of inhibition of antibacterial activity of methanol extracts on *B. cereus* and Figure 4.3.3 show the inhibition zone of antibacterial activity of methanol extract on *K. pneumoniae* with the diameter of 18.33 mm.

Acetone extract produced the largest inhibition zone, 24.00 mm against Gram-positive bacteria which are *B. cereus* and *S. aureus*. On the other hand, the Gram-negative bacteria, *E. coli* was not susceptible to methanol extract with the inhibition zone of 17.00 mm which is the lowest reading among *Y. enterocolitica* and *K. pneumoniae* . In term of antibacterial potential of extract, hexane and chloroform possessed a weak antibacterial activity as their inhibitory zones were relatively small as compared to the other extracts as chow in Figure 4.2.4.

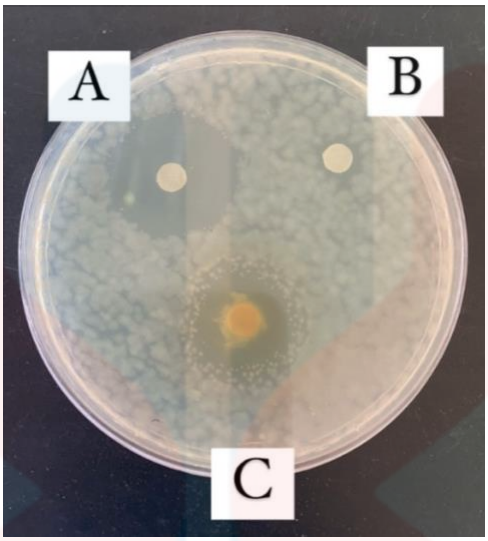

Figure 4.2.2: The inhibition zone of antibacterial activity of methanol extract on *B. cereus*

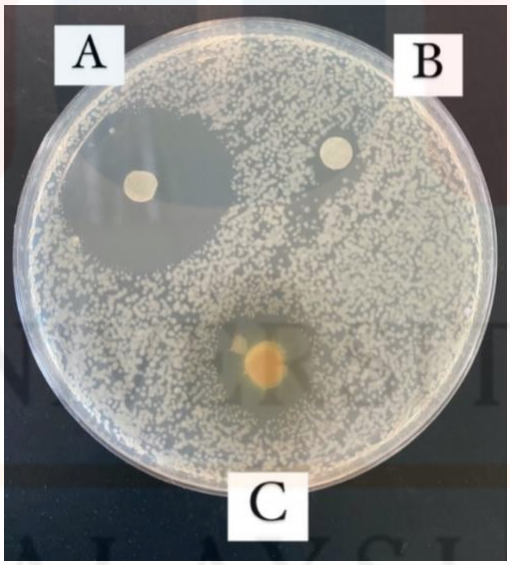

Figure 4.2.3: The inhibition zone of antibacterial activity of methanol extract *on K. pneumoniae*

[A= positive control containing of 30 µg/disc of chloramphenicol, B= negative control containing 1% DMSO, C= methanol extract]

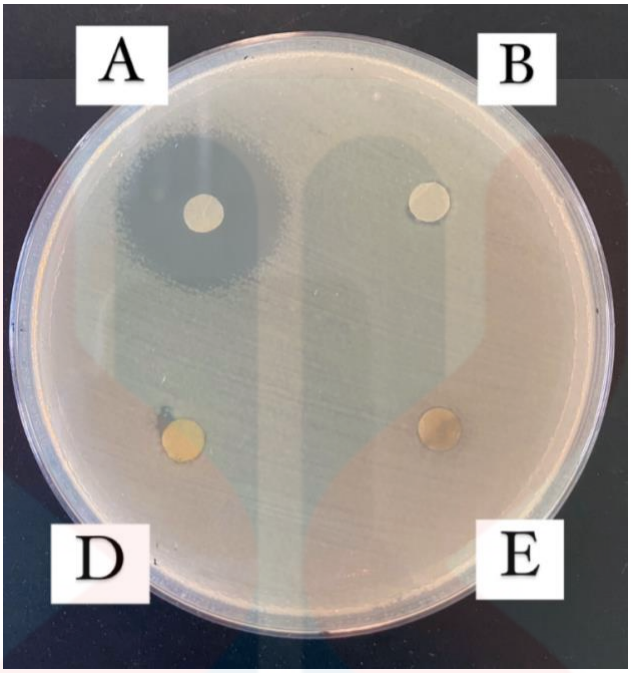

Figure 4.2.4: The inhibition zone of antibacterial activity of hexane and chloroform extract on *E. coli*

[A= positive control containing of 30 µg/disc of chloramphenicol, B= negative control containing 1% DMSO, D= hexane extract, E= chloroform extract]

Since the cell wall of the cell was rigid in nature, the alcoholic solvent was utilized as it has the capability to boost the cell wall permeability and allow for the extraction of large amounts of polar, mid-polar, and low polarity compounds (Yakubu *et al*., 2020). Besides, (Nardiah Rizwana *et al*., 2010) have demonstrated that Gram-positive bacteria are more tolerant to plant extracts than Gram-negative bacteria. These distinctions may be because since Gram-positive bacteria have a single-layer cell wall, whilst Gram-negative bacteria have a multilayered cell wall (Panawala, 2017). The presence of strong peptidoglycan cell wall and teichoic acid at Gram-positive bacteria allows them to stain

whilst, Gram-negative bacteria have a thin peptidoglycan cell wall but an absence of teichoic acid (Dik *et al*., 2018). In addition, Gram-negative bacteria's additional outer membrane, which contains lipopolysaccharides, proteins, and phospholipids, prevents most antibacterial drugs from penetrating or invading first (Lee *et al*., 2019).

4.3 Chemical profiling of antibacterial compound

Thin Layer Chromatography (TLC) is a qualitative technique that has been widely used to distinguish compounds from mixtures that contain several compounds depending on their polarity (Wallace *et al*., 2017). TLC is a popular analytical tool due to its convenience of use, low cost, high sensitivity, and rapidly separation. TLC is used to create well-defined and well-separated spots.

Table 4.3.1: Phytochemical analysis for test compounds on different type of extracts

Extracts	Flavonoids	Alkaloids	Phenols	Lactones	Anthraquinone
Hexane	+	-	+	+	-
Chloroform	+	+	+	+	+
Acetone	+	+	+	+	+
Ethyl Acetate	+	+	+	+	+
Methanol	+	+	+	+	+

Key: (+) present, (-) absence

Table 4.3.1 shows the phytochemical analysis for test compound on different type of extracts. Based on table 4.3.1, hexane extracts show the absence of alkaloids and anthraquinone compounds but the other extracts were having all of the compounds tested. For chloroform, acetone, ethyl acetate, and methanol extracts possessed all of the compounds tested shown from the positive reaction of colour reagent when sprayed on the developed TLC plate. The solvent system used for this test is a mixture of different types of solvent which are non-polar solvent (petroleum ether), mid-polar solvent (ethyl acetate), and polar solvent (methanol) with the ratio of 1:1:1 (v/v/v) (Figure 4.3). The approach can be used to analyze chemicals in plant extracts. The mobile phase in this experiment was a solvent system with several types of solvents with different polarity that give the best separation of the compounds.

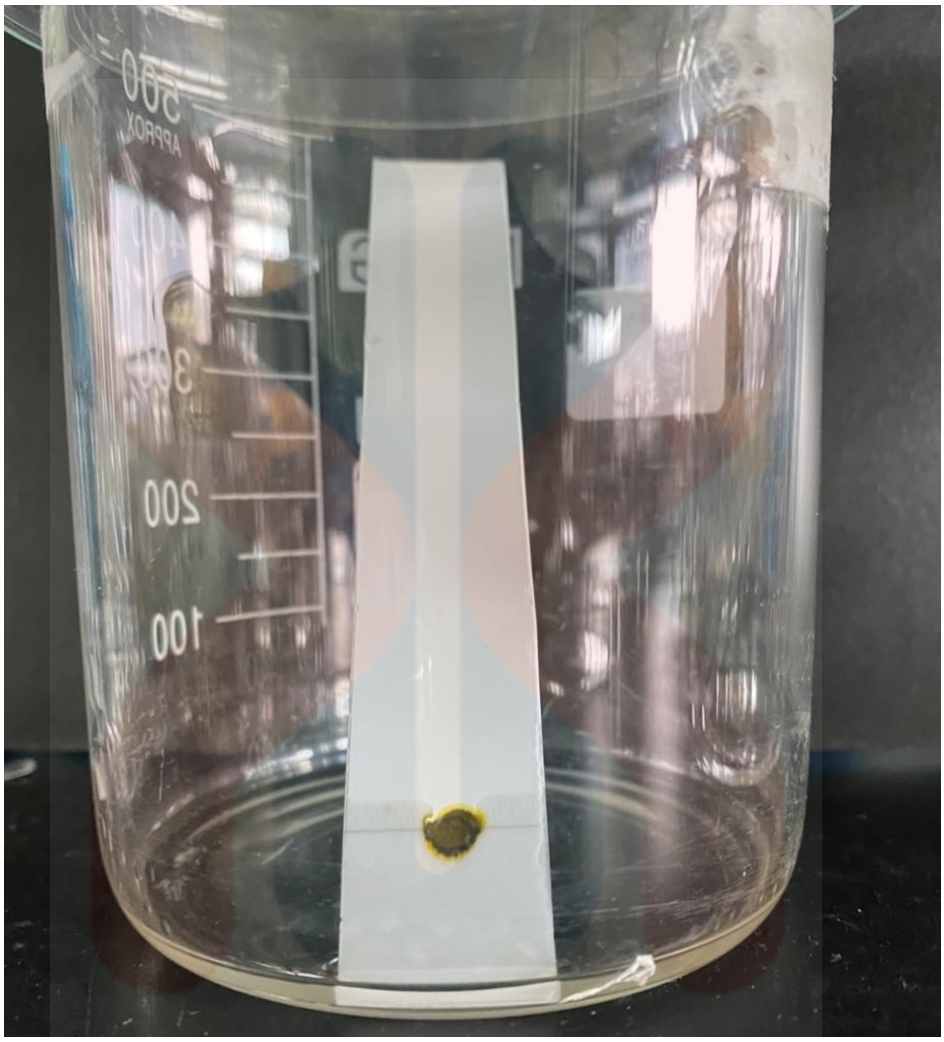

Figure 4.3: Chromatogram developed in a closed chamber.

To separate each compound from a mixture of compounds, TLC method uses polarity interaction between molecules in a mixture and both stationary and mobile phases. This approach is referred to as the transport medium for compounds to be separated since the compounds were moved through the stationary phase by capillary action (Junejo *et al*., 2016). The stationary phase is a sorbent layer that attracts molecule and temporarily binds them. As a result, the molecules move more slowly than compounds that are soluble in the mobile phase (Nahari *et al*., 2019). Hence, the

crude extract was separated into components depending on their polarity and affinity for the stationary phase.

Hexane is the least polar out of the four extracts (Dhana Rangesh Kumar *et al*., 2021), making it incapable of attracting those compounds like alkaloids which are being tested with Wagner's test and also anthraquinones compound. The most active extract is methanol extracts, which the most polar, and it adequately separates components like flavonoids, alkaloids, phenols, lactones, and anthraquinone. It is proof that (Oyeleye *et al*., 2018), solvents extract with a stronger polarity, such as ethanol and methanol are more like to contain secondary compounds. Due to the phytochemical qualities and their variances for each type of extract, it is not surprising that there were disparities in antibacterial activity based on the inhibitory zone around them (Chyau *et al*., 2002).

The chemical profiling of compounds were observed from various extracts of *T. catappa* using different spray reagents. The compound in all five extracts were separated using TLC plate developed using a solvent system of petroleum ether: ethyl acetate: methanol with ration 1:1:1 (v/v/v) prior to spraying with different reagent to observe the presence of a group of organic compound in the extract. When the second TLC plate was sprayed with hydrochloric acid from the first TLC plate which was sprayed with sodium hydroxide, flavonoids components present as shown in Table 4.3.1. Meanwhile, hexane did not produce any positive results. The colour of hexane and chloroform extracts was green, while acetone, ethyl acetate, and methanol extracts were blue-black and purple. The occurrence of phenol compounds in the extracts is indicated by these colours. The developed spot on TLC plate will turn into a brown spots if the lactones presence. Lastly, only hexane did not show the presence

of anthraquinone compared to the other extracts as hexane extract change into colourless when sprayed with 10% potassium hydroxide.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Polar compounds were shown to inhibit Gram-positive and Gram-negative bacteria in this study. Hexane extracts revealed that they were not as potent as methanol extracts in inhibiting antibacterial activity against all of the bacteria tested. *B. cereus* and *S. aureus* were the most susceptible against all extracts. The antibacterial activity of the extracts was concentration dependent, with Gram-positive bacteria were the highest percentage of inhibition by the extracts in this study. In general, Gram-positive bacteria were easier to be inhibited by the extracts compared to Gram-negative bacteria due to the different cell wall components in both groups of bacteria. Gram-negative bacteria possess an outer layer of membrane that act as a barrier for the bioactive compounds to penetrate further into the bacterial cell, whilst the thick peptidoglycan layer in Gram-positive bacteria can easily absorb antibiotic substances that caused them to be more susceptible to the extracts

The types of bioactive molecules varied in different solvents for the same extract, indicating that phytochemical components are solvent dependent. Methanol was found to be a better solvent for extracting secondary metabolites from *T. catappa,* since the highest number of metabolites were present in the extracts, indicating the extracts possess therapeutic qualities. The bioactive compounds provide the extract distinct and effective biological action, making it potent therapeutic agent. These extract were being studied for their therapeutic properties due to the presence of important secondary metabolites that can treat many illnesses where the existing antibiotics available in the market fail to cure due to antibiotic resistance.

Differ extracts had different potential of antibacterial activity against the test bacteria comprising Gram-positive and Gram-negative bacteria. The *T. catappa* leaves under this investigation could be a valuable pharmacological resource and a potential source of novel medications due to its promising potential in antibacterial activity testing. Further research in bioactive compounds is needed to identify the existence of novel compounds in the extracts that possess promising future for development of natural antibiotic agent.

5.2 Recommendation

As a recommendation for future research, the study should be continued with the additional type of bacteria and other groups of pathogenic microbes such as yeast

and fungi to be tested with *T. catappa* extracts. Moreover, further identification and purification on the bioactive compounds in the *T. catappa* leaf extracts should be carried out as an approach to search for novel sources of natural products. The study of chemical profiling in this experiment should be continued with the use of Gas Chromatography Mass Spectrometry (GCMS) and also need to use High-Performance Liquid Chromatography (HPLC) to detect the compound present in each of the extracts more define. The pharmacological studies on *T. catappa* confirm the plant's enormous potential in the treatment of a variety of ailments. Hence, more research and clinical trials are needed for product development in order to strengthen *T. catappa's* use for future generations.

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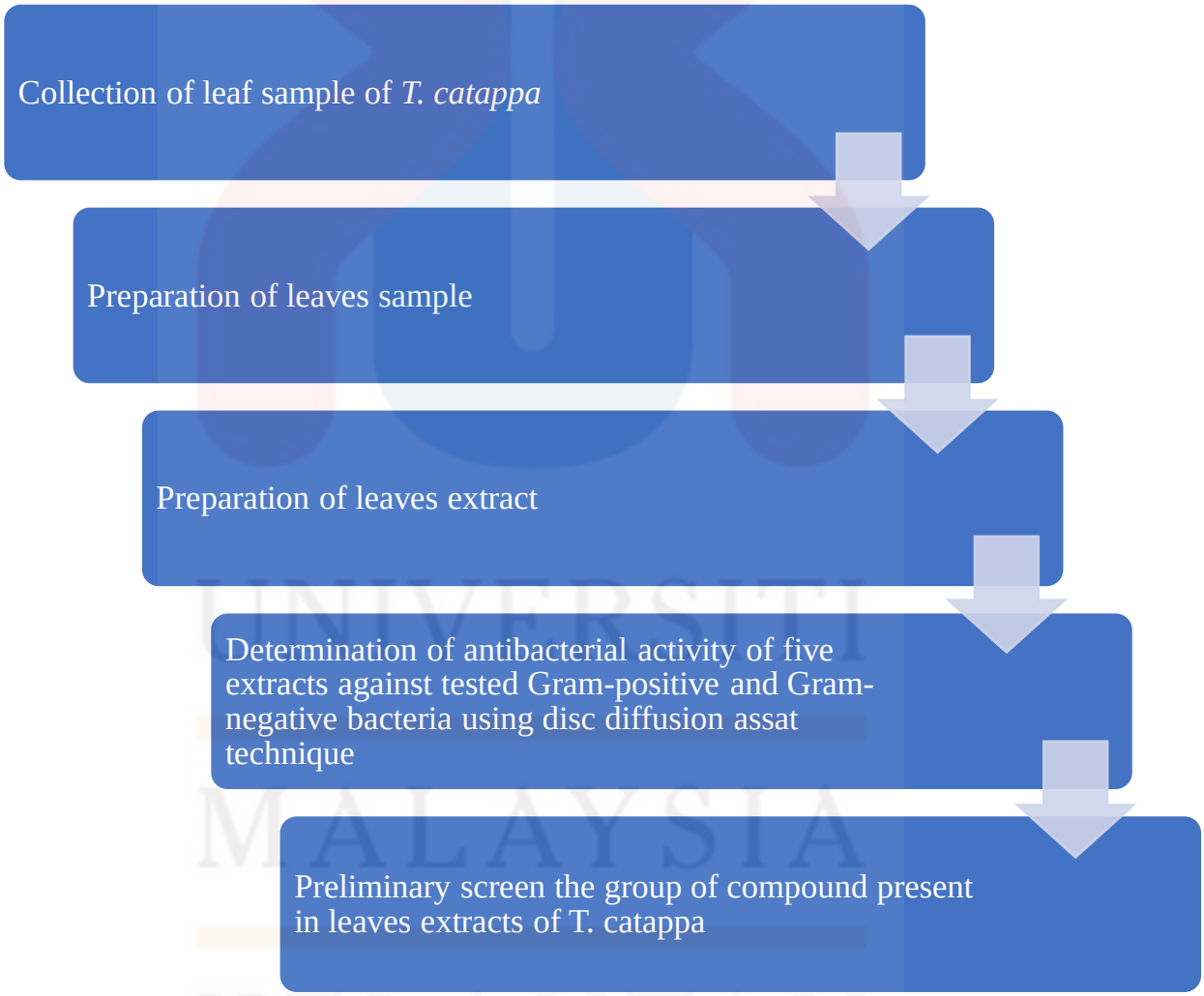
APPENDIX

Flow chart of the experiment



Gantt chart of the experiment

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

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