ANTIMICROBIAL ACTIVITY OF METHANOL EXTRACT FROM *Etlingera punicea* (FAMILY: ZINGIBERACEAE)

By

LAVINYAH ARIMANAN

A report submitted in fulfilment of the requirements for the Degree of Bachelor of Applied Science (Natural Resources Science) with Honours

FACULTY OF EARTH SCIENCE

UNIVERSITI MALAYSIA KELANTAN

2024

DECLARATION

The project report titled "Antimicrobial Activity of Methanol Extract from *Etlingera punicea* (Family: Zingiberaceae)" submitted to Universiti Malaysia Kelantan was a testament to my original research conducted under the supervision of Assoc. Professor. Madya. Ts. Dr. Suganthi A/P Appalasamy. This project was being presented to partially meet the requirements for the Bachelor of Applied Science (Natural Resources Science) degree with Honours. I declare that this project report has not been previously submitted to any other university or institution in pursuit of any degree or certificate. All the work presented in this report is my own, except the references.

Signature :

Name : Lavinyah Arimanan

Date : 8 August 2024

ACKNOWLEDGEMENT

I express my sincere gratitude to Lord Murugan for the divine blessings, opportunities, strengths, and guidance during this journey. Throughout the process of finishing my thesis, his presence offered me courage and insight not only in terms of academic development but also in terms of personal development.

First and foremost, I want to convey my sincere thanks to my project supervisor, Assoc. Professor. Madya. Ts. Dr. Suganthi a/p Appalasamy, for providing me with priceless guidance, tireless encouragement, and constant motivation during every step of the research. Their extensive expertise and experience have played a crucial role in the effective completion of this study.

Also not forget to thank Miss Nivaarani Arumugam, Ph.D. postgraduate student under my supervisor, who has provided an incredible amount of knowledge and helped in this study. Their insightful and supportive comments have greatly assisted in improving the quality of this research work.

I would like to extend my gratitude to my friends Nuratikah Izzati Binti Md Noor Hisham and Muzailinah Binti Moidin for their unwavering support, encouragement, and help in gathering data. I am very grateful to my family for their consistent love, support, and understanding, particularly in the form of financial assistance, across my educational journey. Their support has provided me with strength and motivation.

Finally, I would like to express my sincere thanks to the laboratory assistant of the Faculty of Earth Science at the University of Malaysia Kelantan for providing the essential facilities and resources needed to conduct the experimental study.

Antimicrobial activity of methanol extract from *Etlingera punicea*
(Family: Zingiberaceae)

ABSTRACT

Antimicrobial activity referred to the potential of an element to kill or inhibit the spread of harmful microbes. The medical field utilised antimicrobial activity to develop medical treatments for a wide range of diseases caused by bacteria, viruses, fungi, and parasites. *Etlingera punicea* (Roxb.) R.M.Sm. (1986), was an herbaceous plant that originated from Southeast Asia, especially Sumatra, Indonesia. *E. punicea* was also native and cultivated in Malaysia and Thailand, frequently referred to as “Red Torch Ginger” or “Chalong”. The indigenous peoples traditionally used this plant for therapeutic purposes, especially for its effectiveness in curing stomach aches, fever, and headaches. The objective of the study was to ascertain the minimum inhibitory concentration of methanol extracts obtained from various components of *E. punicea*, such as leaves, pseudostems, and rhizomes, to evaluate their antimicrobial properties. Additionally, the study aimed to compare the inhibitory activity of these methanol extracts against specific microorganisms, including *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative). Several techniques were employed to determine the antimicrobial activity of the methanol extract. These procedures included the use of dilution series, subculturing bacteria, the continuous streaking technique, and the Kirby-Bauer disc diffusion method. There were six distinct concentrations used which were 0 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml, and 10 mg/ml. Next, streptomycin positive control antibiotic was utilised to compare and define the zone of inhibition. As a result, the highest inhibition zone was observed at a 10 mg/ml concentration of the leaf methanol extract of *E. punicea* against *E. coli*. This finding highlights the potential of *E. punicea* leaf extract as an effective antimicrobial agent. The methanol extract of *E. punicea* rhizome showed the lowest inhibition zone against *E. coli* at a concentration of 2 mg/ml, which indicates the Minimum inhibitory concentration (MIC). All concentrations of the three plant components of *E. punicea*, namely the pseudo stem, leaf, and rhizome, illustrated susceptibility to both microorganisms. The concentration of 10 mg/ml of EL extract exhibited the greatest inhibitory zone on *E. coli*, with 11.68 ± 1.386 mm. The lowest inhibitory zone was observed at a concentration of 2 mg/ml of *E. coli* of the ER, with an average of 6.300 ± 0.226 mm. Overall, the leaf extract of *E. punicea* highlighted the greatest amount of effective antibacterial action, resulting in the largest inhibition zone towards both microbes which were *E. coli* and *S. aureus*. This demonstrates that it has the potential to be used as a powerful antibacterial agent in future pharmaceutical studies.

Keywords – Antimicrobial activity, *Etlingera punicea*, Minimum Inhibitory Concentration (MIC), Methanol extract, Inhibition zone, Susceptibility

Aktiviti antimikroba ekstrak metanol daripada *Etlingera punicea*
(Family: Zingiberaceae)

ABSTRAK

Aktiviti antimikroba merujuk kepada potensi unsur untuk membunuh atau menghalang penyebaran mikroba berbahaya. Bidang perubatan telah menggunakan aktiviti antimikroba untuk membangunkan rawatan untuk pelbagai penyakit yang disebabkan oleh bakteria, virus, kulat, dan parasit. *Etlingera punicea* (Roxb.) R.M.Sm (1986), merupakan tumbuhan herba yang berasal dari Asia Tenggara, terutamanya Sumatra, Indonesia. *E. punicea* juga asli dan diusahakan di Malaysia dan Thailand, sering dipanggil “Red Torch Ginger” atau “Chalong”. Orang asli secara tradisional menggunakan tumbuhan ini untuk tujuan terapeutik, terutamanya untuk keberkesanan dalam merawat sakit perut, demam, dan sakit kepala. Tujuan kajian ini ialah untuk menentukan kepekatan penghalang minimum ekstrak metanol yang diperolehi daripada pelbagai komponen *E. punicea*, seperti daun, pseudostems, dan rhizomes, untuk menilai sifat antimikroba mereka. Selain itu, kajian ini bertujuan untuk membandingkan aktiviti menghalang ekstrak metanol ini terhadap mikroorganisme tertentu, termasuk *Staphylococcus aureus* (Gram positif) dan *Escherichia coli* (Gram-negative). Beberapa kaedah telah digunakan untuk menentukan aktiviti antimikroba ekstrak methanol. Prosedur-prosedur ini termasuk penggunaan siri pencairan, bakteria subkultur, teknik streaking berterusan, dan kaedah penyebaran cakera Kirby-Bauer. Terdapat enam kepekatan yang berbeza yang digunakan yang ialah 0mg / ml, 2mg/ml, 4mg/mL, 6mg / mL, 8 mg / ml dan 10 mg/ml. Seterusnya, antibiotik kawalan positif streptomycin digunakan untuk membandingkan dan menentukan zon perencatan. Akibatnya, kawasan perencatan tertinggi diamati pada kepekatan 10 mg/ml ekstrak metanol daun *E. punicea* terhadap *E. coli*. Temuan ini menunjukan potensi ekstrak daun *E. punicea* sebagai agen antimikroba yang berkesan. Ekstrak metanol dari *E. punicea* rizom menunjukkan kawasan inhibisi terendah terhadap *E. coli* pada kepekatan 2 mg/ml, yang menunjukkan kepekatan perencatan minimum (MIC). Semua kepekatan dan tiga komponen tumbuhan *E. punicea*, iaitu batang, daun, dan rizom, menggambarkan kepekaan kepada kedua-dua mikroorganisme. Kepekatan 10 mg/ml ekstrak EL menunjukkan zon perencatan terbesar pada E. coli, dengan 11.68 ± 1.386 mm. Zon perencatan terendah diperhatikan pada kepekatan 2 mg/ml E. coli ER, dengan purata 6.300 ± 0.226 mm. Secara keseluruhan, ekstrak daun *E. punicea* menunjukan jumlah yang terbesar tindakan antibakteria yang berkesan, yang mengakibatkan kawasan penghalang terbesar terhadap kedua-dua mikroba yang merupakan *E. coli* dan *S. aureus*. Ini menunjukkan bahawa ia mempunyai potensi untuk digunakan sebagai agen antibakteria yang kuat dalam kajian farmaseutikal masa depan.

Kata kunci – Aktiviti antimikrobial, *Etlingera punicea*, Kepekatan Perencatan Minimum (MIC), ekstrak metanol, Zon perencatan, Kecenderungan

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

E. punicea	*Etlingera punicea* (Roxb.) R.M.Smith
MCI	The minimum inhibitory concentration
E. coli	*Escherichia coli*
FSB	Faculty of Earth Science
UMK	Universiti Malaysia Kelantan
S. aureus	*Staphylococcus aureus*
ANOVA	Two-way analysis of variance
SPSS	IBM® Software (SPSS®) Statistics version 21
SD	Standard deviation

LIST OF SYMBOLS

EP	*Etlingera punicea* Pseudostem
EL	*Etlingera punicea* Leaf
ER	*Etlingera punicea* Rhizome
EP2	*Etlingera punicea* Pseudostem duplicated
EL2	*Etlingera punicea* Leaf duplicated
ER2	*Etlingera punicea* Rhizome duplicated

1.0 INTRODUCTION

1.0 Background of study

Taxonomy (Linnaeus, 1758) refers to the study of living organisms including animals, plants, microorganisms, and humans to classify them into different categories for future research and identification. Binomial nomenclature (Linnaeus, 1758) was the process used to determine the scientific name for any species (Rouhan & Gaudeul, 2013). *Etlingera punicea* (Roxb.) R.M.Smith (1986), frequently known as “Red Torch Ginger” or “Chalong”, was an herbaceous species in the order Zingiberales (Grisebach, 1854), family Zingiberaceae (Martinov, 1820) and genus *Etlingera* (Giseke, 1792). The Zingiberales classification encompassed two families, namely ginger and banana. In this group, there were eight distinct families, of which four were classified under the ginger family, Marantaceae (Brown, 1814), Cannaceae (Juss, 1789), Zingiberaceae (Martinov, 1820), and Costaceae (Nakai, 1941), while the remaining four were categorized as part of the banana family, Strelitziaceae (Hutchinson, 1934), Lowiacceae (Ridley, 1924), Heliconiaceae (Vines, 1895) and Musaceae (Juss, 1789). Zingiberaceae which were commonly referred to as ginger, exhibit robust aromatic and medicinal properties. There were approximately 1600 species from 52 genera recognized around the world and about 1000 species discovered in Asia (Xu & Chang, 2017). Around 19 genera with 200 species of Zingiberaceae existed in Peninsular Malaysia (Akram et al., 2023).

Genus *Etlingera* (Giseke, 1792) was a medicinal plant native to the Indo-Pacific region, containing more than 100 species. *Etlingera punicea* (Roxb.) R.M.Smith (1986), was first described by William Roxburgh a Scottish botanist, and given its current name by Rosemary Margaret Smith a British botanist. *E. punicea* was a robust perennial herb and up to 7 meters tall with slender rhizomes. *E. punicea* was native to Sumatra, Indonesia, and cultivated in Malaysia and Thailand (Appalasamy et al., 2020). However, some records indicated that *E. punicea* grew naturally in multiple regions of Malaysia, especially Pahang, Kelantan, Sarawak, and Terengganu (Nagappan et al., 2019). Therefore, *E. punicea* might be both native and cultivated in Malaysia, depending on the location and source. To conduct this research, specimens of *E. punicea* were gathered from Lata Janggut located in the Jeli District of Kelantan. In Thailand, the rhizome serves as a condiment and an ingredient in noodles and curry meals (Ud-Daula & Basher, 2019). *Etlingera* plants have been extensively used as spices, vegetables, and medicine since ancient times.

The power of certain plant compounds to prevent or kill the growth of microorganisms including bacteria, fungi, viruses, and a few parasites can be described as antimicrobial activity. Plants established several defence systems to protect against harmful pathogens over millions of years, and a few of these methods contained the formation of antimicrobial chemicals (Beristain-Bauza et al., 2019). These natural antimicrobial chemicals could be discovered in a variety of plant components, including leaves, roots, stems, inflorescences, and fruits. Many plants, particularly fragrant herbs such as oregano, thyme, ginger, and tea tree, contained methanol extract, which was high in volatile substances with antibacterial action (Savoia, 2012).

These extracts were widely used in herbal treatments and as organic food preservatives (Ud-Daula et al., 2016). *E. punicea* was one of these plants, and the methanol extract was used in this research by several methods such as dilution series, subculturing bacteria, and the Kirby-Bauer disc diffusion method. The process of microbial culture involves the regulated reproduction of microbial organisms inside a specified culture medium, enhanced by laboratory conditions. The use of this diagnostic procedure was fundamental and served as a primary research technique in the field of molecular biology (Kim et al., 2017). The disc diffusion method, commonly known as the Kirby-Bauer test, was widely employed in microbiology and clinical microbiology for determining the susceptibility or resistance of microorganisms to antimicrobial agents.

This technique assists in analyzing the effectiveness of various medicinal products against strains of bacteria (Ud-Daula et al., 2016). The Kirby-Bauer disc diffusion method was a commonly used laboratory technique in microbiology to determine the minimum inhibitory concentration (MIC) of an antibacterial agent against a certain microorganism, such as bacteria, fungi, or other pathogens (Rama, 2012). The minimum inhibitory concentration (MIC) was the lowest concentration of an antimicrobial substance which effectively prevents the visible development of a microorganism (Kowalska-Krochmal & Dudek-Wicher, 2021).

1.1 Problem statement

Antimicrobial agents could be discovered throughout many plants. These natural antimicrobial chemicals aid plants in protecting themselves against various microbes such as bacteria, fungi, and viruses. *Etlingera punicea* (Roxb.) R.M.Smith (1986), was a tropical plant that contains antimicrobial properties against bacteria and fungi. Indigenous people used this herbaceous plant as a source of traditional medicinal properties to treat fever and haemorrhoids during antiquity (Nagappan et al., 2019). Furthermore, each component of a plant contains a different quantity of bioactive compounds, including leaves, pseudo stems, inflorescences, and rhizomes (Subramaniam et al., 2012).

These multiple herbaceous plant parts could be applied in various traditional medical treatments. Limited research was conducted on *E. punicea*, despite its proven antimicrobial properties. The present collection of research provides limited and unclear details about the antimicrobial activities of *E. punicea*, which creates an important information gap regarding its ability to serve as a source of new antimicrobial agents. The requirement for the development of efficient and harmless antimicrobial agents has become increasingly crucial, particularly in consideration of the increasing concerns surrounding antibiotic resistance. Moreover, there was a lack of information available on comparing the minimal inhibitory concentration (MIC) and potential inhibition bacteria activity of *E. punicea* extracts from different plant parts for their antimicrobial activity.

The minimum inhibitory concentration (MIC) was utilised as a quantitative indicator of the effectiveness of antimicrobial medicines (Ndenge, 2021). The purpose of this investigation was to fill this research gap through conducting an extensive study of the antimicrobial effects of *E. punicea* extracts on a range of microbes such as *Staphylococcus aureus* and *Escherichia coli*. The research also identified the minimum inhibitory concentration of these extracts, hence resulting in useful data related to the possible usage of *E. punicea* as a source of new antimicrobial compounds. The findings of this study had the potential to make a significant contribution towards the advancement of alternative therapeutic approaches for treating illnesses caused by antibiotic-resistant bacteria.

1.2 Objectives

- To compare the inhibition activity of extracts from various parts of *Etlingera punicea* (Roxb.) R.M.Smith (1986) using selected microorganisms such as *Staphylococcus aureus* and *Escherichia coli.*
- To determine the minimum inhibition concentration of extracts from various parts of *E. punicea* for antimicrobial activity.

1.3 Scope of study

The research was focused mainly on *Etlingera punicea* (Roxb.) R.M.Smith (1986), a perennial herb in the family Zingiberaceae. The research study was conducted in the Microbiology and Biochemistry Laboratory, Faculty of Earth Science (FSB), at Universiti Malaysia Kelantan (UMK), campus Jeli. *E. punicea* samples were collected in Lata Janggut, Jeli District, Kelantan.

This research utilised the three components of *E. punicea*, including the leaves, pseudo-stems, and rhizomes. In essence, the stock solution preparation and experimental procedures were carried out at the Microbiology and Biochemistry Laboratory, Faculty of Earth Science (FSB) at the Universiti Malaysia Kelantan (UMK), Campus Jeli. A variety of methods were employed in this research including serial dilution, bacteria culture, and the Kirby-Bauer disc diffusion techniques. These methods were used to evaluate the antimicrobial activity of the methanol extraction from different parts of *E. punicea.*

1.4 Significant of study

Malaysia was recognised due to its outstanding biodiversity, which includes an extensive variety of ecosystems and wildlife (Sahri et al., 2012). This research data would help to demonstrate the significance of conserving biodiversity by establishing the medicinal properties of native plants like *Etlingera punicea* (Roxb.) R.M.Smith (1986). Furthermore, Malaysians have a long history of employing herbal remedies in traditional medicine. The discovered of strong antimicrobial compounds in *E. punicea* might support and improve the traditional application of plants in herbal medicine. For instance, when the extract and its components indicate significant antibacterial action, they might be turned into new therapeutic agents. This might aid in Malaysia healthcare system by providing alternative treatments against microbial diseases.

2.0 LITERATURE REVIEW

2.1 Order Zingiberales (Grisebach, 1854)

Etlingera punicea (Roxb.) R.M.Smith (1986) belongs to the order Zingiberales (Grisebach, 1854), family Zingiberaceae (Martinov, 1820) and genus *Etlingera* (Giseke, 1792). Zingiberales represent a collection of plant species that encompasses gingers, bananas, heliconias, and prayer plants. These kinds of vegetation could be discovered in the tropical areas of Africa, America, Asia, and the South Pacific (Sass et al., 2016). These herbaceous plants play an important ecological role within their native ecosystems. These plants also serve as a source of food, spices, medicinal properties, and ornamentals. The Zingiberales were classified into two groups which include banana families and ginger families. It consists of eight families including Strelitziaceae (Hutchinson, 1934), Lowiacceae (Ridley, 1924), Heliconiaceae (Vines, 1895), Musaceae (Juss, 1789), Marantaceae (Brown, 1814), Cannaceae (Juss, 1789), Zingiberaceae (Martinov, 1820), and Costaceae (Nakai, 1941). The largest family was Zingiberaceae with over 1000 species and the smallest family was Lowiacceae and Cannaceae. Members of Zingiberales were a prominent component of humid tropics forest understory vegetation and had developed numerous pollination strategies. They might be found in tropical and subtropical environments throughout the world, and fossil records extend back millions of years. The Zingiberales also referred to as the Scitamineae, are a group of monocotyledons. These kinds of vegetation usually grew in tropical areas and had a homogeneity similar to grasses, palms, or orchids. This group had already been recognized by taxonomists as a distinct entity for a long time by Tomlinson in 1962.

Zingiberales, an order of flowering plants, was characterized by their herbaceous habit, distichous phyllotaxy, large petiolate leaves, and showy bracteate inflorescences. Figure 2.1 illustrates the cladogram of Zingiberales, which was categorized based on the arborescent growth types shown by the various species. They had large petiolate leaves with long stalks attached to the stem, often exhibiting transverse venation for structural support (Sass et al., 2016). Zingiberales were known for their striking inflorescences, which were clusters of flowers surrounded by colourful bracts. Some species had modified stamens and staminodes, sterile structures that could attract pollinators or provide additional support to the flower. Asymmetric guard cells, specialized cells that control the opening and closing of stomata on the leaf surface, were shared by most members of the order. However, there might be differences between families, these characteristics collectively constitute the order Zingiberales and separate it from other plant groups (Kress, 2013).

Bentham and Hooker introduced the first classification of Zingiberales. They discovered four tribes within the Scitamineae family, including Zingibereae, Maranteae, Canneae, and Museae (Bentham & Hooker, 1883). One important development was the analysis of morphological and anatomical characteristics in determining the evolutionary relationships between the different order groups. An innovative classification system regarding the Zingiberales was being developed based on the cladogram, which identifies eight families (Kress, 2013). This modified categorization more accurately illustrates evolutionary relationships and assists in understanding the diversity and characteristics within the multiple families of order.

According to the cladogram, the Musaceae, Strelitziaceae, and Lowiaceae were closely connected, whilst Zingiberaceae and Costaceae represented a group (Simpson, 2019). The Cannaceae and Marantaceae families were also related. This improved knowledge of the relationships between the families led to a redesigned phylogenetic classification of the Zingiberales.

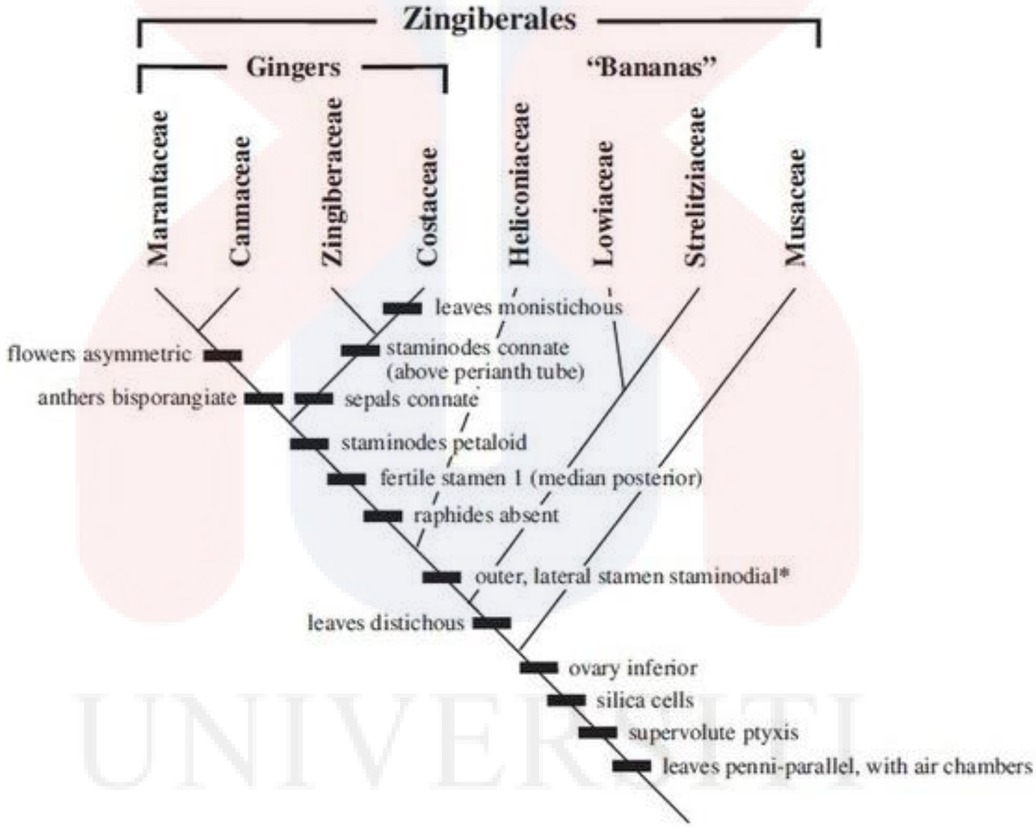

Figure 2.1: Cladogram of the Zingiberales (Simpson, 2019)

2.2 Family Zingiberaceae (Martinov, 1820)

Zingiberaceae, also known as the ginger family and the largest family within the order Zingiberales. It can be found all over the world, mostly in tropical and subtropical places such as America, Indonesia, Indochina, Malaysia, and Thailand (Uma & Muthukumar, 2014). Malaysia had discovered 750 species and 31 genera of Zingiberaceae plants. At the same time, approximately 200 species from 19 genera had been documented in Peninsular Malaysia (Akram et al., 2023). According to various taxonomic classification techniques, this family contains various number of subfamilies and genera. However, the taxonomic classification of Zingiberaceae remains debated, specifically the classical taxonomy. Some Zingiberaceae species contain cryptic morphologies, making it difficult to identify them by classical taxonomy, which was related to the special morphological traits of a tribe or species.

The distinguishing features of the ovary, including locules, placentation, staminodes, fertile anther modifications, rhizome, and shoot-leaf orientation, had been employed in traditional taxonomy classification and identify the four tribes including Alpinieae, Hedychieae, Globbeae, and Zingiberaceae (Xu & Chang, 2017). Zingiberoideae, Alpinioideae, Siphonochiloideae, and Tamijioideae were the four subfamilies. The Zingiberoideae subfamily was widely regarded as the largest and most diverse, comprising over 40 genera and around 1000 species (Uma & Muthukumar, 2014). A diverse array of herbs and spices with medicinal properties were commonly utilised, including ginger, turmeric, and cardamom. The subfamily Alpinioideae had approximately 15 genera with over 300 species, including galangal, grains of paradise (Aframomum), and several kinds of ornamental plants with spectacular blooms.

The Siphonochiloideae subfamily comprises only one genus, *Siphonochilus*, containing about 10 species. It features tubular blooms with a long corolla tube and was native to Africa. Tamijia had 1 genus with 2 species that are indigenous to Borneo and flowers with a large labellum (Akram et al., 2023). There were several major genera in the Zingiberaceae family, including *Alpinia*, *Etlingera*, *Curcuma*, *Globba*, *Zingibe*, *Renealmia*, *Riedelia*, *Amomum*, *Aframomum*, *Boesenbergia*, *Hedychium*, *Hornstedia*, and *Meisteria* as shown in Figure 2.2. *Alpinia* was the largest genera in Zingiberaceae with around 200 species. The second largest genera was *Etlingera* with around 110 species (Nagappan et al., 2019). Zingiberaceae display distinctive characteristics, such as two nectar-producing glands found at the bottom of the style. These glands create nectar, which attracts pollinators to the blooms (Appalasamy et al., 2023). The Zingiberaceae family has been identified by the presence of the labellum, which is formed by the combination of two staminodes located on the inner staminal spiral. The Zingiberaceae plant provided essential oils, which had aromatic properties and were frequently used for both medicinal and culinary purposes. Zingiberaceae were classified according to flower and vegetative characteristics. Zingiberaceae constitute a monophyletic group, which means it includes all descendants of a common ancestor. It can be considered an element within the larger monophyletic order Zingiberales, which additionally contains the families Costaceae, Marantaceae, and Cannaceae (Zahara, 2020).

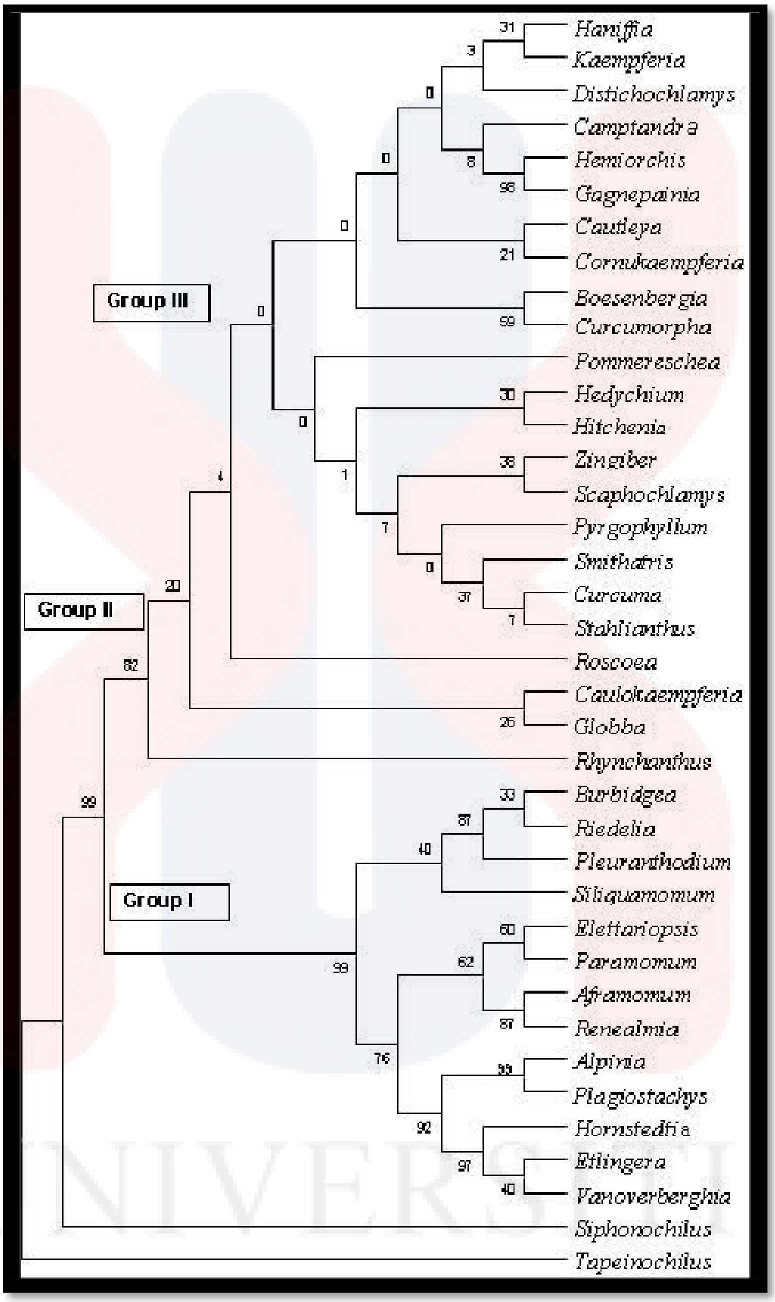

Figure 2.2: Phylogenetic tree of the family Zingiberaceae

(Selvaraj et al, 2008)

2.3 Genus *Etlingera* (Giseke, 1792)

Etlingera was the second-largest genus in the Zingiberaceae family from the tribe Alpinieae (Chan et al., 2013). This group of plants encompasses approximately 100 species and was commonly grown around humid forests. These plants are native to the Indo-Pacific region as well as used for flavour, culinary, ornamental, and medicinal properties. *Etlingera* was a notable genus in Malaysia that was traditionally used for a variety of purposes by the indigenous people (Khaleghi et al., 2012). Different parts of each *Etlingera* species were used for their specific properties. For example, the popular technique was to rip off and consume the inner sheaths of certain species of leafy shoots. These components of plants can be consumed raw or cooked as vegetables (Ud-Daula et al., 2019).

The immature flower buds from Torch Ginger provide a unique aromatic flavour that was commonly utilised in Singapore, Malaysia, and Thailand. Rojak and laksa were two popular meals that use the flower bud. It was also capable of being used as an additional garnish for sour-savory soups (Subramaniam et al. 2012). Some species were used to treat headaches and stomach pain. A particular species, having huge distinctive red marks, was often used directly to relieve itchiness and other types of skin disorders. It was abundantly cultivated and established as an ornamental plant in tropical gardens, where the flowers can be purchased as cut flowers. There were more than 15 species of *Etlingera* that exist in Malaysia (Chan, 2013).

Etlingera species produce leafy shoots which could grow up to as much as eight meters high, and bases which were so strong. These shoots can form clumps or contain lengthy creeping rhizomes, and their leafy shoots can extend more than a meter apart. The shoots of the inflorescence were brief and did not come out of the ground. The flowering structures of *Etlingera* were identified through bright red labella, which resemble petals and extend outward from the base of the plant. The flower tubes and ovaries were located below ground level (Ud-Daula & Basher, 2019).

2.4 Morphology of *Etlingera punicea* (Roxb.) R.M.Smith (1986)

Etlingera punicea (Roxb.) R.M.Smith (1986), was initially documented by William Roxburgh, a prominent botanist from Scotland. Later, it was officially given its current name by Rosemary Margaret Smith, a distinguished botanist hailing from Britain. *E. punicea* showed morphological features such as rhizomes which extend up to a maximum length of 12 cm under the soil surface, with a diameter of 3.0 cm. The rhizome was overlapping scale leaves, and its appearance was a combination of green with a shade of pink on a dull red background, as seen in Figure 2.3 (a). It was a tall forest plant that could reach heights of up to 6 meters (Ud-Daula & Basher, 2019). The leafy shoots had a height ranging from 2 to 5 meters and were seen to be either widely dispersed, with a spacing of 11 to 40 centimetres, or closely packed (Nagappan et al., 2019). Figure 2.3 (b) illustrates the reddish-brown abaxial surface of the leaves of *E. punicea*. when this plant was young, its lower part exhibited an alluring shade of reddish-brown, which eventually transformed into a dazzling emerald colour (Darus, 2020).

This plant could easily be identified due to its stunning shiny deep green leaves as demonstrated in Figure 2.3 (c). In Figure 2.3 (d) shows the inflorescence of *E. punicea* and resembles a deep cup with round, sterile bracts that dramatically recurved and consist of subtruncate apices. The species contains few flowers, normally about 1-3, and the edge of each flower has a yellow centre.

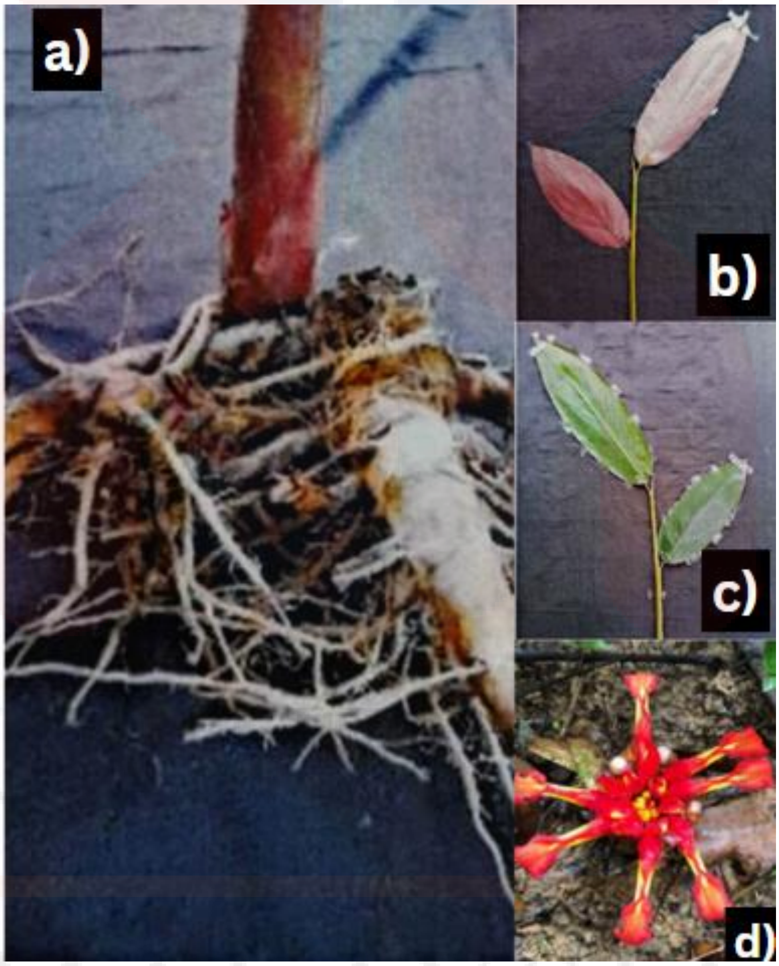

Figure 2.3: The various parts of the plant *Etlingera punicea* a) *E. punicea* rhizomes that were green-tinged with a pale pink or reddish colour and had scales that overlap the leaves b) The leaves of *Etlingera punicea* had a reddish-brown abaxial surface c) The adaxial surface of *Etlingera punicea* leaves displayed slightly elevated lateral veins and was completely green in colour d) The inflorescence of *E. punicea* had vibrant red bracts that surrounded with pale yellow. (Darus, 2020)

2.5 Distribution of *Etlingera punicea* (Roxb.) R.M.Smith (1986)

Etlingera punicea (Roxb.) R.M.Smith (1986), was distributed across various Southeast Asian countries, especially Thailand, Malaysia, and Indonesia. *E. punicea* was native to Sumatra, the largest island of the Sunda Islands in Western Indonesia (Appalasamy et al., 2022). *E. punicea* grows during the warm and humid temperatures frequently discovered in this area and prefers well-drained and fertile soils which are richest in organic material. *E. punicea* commonly occurs in lowland rainforests and alongside riverbanks due to constantly elevated soil moisture levels, without being waterlogged (Lim, 2012). *E. punicea* has changes in development characteristics and chemical composition based on the soil texture and environmental factors in various countries. The composition of soil, which includes elements such as pH, nutrient content, and moisture levels, has a substantial impact on the growth of plants and the formation of bioactive compounds. Soils that contain a high concentration of organic matter and nutrients have a positive effect on the growth and overall well-being of plants, resulting in more colourful flowers and greenery. Moreover, the existence of specific minerals or organic compounds in the soil might influence the level and diversity of vital oils and other phytochemicals generated by the plant. The variances in geographical areas can lead to differences in the flavour, aroma, and potential therapeutic effects of *E. punicea.*

2.5.1 Distribution of *Etlingera punicea* (Roxb.) R.M.Smith (1986) in Malaysia

Figure 2.4 indicates the geographic distribution of *E. punicea* in Malaysia. The geographical distribution of these entities was primarily located within five Malaysian states, namely Sarawak, Sabah, Pahang, Terengganu, and Kelantan. *E. punicea* was the one frequently discovered in wet and shady areas of primary and secondary rainforests. (Appalasamy et al., 2020). *E. punicea* was mostly found in Fraser's Hill and Tanah Rata, located in Malaysia.

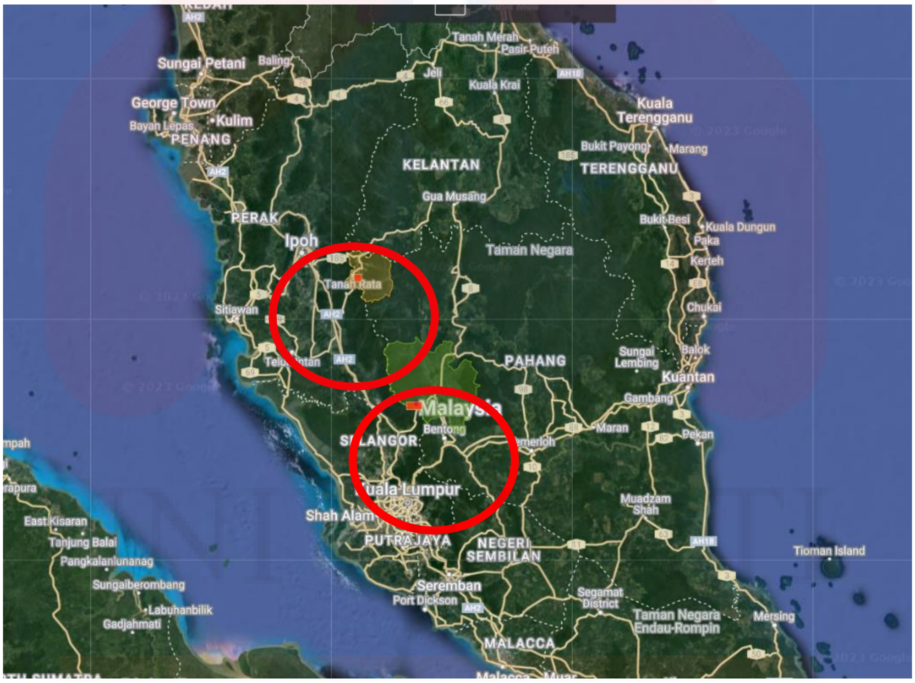

Figure 2.4: Map distribution of *Etlingera punicea* (Roxb.) R.M.Smith (1986)in Malaysia primarily located at Fraser Hill and Tanah Rata (*Etlingera Punicea*, n.d.)

2.5.2 Distribution of *Etlingera punicea* (Roxb.) R.M.Smith (1986) in Indonesia

E. punicea was discovered in five distinct regions in Indonesia as shown in Figure 2.5. North Sumatra, West Sumatra, West Java, Central Java, and East Java. *E. punicea* was found primarily around West Sumatra, including in the areas of Padang, Bukittinggi, and Payakumbuh. *E. punicea* had the ability to grow in moist, shady areas, logging roads, and riverbanks.

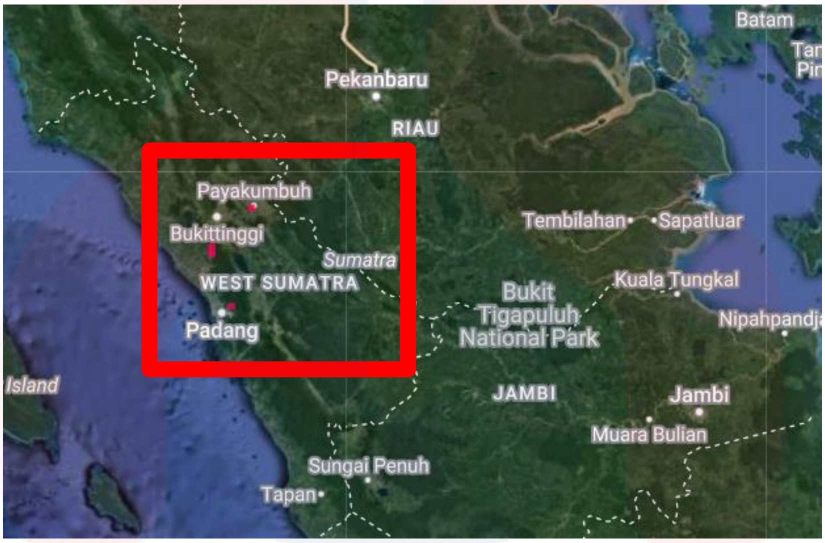

Figure 2.5: A map showed the distribution of *Etlingera punicea* (Roxb.) R.M.Smith (1986) in Indonesia west Sumatra mainly in payakumbuh, bukittinggi and padang (*Etlingera Punicea*, n.d.)

2.6 The selected microbial strains were *Escherichia coli* and *Staphylococcus aureus*.

Microorganisms were tiny living things that could not be observed with the unaided human eye. Despite their small size, they played crucial roles in a wide range of ecological, industrial, and biological processes. Microorganisms were classified into various categories, including bacteria, viruses, fungi, archaea, and protists (Blagodatskaya & Kuzyakov, 2013).

Bacteria were microscopic unicellular organisms that were prevalent across the planet and served an essential role in the planet. Certain bacteria were capable of thriving in environments with extreme temperatures and pressures. Bacteria manifest in various configurations, including spherical, rod-shaped, and spiral structures (Kachhawa, 2017). Although certain strains of bacteria were potentially pathogenic, it was important to note that most of these microorganisms played vital roles in a variety of environmental and biological systems. Bacteria appeared more than 3 billion years ago and have since become the most predominant organisms on earth, having had an important effect on the environment (Alegado & King, 2014). This study employed Gram-positive and Gram-negative bacteria, specifically *Escherichia coli* and *Staphylococcus aureus*.

2.6.1 *Escherichia coli* bacteria

Escherichia coli was a type of bacteria with a rod-shaped structure and was classified as Gram-negative. It has been extensively researched due to its rapid growth rate under optimal conditions, with a doubling time of twenty minutes. The ideal development conditions for *E. coli* are a temperature of thirty-seven degrees Celsius, requiring oxygen, and a pH level of seven. *E. coli* served as a host organism for various gene modification strategies (Jang et al., 2017). Usually discovered in the lower intestine of warm-blooded species, which were endotherms. Certain strains of bacteria used to contribute to the normal flora of the stomach and were beneficial to their hosts by generating vitamin K2 (Allocati et al., 2013). Although the majority of *E. coli* strains were not harmful to human health, certain serotypes were known to have caused food poisoning.

Table 2.1 displays the taxonomic categorization of *E. coli* which presents comprehensive information regarding its hierarchical place throughout the biological taxonomy framework, including its domain, kingdom, phylum, class, order, family, genus, and species (Faner et al., 2017). Figure 2.6 illustrates the *E. coli* bacteria, identified by their tiny rod shape, pink colour, and gram-negative attributes, as observed under a microscope at a hundred times magnification.

Table 2.1: The taxonomic classification of *Escherichia coli* (Faner et al., 2017)

Taxonomic Rank	Taxon
Domain	Bacteria
Kingdom	Eubacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*E. coli*

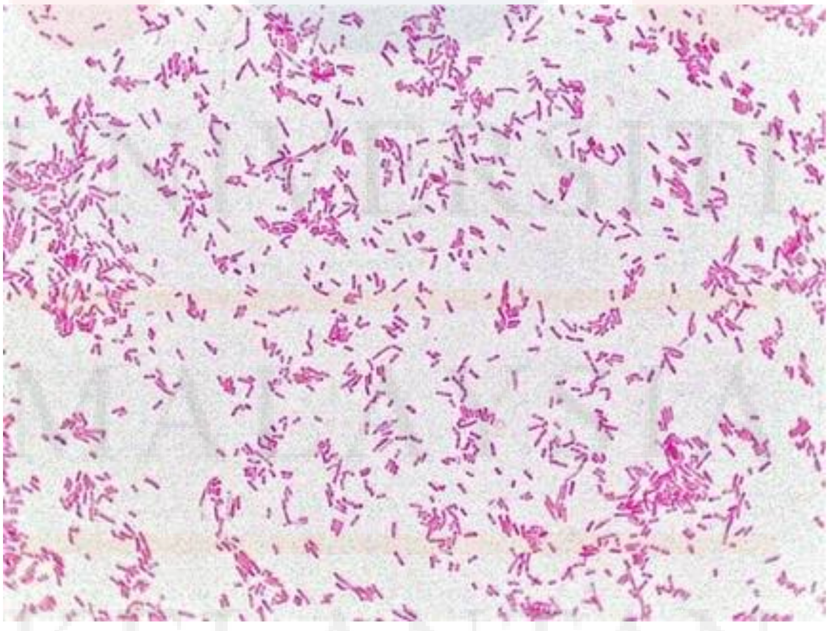

Figure 2.6: The *E. coli* bacteria, which are small rods with a pink colour and gram-negative features, under a microscope at a magnification of 100X (Anwar et al., 2022).

2.6.2 *Staphylococcus aureus* bacteria

Staphylococcus aureus was a kind of gram-positive bacteria, a spherically shaped bacteria which was frequently discovered in the human body. *S. aureus* was capable of surviving within a temperature range of seven to fifty-eight degrees Celsius, with its ideal growth occurring at thirty-seven degrees Celsius. It might lead to several kinds of illnesses, from a skin infection to more serious conditions including endocarditis and toxic shock syndrome (Lee et al., 2018). The bacteria mostly inhabited the nasal membrane along with the surface of warm-blooded animals, including humans. These organs were also used as the major defence towards disease.

Nonetheless, once the bacteria got into the deeper tissues, the primary function of the natural protection of the organism was carried out by macrophages. Usually, when there was a continuing infection, macrophages and other lymphocytes utilised harmful reactive oxygen species including hydrogen peroxide, superoxide, and hydroxyl radicals to eliminate the absorbed bacteria (Otto, 2014). Table 2.2 shows the taxonomic classification of the *S. aureus* bacteria, demonstrating its hierarchical categorization ranging from domain to species (Park & Seo, 2019). This classification contains significant hierarchical levels, including the domain Bacteria, the phylum Firmicutes, and the class Bacilli. Furthermore, it underlines the taxonomic classification of the order Bacillales, the family Staphylococcaceae, and the genus *Staphylococcus*. The comprehensive classification system aids in comprehending the links between evolution and the biological attributes of *S. aureus* (Myles & Datta, 2012).

Figure 2.7 displays *Staphylococcus aureus* as observed via a microscope after undergoing the Gram stain procedure (Joshi et al., 2014). The bacteria appear gram-positive, demonstrating a unique purple colouration, and are characterized by their spherical shape. The staining technique, pioneered by Hans Christian Gramme in 1884, continues to play a crucial role in categorizing bacteria according to their cell wall composition.

Table 2.2: The taxonomic classification of *Staphylococcus aureus* (Park & Seo, 2019).

Taxonomic Rank	Taxon
Domain	Bacteria
Kingdom	Eubacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Staphylococcaceae
Genus	*Staphylococcus*
Species	*S. aureus*

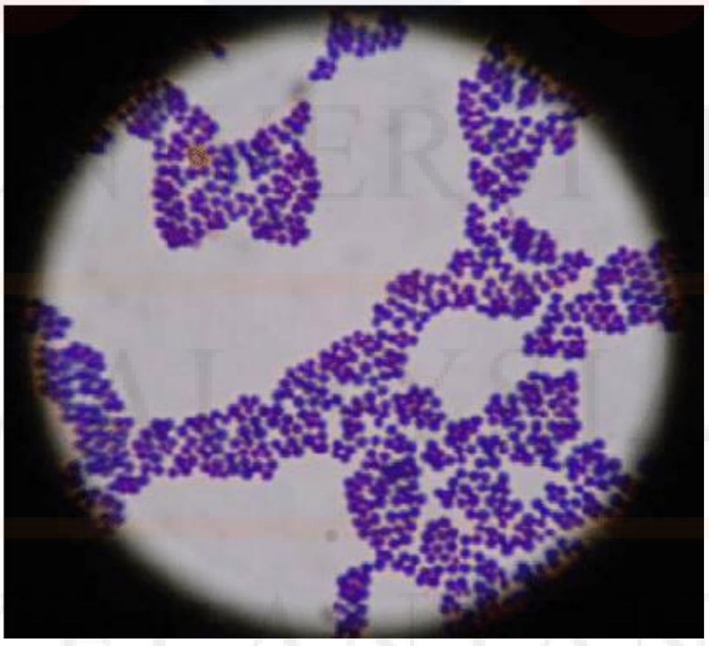

Figure 2.7: *Staphylococcus aureus* under the microscope following the Gram staining procedure, which displayed purple-coloured gram-positive bacteria with spherical shapes (Joshi et al., 2014).

2.7 Minimum Inhibitory Concentration (MIC)

In the field of microbiology, the term "MIC" refers to the Minimum Inhibitory Concentration. This term is widely used to determine the lowest concentration in mg/ml of a particular antimicrobial agent that effectively inhibits the growth of a microorganism (Kowalska−Krochmal & Dudek-Wicher, 2021). Microbiology laboratories frequently conduct MIC testing to evaluate the efficacy of antimicrobial agents against specific strains of bacteria. This testing is an essential aspect of microbiological analysis, as it helps healthcare providers determine the most suitable antibiotic therapy for the treatment of illnesses (Elshikh et al., 2016).

MIC analysis aids clinicians in selecting the optimal antibiotic therapy for infections caused by bacteria. Clinicians can choose the most suitable antibiotic with the lowest concentration essential to kill bacterial growth by identifying the minimum inhibitory concentration (MIC) of several antibiotics against the infectious bacteria. These assist in minimising the chances of treatment failure and the emergence of resistant antibiotics (Van Hal et al., 2012). Various techniques can be employed to determine MIC values, each has its own merits and drawbacks. Examples include the Broth Dilution Method, Agar Dilution, disk diffusion, Gradient Diffusion Methods, and E-Test. (Kowalska-Krochmal & Dudek-Wicher, 2021)

3.0 MATERIALS AND METHOD

3.1 The dilution of *Etlingera punicea* (Roxb.) R.M.Smith (1986) crude extract from different plant parts

The extracts of *Etlingera punicea* (Roxb.) R.M.Smith (1986) was obtained from (Alif Syazwan, 2023, UMK unpublished) in extract form. These prepared extracts included different parts of *E. punicea* such as pseudostem, rhizome, and leaves. The stock solution was prepared by diluting 0.05 gram of leaf extract in four millilitres of methanol (HmbG, Malaysia). Then, the stock solution was completely mixed using a vortex machine provided by Universiti Malaysia Kelantan (UMK), Jeli Campus. The volume was adjusted in order to achieve the required concentrations of 0 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml, and 10 mg/ml respectively. The previously described method was utilised for the two remaining parts of *E. punicea*, namely the pseudostem and rhizomes. Next, the stock solution and the newly prepared concentrations were preserved in a refrigerator at a temperature of four degrees Celsius until it was required for future usage (Yassen, 2016).

Figure 3.1: Vortex mixer machine (Universiti Malaysia Kelantan UMK, Jeli Campus)

3.2 The disk filter paper was prepared with a diameter of 6 mm.

This study utilised the Whatman® filter paper no. 1 from Sigma Aldrich in Damstadt, Germany, to determine the inhibition zone. The filter paper was provided by the Biochemistry and Microbiology Laboratory, of the Faculty of Earth Science, Universiti Malaysia Kelantan, Campus Jeli. The round-holed puncher was used to punch the filter paper to a diameter of 6 mm. Then, it was placed into a sterile petri dish and wrapped with aluminium foil paper. Finally, the covered petri dish was sterilized using an autoclave and placed in an oven at forty degrees Celsius for further usage.

3.3 The nutrient agar plate was prepared for the cultivation of microorganisms.

The agar media preparation process was performed using laminar flow, which ensures a sterile environment and conditions. nutrient agar was a part of this research, and this medium was frequently employed in the cultivation of bacteria. The process of preparing nutrient agar was conducted within the Biochemistry and Microbiology Laboratory of the Faculty of Earth Science, Universiti Malaysia Kelantan, Campus Jeli. A quantity of twenty-eight grams of agar powder (OXOID, England), was mixed with one litre of distilled water in a sterile media bottle. In order to dissolve the agar, a glass rod was used to stir the solution. The nutrient agar medium mixture was sterilized using an autoclave machine (Tomy, Japan) at one hundred twenty-one degrees Celsius for two hours.

After that, it was cooled in laminar flow for fifteen minutes at room temperature. Next, twenty mL of nutrient agar solution was measured using a clean measuring cylinder and poured into each petri dish in a glass. Within the laminar flow, plates of nutrient agar were placed and chilled until they solidified at twenty-seven degrees Celsius. A tightly sealed petri dish was utilized to prevent the penetration of air into the dish. The sealing process was done with great care to ensure that the dish was completely airtight (Basu et al., 2015).

3.4 The antimicrobial testing by disk diffusion assay

3.4.1 The stock culture was prepared of using *Staphylococcus aureus* and *Escherichia coli* bacteria

This study utilised *Escherichia coli* and *Staphylococcus aureus* as microorganisms, representing Gram-negative and Gram-positive bacteria, respectively. These strains were supplied by the Microbiology and Biochemistry Laboratory, FSB, Universiti Malaysia Kelantan (UMK), Jeli Campus. The streaking technique (Koch,1881) was employed to perform the bacterial culture. The entire procedure was conducted properly within a laminar airflow cabinet. Before streaking a culture tube specimen, metal transfer loops were sterilized by burning the wire loop in the bright blue region of a Bunsen burner immediately above the inner flame until red-hot. After the sterilization process, the inoculating loop was let to cool down. Next, the one hundred microliters of *Escherichia coli* bacteria were transferred onto the newly prepared nutrient agar medium in a zig-zag formation using the inoculating loop. The microorganism was streaked from the edge (A) towards the edge (B) of the nutrient agar plate and utilised an inoculating loop which was shown in Figure 3.1.

The same inoculum loop was employed, and the process of streaking was replicated twice on the same nutrient agar plate. The technique can be referred to as the continuous streaking method (Jiang et al., 2016). After that, the same steps mentioned above were repeated for the *Staphylococcus aureus* strain.

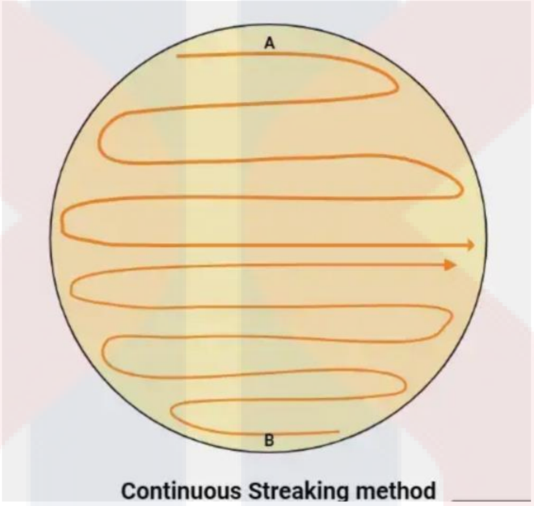

Figure 3.2: The continuous streaking method for microorganism culture (Tankeshwar, 2022)

3.4.2 The filter paper discs were prepared using the crude extract of *Etlingera punicea* (Roxb.) R.M.Smith (1986)

The procedure of preparing dried filter discs was conducted. Whatman® filter paper no. 1 from Sigma Aldrich in Damstadt, Germany, the filter paper was used for preparing the discs, which had a diameter of around six millimetres. The disk filter paper was carefully positioned into a nutrient agar Petri dish. Then, the stock solution of the *E. punicea* leaves extract, which has a concentration of 2 mg/ml, was transferred using a micropipette onto the disc filter paper. In this experiment, several concentrations of three component extracts of *E. punicea* were utilised.

The technique mentioned above was repeated for the remaining two components of *E. punicea*, which are rhizomes and pseudostem, at different concentrations of 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml, and 10 mg/ml. These concentrations were accurately measured and transferred onto filter paper discs using a micropipette (Redfern et al., 2014).

3.4.3 Disc diffusion assay to evaluate the efficiency of antimicrobial agents

The sterile forceps were utilized to pick the discs, then placed on the surface of nutritional agar that had been previously cultured with Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus*, and *Escherichia coli* (Mostafa et al., 2018). The diffusion test was performed in duplicate for each concentration. The plates were incubated in a controlled environment at a temperature of thirty-seven degrees Celsius for a duration of twenty-four hours. In one petri dish, the *E. punicea* extract leaves were divided into six distinct concentrations as indicated in Figure 3.3. Additionally, a positive antimicrobial susceptibility test disc containing streptomycin at a concentration of 10mg/ml was included. This procedure was applied to all three elements of *E. punicea*. The antimicrobial activity was examined and recorded by calculating the lengths of inhibition zones in millimetres using Digital Vernier Calipers (Balouiri et al., 2016).

Figure 3.3: The arrangement of the filter disc paper on the nutrient agar medium. (Chew et al., 2018)

3.4.4 The measurement of the inhibition zone by using a Digital Vernier Calipers

In order to measure the inhibition zone, the diameter of the inhibition zone was calculated in millimetres (mm) using a digital vernier calliper. As illustrated in Figure 3.4, the diameter of the clear zone was discovered through direct observation, without any optical devices, by placing digital vernier callipers on the enclosed petri dish. The diameter of the inhibition zone was determined by measuring the distance from point A to point B, as illustrated in Figure 3.5.

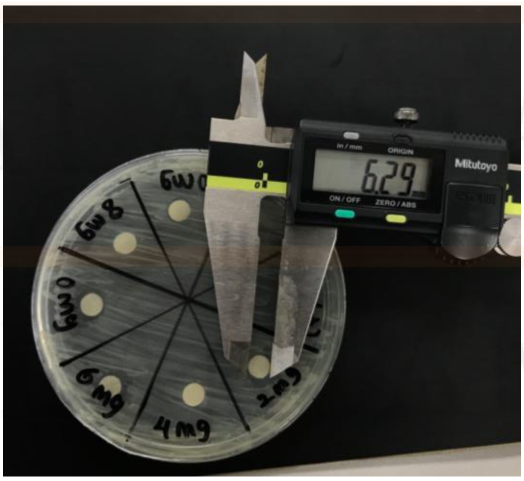

Figure 3.4: The measurement of the diameter inhibitory zone by digital vernier calliper

(Bhargav et al., 2016)

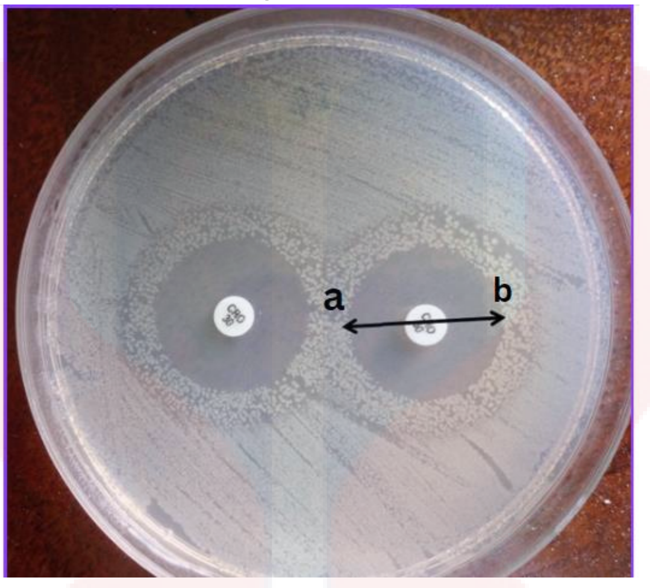

Figure 3.5: The image illustrates the measurement of the inhibition zone diameter to determine antimicrobial activity using the Kirby–Bauer disk diffusion method.

3.5 Statistically analysed for antimicrobial activities in *Etlingera punicea* (Roxb.) R.M.Smith (1986) different parts

The statistical analysis was utilised to discover the data related to the evaluation of antibacterial activity against *Etlingera punicea* (Roxb.) R.M.Smith (1986) crude extract using three different compounds at five various concentrations. These data were examined using a two-way analysis of variance (ANOVA) (Cornell, Snedecor, & Cochran, 1981) of the post hoc Tukey test. These statistical analyses will be performed using IBM® Software (SPSS®) Statistics version 21 (Abers et al., 2021). The experiment was conducted using duplicated for each concentration, and the mean values were determined to evaluate the outcomes (Naveed et al., 2013).

The comparison of the inhibition activity of extracts from various parts of *E. punicea* using selected microorganisms such as *Staphylococcus aureus* and *Escherichia coli* was assessed using a post hoc Tukey test with a significant level of 0.05%. The presence of combining lower-case letters indicates that there is no statistically significant difference ($p>0.05$). The absence of different lowercase letters in columns indicates the presence of statistically significant differences, with a significance level of $p < 0.05$. The mean of data was considered the null hypothesis was rejected, which is no significant difference in the antimicrobial activity between the extracts from various parts of *E. punicea*.

Comparison analysis was determined using the mean and standard deviation. The analysis of variance (ANOVA) was employed to establish the minimal inhibitory concentration of extracts derived from different sections of *E. punicea*, using six distinct concentrations. The results and conclusions were to determine if there are significant differences in Minimum Inhibitory Concentration (MIC) across extracts derived from different parts of *E. punicea* at various levels of concentration.

4.0 RESULTS AND DISCUSSION

4.1 The preparation of stock solution *Etlingera punicea* (Roxb.) R.M.Smith (1986) from different plant parts.

The *Etlingera punicea* (Roxb.) R.M.Smith (1986) crude extract was a composite blend of natural elements including flavonoids, terpenoids, and alkaloids, which had the potential to provide health benefits. These elements displayed antioxidant, anti-inflammatory, and antibacterial properties (Syazwan, 2023 [unpublished thesis UMK]). When conducting an analysis of crude extracts, it was vital to understand that each extract derived from natural sources, namely plants, animals, or minerals, possessed unique features. These characteristics encompassed a range of qualities, including texture, colour, and aroma (Stéphane et al., 2022). Although there were similarities in order, family, or genus, each plant species exhibited unique and distinguishing characteristics in the crude extract.

E. punicea had specific traits in the crude extract that differentiated it from other plant species as well as within various plant parts such as pseudo stem, leaves, and rhizomes. For example, the research conducted by Shahid-Ud-Daula et al. (2019) discovered that the leaf moisture level of *Etlingera fimbriobracteate* was considerably lower in comparison to the moisture content of the stems and rhizomes. Several factors influenced the different plant parts, causing variations in the extracts. There were differences in physiological and metabolic processes, the chemical composition and structure of tissues, as well as variations in surrounding and climatic factors. This was primarily influenced by the processes of absorption, transpiration, and utilisation (Chavarria and dos Santos, 2012).

Table 4.1 displays the attributes of the crude extract obtained from *E. punicea* for three components, including their abbreviations, texture, colour, and odour. Based on Table 4.1, the crude extract derived from the pseudostem of *E. punicea* demonstrated a texture characterised by a high degree of viscosity and a semi-liquid consistency. However, the leaves and rhizomes had a watery consistency with noticeable viscosity. The temperature and pressure settings utilized during the extraction process impacted the physical structure of the extracted substances, causing certain compounds to maintain a semi-liquid form even after the removal of the solvent (Li et al., 2022). In addition, some naturally occurring chemicals, such as oils or gums, inherently possessed semi-solid or viscous properties. The bioactive components formed complex structures or interacted with water molecules, leading to higher viscosity, mainly polysaccharides, proteins, and glycoproteins, which usually had large molecular weights (Laxmi & Begum, 2022).

According to Table 4.1, the extract obtained from the pseudostem of *E. punicea* displayed a reddish-brown hue, whereas the leaves exhibited a brownish-black colour, and the rhizome manifested a combination of golden and reddish-dark brown shades. The distinctive colour variations exhibited by plants were attributed to the varied phytochemical compositions that existed in different parts of the plant. These phytochemicals contributed to the plant overall aesthetic appeal and potential utility in various applications (Okorie et al., 2020). The *E. punicea* pseudostem extract was characterized by a delightful and intense fragrance, which was both spicy and flowery based on Table 4.1. Then, the leaves exuded a fresh, green, and herbaceous fragrance that was naturally appealing. Lastly, the rhizome emanated a potent and spicy scent that was distinct and discernible.

According to the research article, the crude extract exhibited the presence of volatile organic molecules, which contributed to its odour (Hamma et al., 2020). Limited solubility of the crude extract in methanol was observed during dilution. Vortex blending was employed to facilitate dissolution. Certain elements of the crude extract naturally exhibited limited solubility in methanol because of their chemical properties or molecular structure. For optimal dispersion, it was essential to increase the mixing period or use more vigorous agitation when utilizing a vortex machine (Dhawan & Gupta, 2017). The crude extract of *E. punicea* was properly diluted and the concentration has been adjusted. As illustrated in Figure 4.1 (a), alterations to the concentration caused the stock solution of *E. punicea* leaf to obtain a dark black-brown shade. The black-brown colour transformed into a dark brown shade when the concentration dropped from 10 mg/ml to 2 mg/ml.

Changing the concentration of the stock solution was expected to have an impact on the density of the pigments or chemicals present in the solution (Payne et al., 2024). Higher concentrations could have led to a greater abundance of chemical components, resulting in a lighter or paler colour. On the other hand, as the concentration reduced, there was a lower number of pigment molecules per unit volume, resulting in a deeper and more saturated golden-brown colour (Ngamwonglumlert et al., 2017). After adjusting the concentration, it was noted that the stock solution derived from the rhizome of *E. punicea* exhibited a unique colouration that closely resembled the range of golden light brown hues. When the concentration of the solvent dropped from 10mg/ml to 2mg/ml, there were recognised colour changes demonstrated in Figure 4.1 (b), which ranged from a golden light brown hue to a pale golden white.

When adjusting the concentration, the stock solution pseudo stem of *E. punicea* displayed a unique golden-brown colour. Furthermore, noticeable colour changes were seen in the concentration range of 10 mg/ml to 2 mg/ml, transforming from a golden brown to a pale golden-brown hue illustrated in Figure 4.1(c). Methanol was frequently utilized as a negative control in antimicrobial activity experiments due to the absence of substantial antibacterial properties at common concentrations. The diagram presented in Figure 4.1 (d) demonstrated the application of methanol as a negative control agent, with its concentration of 0 mg/ml, to evaluate its efficacy against *E. coli* and *S. aureus* microorganisms for antimicrobial activity assessment. In addition, the use of methanol as a negative control enabled researchers to evaluate the fundamental microbiological development in the absence of any effective antimicrobial substance, thereby establishing a standard for comparing the experimental findings (Valle et al., 2016). Investigators could evaluate the impact of a chemical by comparing its antimicrobial activity and methanol, which served as the solvent control. This comparison helped identify whether the chemical had a significant result (Voukeng et al., 2012).

Table 4.1: The characteristics of the crude extract of *Etlingera punicea*

Parameter	The components of *Etlingera punicea* Plant		
	Pseudostem	Leaf	Rhizome
Abbreviations	EP = *Etlingera punicea* Pseudostem	EL = *Etlingera punicea* Leaf	ER = *Etlingera punicea* Rhizome
Texture	High viscosity and semi-liquid	Noticeable viscosity and watery consistency	Noticeable viscosity and Watery consistency
Colour	Reddish-brown	Brownish black	Golden and reddish-dark brown
Odor	Spicy and flowery	Fresh, green, and herbaceous fragrance	Spicy and pungent scent

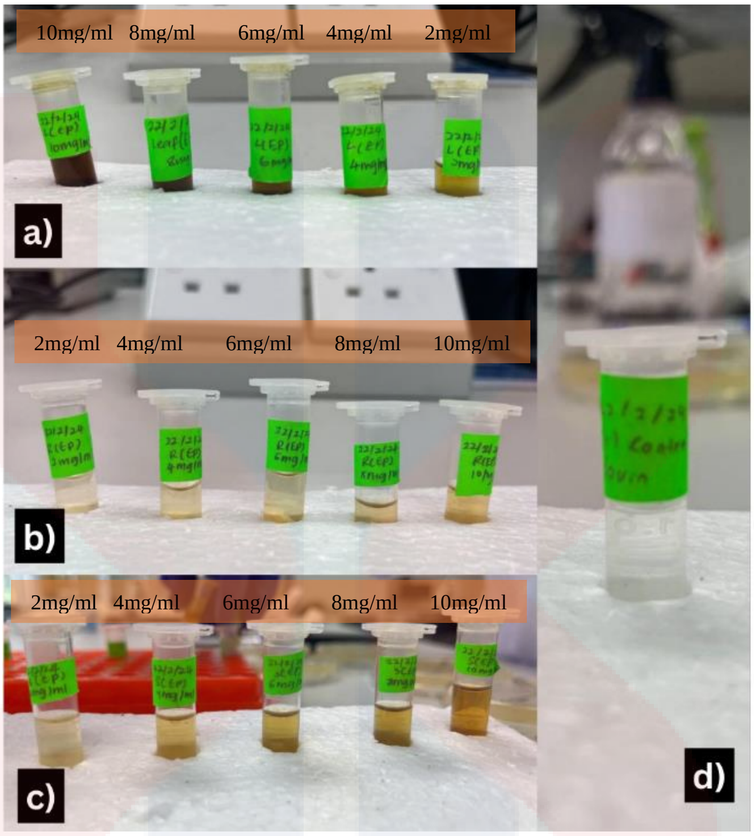

Figure 4.1: The prepared stock solution of *E. punicea* plant parts a) Leaf solution 10 mg/ml to 2 mg/ml, b) Rhizome solution 2 mg/ml until 10 mg/ml, c) Pseudostem solution 2mg/ml to 10 mg/ml, d) methanol as a negative control with a concentration of 0 mg/ml

4.2 The antimicrobial activity of *Etlingera punicea* (Roxb.) R.M.Smith (1986) crude extract from different plant parts

According to the outcomes of the experiment, an agar disc diffusion experiment was employed to assess the antimicrobial activity against *Staphylococcus aureus* (a Gram-positive bacteria) and *Escherichia coli* (a Gram-negative bacteria). Based on the results stated in Table 4.2, extracts from three distinct sections of the *Etlingera punicea* (Roxb.) R.M.Smith (1986) plants were found to be effective against the bacterial strain. Using a disc diffusion assay, the susceptibility of crude extracts of three different plant parts from *E. punicea* was evaluated at six different concentrations which were 0 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml, and 10 mg/ml.

Both *E. coli* and *S. aureus* displayed susceptibility to all concentrations of the three plant parts of *E. punicea*, including the pseudo stem (EP), leaf (EL), and rhizome (ER). Methanol was performed as the negative control, revealing no inhibitory zones for all components and microorganisms tested. The experiment results indicated that the solvent utilised did not display any antibacterial properties, and there were no signs of contamination or other influencing factors. Various similarities and differences were discovered when comparing *E. punicea* pseudostems (EP) with two microorganisms. All concentrations of EP showed substantial similarity to each other, except for the 10 mg/ml concentration of *S. aureus*. The second similarity observed was the 4 mg/ml concentration, which showed the highest inhibition zone compared to the 6 mg/ml and 8 mg/ml concentrations in both microbes.

In *E. coli*, the concentration of 4 mg/ml indicated a mean of 8.185 mm with a standard deviation (SD) of 1.619 mm, in *S. aureus* the mean was 8.375 with an SD of 1.407. Notably, in the case of the EP of *E. coli* displayed the highest level of inhibition zone at concentrations 4 mg/ml and 10 mg/ml, with both concentrations having an identical diameter mean of 8.185. However, they varied in terms of standard deviation, with the concentration of 4 mg/ml having 1.619 SD and a concentration of 10 mg/ml exhibiting 0.375 SD. The 10 mg/ml concentration had a smaller average SD indicating greater consistency in the results compared to the 4 mg/ml concentration (Chandana et al., 2024). In the case of *S. aureus* of EP, the concentration of 4 mg/ml was greater than the concentrations of 6 mg/ml and 8 mg/ml, except the concentration of 10 mg/ml.

When comparing all values in the EP extract, the lowest concentration of 6 mg/ml was reported in *E. coli* with a mean of 7.160 and SD of 0.608. The streptomycin positive control for EP in the *E. coli* illustrated the highest inhibition zone compared to *S. aureus*. In *E. coli*, the mean inhibition zone for streptomycin was 17.855 with a standard deviation of 6.541, while in *S. aureus*, the mean was 15.745 with a standard deviation of 1.803. However, at a 10 mg/ml concentration of EP, *S. aureus* had higher effectiveness than *E. coli*. The overall concentration of EP extract exhibits a higher inhibitory zone in *S. aureus* compared to *E. coli*. The antibacterial activity of the EP extract is greater against *S. aureus* than *E. coli* because of the structural differences between the Gram-positive and Gram-negative bacteria. Gram-positive bacteria contain a thick peptidoglycan layer but a poor outer membrane, making them susceptible to antimicrobial substances. Gram-negative bacteria have an outer membrane that serves as a barrier to several chemicals, which leads to limiting their effectiveness (Nicolas et al., 2019).

The leaf of *E. punicea* (EL) showed changes in both concentration and microbes. When comparing the concentrations of two bacteria in the EL extract, the findings show similarities, excluding the 10 mg/ml concentration in *E. coli*. Nevertheless, the 10 mg/ml concentration of EL extract in *E. coli* was statistically significant compared to the streptomycin, which serves as the positive control. The concentration of 10 mg/ml of EL extract in *E. coli* presented the highest inhibition zone compared to all other concentrations in the EL extract. The mean inhibition zone was reported as 11.680 with a standard deviation of 1.386. This concentration was closely similar to streptomycin in both microbes which were in *E. coli* the mean was 17.320 with SD 3.578 and for *S. aureus* 16.335 with SD 2.892.

The smallest inhibitory zone spotted in the EL extract was at a concentration of 2 mg/ml in *S. aureus*, with a mean value of 7.235 and SD 1.082. The inhibition zone of streptomycin in *E. coli* was significantly greater compared to *S. aureus*, with mean values of 17.320 and 16.335, and standard deviations of 3.578 and 2.892, respectively. When comparing the concentration of 10 mg/ml of EL extract between both microorganisms, it was found that *E. coli* had the highest recorded result versus *S. aureus.* When examining the total concentration of Two microorganisms, it was noticed that the EL extract of *E. coli* demonstrated the largest inhibitory zone compared to *S. aureus.* This indicates that the EL extract is powerful against *E. coli.* Next, the rhizome of *E. punicea* (ER) illustrates both similarities and differences when compared completely. Apart from the 10 mg/ml concentration in *E. coli*, the comparison of overall concentrations of ER extract for both microbes highlights similarities.

The 10 mg/ml concentration of ER extract in *E. coli* shows the highest inhibition zone compared to other concentrations of ER extract. The 10 mg/ml concentration of ER extract demonstrated similarity to streptomycin in *E. coli*, but it did not exhibit a similar pattern to streptomycin in *S. aureus.* The average value for a concentration of 10 mg/ml of ER extract was 9.340±0.707, while the mean value of streptomycin positive control for *E. coli* was 15.575±0.700. Then, the ER extract demonstrated the lowest concentration of 2 mg/ml in *E. coli*, with a mean value of 6.300 and SD of 0.226. However, the comparison of inhibition results between the streptomycin for both microbes displayed comparable outcomes, and the highest level of inhibition was reported in *S. aureus* with a mean of 17.935 and SD 2.906.

The concentration of 10 mg/ml ER extract of *E. coli* produced the largest inhibitory zone compared to the *S. aureus*. In addition, the extract of ER has a greater potential for reaction in *E. coli* compared to *S. aureus*, which is demonstrated by the contrasting effects of all concentrations. Among all the comparisons of concentration with three different parts of *E. punicea*, the concentration of 10 mg/ml was the most effective. The concentration is substantially higher throughout three plant parts with two microorganisms and six different concentrations. When compared to the positive control streptomycin, it is demonstrated that they are nearly identical. The highest level of inhibitory zone was reported at a concentration of 10 mg/ml of EL extract on *E. coli* which was the mean was 11.680 with SD was 1.386.

The results highlighted the reliability and efficacy of the leaf extract as an antibacterial agent, as well as the consistency of the experimental procedure. Despite the limited number of bioactive chemicals found in the leaf, which was 15, these compounds exhibit strong effectiveness against the gram-negative bacteria (Syazwan, 2023 [unpublished thesis UMK]). The leaf extract of *E. punicea* comprises a highly powerful flavonoid compound compared to alkaloid and terpenoid. Flavonoids are a wide-ranging collection of phytonutrients, which are plant compounds, that may be found in nearly all fruits and vegetables. They have become recognised for their strong anti-inflammatory characteristics (Wulandari et al., 2016). These molecules are essential for the defence mechanism of plants, protecting it from harmful substances and improving the vibrant colours of various fruits and flowers. Flavonoids have become popular in the field of human nutrition due to potential health advantages, which include anti-inflammatory, antiviral, and anticancer properties (De Luna et al., 2020).

These compounds are divided into various subcategories, such as flavonols, flavones, flavanones, and anthocyanins, each showing unique biochemical properties. Consistently eating foods that are high in flavonoids, such as berries, citrus fruits, tea, and dark chocolate, is linked to a reduced risk of developing chronic diseases. This has produced significant attention from researchers in the fields of nutrition and medicine (Saeed et al., 2012). Then, the lowest level of the inhibition zone was recorded at a concentration of 2 mg/ml of *E. coli* of the ER, with a mean of 6.300 and a standard deviation of 0.226. According to Table 4.2, there were significant similarities identified across the lowercase letters, indicating no significant difference with a p-value of more than 0.05.

When the different lowercase letters in columns represent statistically significant differences with p value less than 0.05. When comparing overall, the highest concentration of 10 mg/ml of EL extract at *E. coli* showed substantial similarity to the inhibitory effects reported in seven different conditions. The concentration of 10 mg/ml was indicated by the lowercase letter "de" in Table 4.2, which was identical to the streptomycin positive control in the EP plate of *S. aureus* and the ER plate of *E. coli* bacteria. The lowercase letter "e" indicates similarities to four different conditions of streptomycin, including the EP and EL plate of *E. coli*, and the EL and ER plate of *S. aureus*. Then, the lowercase letter "d" denotes a similar outcome to a concentration of 10 mg/ml of ER extract in *E. coli*, with a mean value of 9.340±0.707. Additionally, this 10 mg/ml concentration of ER extract is associated with another lowercase letter which was the "C".

This lower-case letter corresponds to three conditions including the 10 mg/ml concentration of EP extract in *S. aureus*, the 8 mg/ml concentration of EL extract in *E. coli*, and the 10 mg/ml concentration of EL extract in *S. aureus*. The lowest concentration that was compared to the overall components of *E. punicea* with two microorganisms was the 2 mg/ml concentration of ER extract in *E. coli*. The findings revealed that this concentration was signified by the letter 'b', and it displayed statistical similarity to another group of data. The similarity among the concentrations and the related value for inhibition indicates a possible structure or relationship. The streptomycin positive control exhibited a greater inhibitory zone compared to all tested concentrations, even though the 10 mg/ml concentration closely resembled the streptomycin. These findings indicate that a higher concentration of 10 mg/ml was even more effective in inhibiting bacterial growth.

Moreover, there was a clear positive link between the concentration and the size of the inhibition zone, suggesting that as the concentration increases, the inhibition zone also expands which was displayed in Figure 4.2 and Figure 4.3 (Daniel-Jambun et al., 2017). Figure 4.2 illustrates the inhibition zone of *E. punicea* extract against *E. coli* gram-negative bacteria with three different components and duplicated Petri dishes which were a) pseudostem (EP1), b) duplicated pseudostem (EP2), c) Leaf (EL1), d) duplicated leaf (EL2), e) Rhizome (ER1) f) duplicated of rhizome (ER2). Then, figure 4.3 demonstrated the inhibition zone also of *E. punicea* with three different parts but against the *S. aureus* gram-positive bacteria and the duplicated Petri dishes.

Not only that, Figure 4.2 and Figure 4.3 displayed the replicated procedure highlighting the similarity of the inhibition zone creation, demonstrating that there were no noticeable differences in the inhibition zone throughout the duplicated Petri dishes. The methanol extract of *E. punicea* was employed in this study due to the effectiveness of methanol as a solvent for extracting various bioactive components. The chemicals, which include alkaloids, flavonoids, and phenolics, frequently have antibacterial characteristics. Methanol has the ability to penetrate the cell walls of plants and dissolve molecules that are both polar and non-polar. This characteristic makes it appropriate to perform a thorough analysis of the chemical components present in *E. punicea*. The methanol leaf extract of *E. punicea* provides the largest inhibition zone against both *E. coli* and *S. aureus*, indicating the strongest antimicrobial activity. This demonstrates that the leaf extract has efficient antibacterial properties against both gram-negative and gram-positive microorganisms. This leaf extract has the potential to be utilised as an antimicrobial agent in future pharmaceutical research.

Table 4.2: Mean ± standard deviation values of zones of inhibition (mm) of *Etlingera punicea* plant against *Escherichia coli* and *Staphylococcus aureus.*

Zone of inhibition (mm)				
Microorganism	Concentration (mg/ml)	Parts of *Etlingera punicea* Plant		
		Pseudo stem	Leaf	Rhizome
		Mean ± SD	Mean ± SD	Mean ± SD
E. coli	0 (Negative control= Methanol)	0.000±0.000 [a]	0.000±0.000 [a]	0.000±0.000 [a]
	2	7.295±0.544 [b]	7.325±0.841 [b]	6.300±0.226 [b]
	4	8.185±1.619 [b]	7.755±0.856 [b]	6.830±0.523 [b]
	6	7.160±0.608 [b]	8.525±0.276 [b]	8.050±0.495 [b]
	8	7.775±0.106 [b]	8.900±0.933 [bc]	8.695±0.007 [b]
	10	8.185±0.375 [b]	11.680±1.386 [de]	9.340±0.707 [cd]
	Streptomycin (Positive control = 10 mg/ml)	17.855±6.541[e]	17.320±3.578[e]	15.575±0.700 [de]
S. aureus	0 (Negative control= Methanol)	0.000±0.000 [a]	0.000±0.000 [a]	0.000±0.000 [a]
	2	7.395±1.407 [b]	7.235±1.082 [b]	6.910±0.042 [b]
	4	8.375±0.940 [b]	8.555±2.779 [b]	7.525±0.643 [b]
	6	7.955±1.351 [b]	7.880±0.170 [b]	8.220±0.028 [b]
	8	8.245±1.478 [b]	8.180±0.240 [b]	8.430±0.693 [b]
	10	8.860±2.093 [bc]	8.875±0.573 [bc]	8.470±0.735 [b]
	Streptomycin (Positive control = 10 mg/ml)	15.745±1.803[de]	16.335±2.892[e]	17.935±2.906[e]

The ANOVA post hoc Tukey test was used to determine significant similarities and differences between the mean values of the inhibition zone. The similar lower-case letters indicate that there is no statistically significant difference ($p>0.05$). Different lower-case letters in columns indicate statistically significant differences ($p < 0.05$).

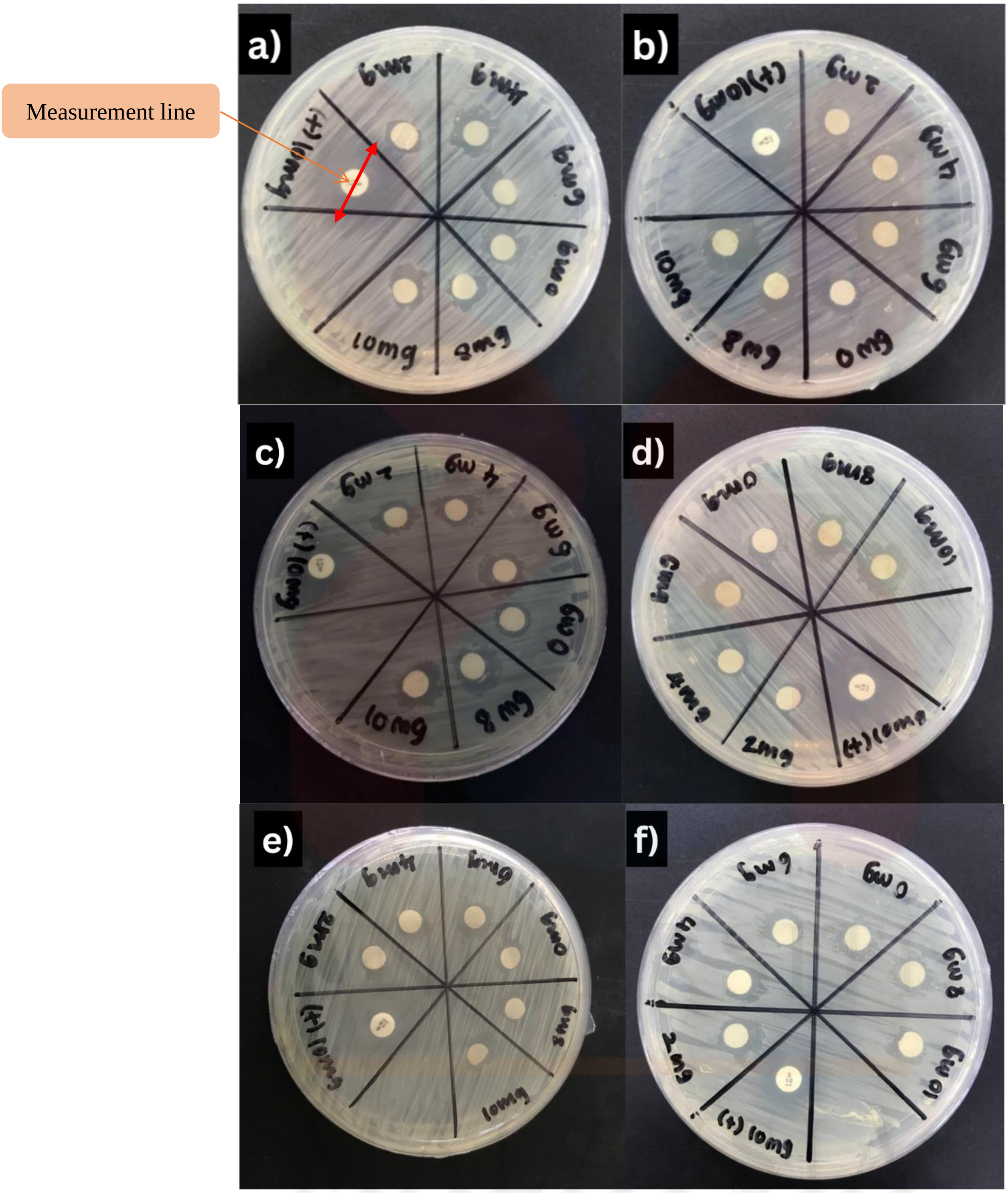

Figure 4.2: The zone of inhibition of the extract of *E. punicea* was observed in the Petri dish against *Escherichia coli*, a) pseudostem and highlight the measurement line of inhibition zone (EP1), b) duplicated of pseudostem (EP2), c) Leaf (EL1), d) duplicated of Leaf (EL2), e) Rhizome (ER1) f) duplicated od rhizome (ER2)

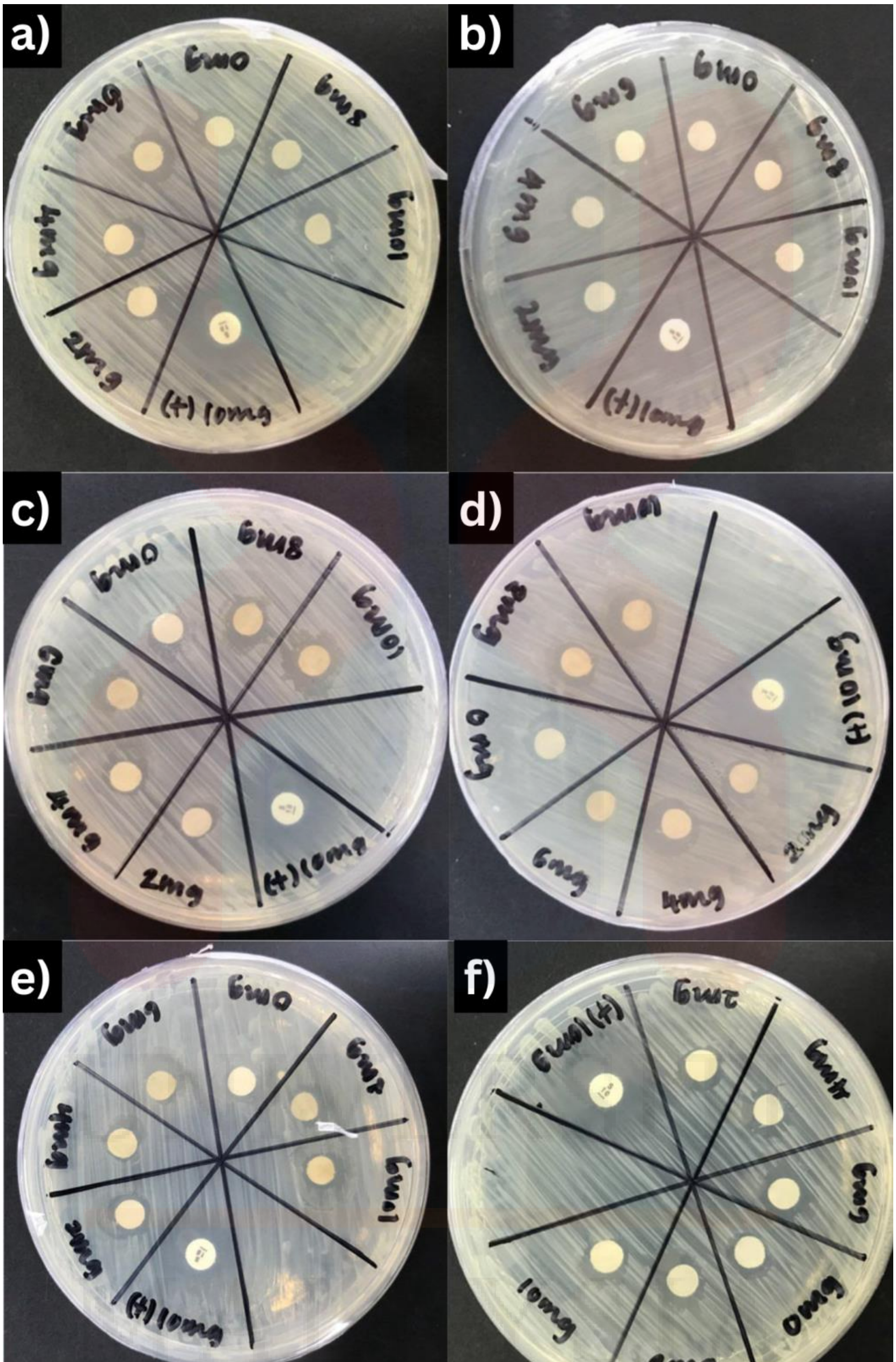

Figure 4.3: The zone of inhibition of extract *E. punicea* was observed in the Petri dish against *S. aureus,* a) pseudostem (SP1), b) duplicated petri dish of pseudostem (SP2), C) leaf (SL1), d) duplicated leaf (SL2), e) rhizome (SR1), f) duplicated (SR2)

4.3 Minimum Inhibitory Concentration (MIC)

The Minimal Inhibitory Concentration (MIC) was the lowest concentration of an antimicrobial agent that inhibited the observable growth of a microbe after incubation for 24 hours (Mouton et al., 2018). This measurement was a crucial factor in evaluating antimicrobial susceptibility which helped in determining the effectiveness of an antimicrobial agent versus a specific bacterium (Parvekar et al., 2020). In this study, the 2 mg/ml concentration was related to the minimum inhibitory concentration (MIC). This concentration demonstrates susceptibility to the three components of *E. punicea*. This concentration was found to effectively prevent microbial growth. This outcome shows that a small quantity of the extract, when dissolved in methanol, was sufficient to demonstrate an antibacterial impact. The high efficacy of *E. punicea* at this concentration indicates the presence of powerful bioactive chemicals, which have the potential to be used in the development of natural antibacterial drugs.

According to the research paper Modulatory antimicrobial activity of *Piper arboreum* extracts, the minimal inhibitory concentration (MIC) against *C. krusei* was determined at 1024 µg/mL, or 1.024 mg/ml (Tintino et al., 2014). When comparing the two results, the *E. punicea* extract was closely similar and effective in inhibiting the growth of microbes. A study demonstrated the antimicrobial activity of the crude extract, fractions, and isolation of zerumbone from the rhizomes of *Zingiber roseum*, the minimum inhibitory concentration (MIC) value for zerumbone ranged from 64 µg/mL (0.064 mg/mL) against several fungi, gram-negative and positive bacteria (Al-Amin et al., 2019).

When comparing the study papers, the efficacy of the zerumbone component from *Zingiber roseum* was greater than that of *E. punicea*. Zerumbone had a much greater level of antimicrobial activity, successfully inhibiting a broad variety of harmful microorganisms. Zerumbone refers to an organic substance obtained from botanical sources. More precisely, it is a kind of organic compound called sesquiterpene that is present in the rhizome of the Zingiber zerumbet plant, which is usually referred to as shampoo ginger or pinecone ginger (Girisa et al., 2019). Lastly, the study report entitled Antioxidant and Antimicrobial Activity of Extracts from Various Parts of *Etlingera sayapensis* (Zingiberaceae) indicates that the minimum inhibitory concentration (MIC) of the stem extract against the *Candida parapsilosis* fungus was determined at 1.04 mg/ml (Mahdavi et al., 2017). When comparing to *E. punicea*, it was found that they were nearly related due to their closely similar concentrations.

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The research successfully demonstrated the antimicrobial activity of the extract derived from *Etlingera punicea* (Roxb.) R.M.Smith (1986) by comparing the inhibitory potential of its three plant components namely the pseudostem (EP), leaf (EL), and rhizome (ER). The research study outcomes demonstrated that six concentrations showed susceptibility to three components of *E. punicea* against two types of microorganisms. The three parts of *E. punicea* extract demonstrate significant potential as natural antibacterial agents due to their efficient inhibition of the growth in *Staphylococcus aureus* (gram-positive bacteria) and *Escherichia coli* (gram-negative bacteria).

Next, the highest inhibitory zone was recorded at 10 mg/ml concentration of EL extract towards *E. coli* with a mean of 11.680 and an SD of 1.386. The lowest concentration was observed at 2 mg/ml of ER extract at *E. coli* with a mean of 6.300 and SD 0.226. Based on the evidence presented, it was concluded that the methanol leaf extract of *E. punicea* revealed better effectiveness compared to EP and ER. The Minimum Inhibitory Concentration (MIC) of this study was measured at a concentration of 2 mg/ml and showed susceptibility to all three parts of *E. punicea.* The *E. punicea* extract represented the greatest inhibitory zone against gram-negative bacteria, specifically *E. coli*, compared to gram-positive bacteria such as *S. aureus*.

The chemical features of the substances in the *E. punicea* extract could lead to a more efficient interaction with the parts of gram-negative bacteria because of variations in pH, ion concentration, or other microenvironmental elements present within the bacterial cell walls. In addition, methanol was also a polar solvent with the ability to extract an extensive range of bioactive chemicals, including compounds with antimicrobial traits. Finally, the leaf extract of *E. punicea* exhibits a higher level of antibacterial activity when compared to the pseudostem and rhizome.

5.2 Recommendation

Further research can be conducted by adjusting the concentration to determine the most effective antimicrobial activity in both the smallest and largest inhibitory zones. The difference between the highest and lowest concentrations of *E. punicea* commonly consists in their relative efficacy in inhibiting or destroying microbes. Increased concentrations generally result in more powerful antimicrobial actions, which may lead to bigger inhibitory zones or reduced microbial populations. Conversely, lower doses might prove less effective although provide benefits in terms of safety and cost-effectiveness. For example, a study can be carried out to investigate the effectiveness of lower concentrations at 0.05 mg/ml or 1mg/ml to determine the minimum inhibitory concentration (MIC) of *E. punicea* using the disk diffusion technique. Other than the disc diffusion method, there are several additional techniques available to determine the minimum inhibitory concentration (MIC) of antimicrobial activity. These techniques, which could potentially be used in future studies, include the Gradient Diffusion Method, Agar Well Diffusion Method, and Microbroth Dilution Method.

Apart from that, chemical compounds could be isolated from the extract of *E. punicea* for antimicrobial properties. Isolating active components from plant extracts could result in improved potency or effectiveness compared to the raw extract. The reason for the difference is that the developed version of the molecule usually has a greater concentration, which results in a stronger antibacterial activity. In addition, removing inactive or antagonistic chemicals might decrease contamination and increase the overall effectiveness of the antimicrobial substance. In the present research, the leaf extract exhibited the largest inhibition zone, displaying a powerful inhibitory action. Furthermore, the flavonoid ingredient can be extracted from the leaf extract. Flavonoids have received awareness for their varied antidiabetic effects and are typically recognised as harmless with very few negative consequences when used for diabetes treatment.

6.0 REFERENCES

Abers, M., Schroeder, S., Goelz, L., Sulser, A., St. Rose, T., Puchalski, K., & Langland, J. (2021, April 17). Antimicrobial activity of the volatile substances from essential oils. *BMC Complementary Medicine and Therapies*, 21(1).

Akram, S., Khamis, S., Zulkifly, S., Talib, R., & Ab Ghani, N. I. (2023). Phylogeny Study of 20 Selected Species of Zingiberaceae from Ex situ Collections in Peninsular Malaysia. *Pertanika Journal of Tropical Agricultural Science*, 46(1).

Alegado, R. A., & King, N. (2014). Bacterial influences on animal origins. *Cold Spring Harbor perspectives in biology*, 6(11), a016162.

Allocati, N., Masulli, M., Alexeyev, M. F., & Di Ilio, C. (2013). *Escherichia coli* in Europe: An Overview. *International Journal of Environmental Research and Public Health/International Journal of Environmental Research and Public Health*, 10(12), 6235–6254.

Anwar, M. A., Aziz, S., Ashfaq, K., Aqib, A. I., Shoaib, M., Naseer, M. A., & Mahmood, A. (2022). Trends in Frequency, Potential Risks and Antibiogram of *E. coli* Isolated from Semi-Intensive Dairy Systems. *Pakistan Veterinary Journal*, 42(2), 167.

Al-Amin, M., Siddiqui, M. A., Ruma, S. A., Eltayeb, N. M., Sultana, G. N. N., Salhimi, S. M., & Hossain, C. F. (2019). Antimicrobial activity of the crude extract, fractions and isolation of zerumbone from the rhizomes of Zingiber roseum. *Journal of Research in Pharmacy*, 23(3), 559-66.

Appalasamy, S., Arumugam, N., Zamri, N. S. A., Fadhlina, A., Kumaran, J. V., & Subramaniam, S. (2022). First Report on Wild Ginger (Family: Zingiberaceae) Species Composition with New Records in Limestone Forests of Kelantan, Peninsular Malaysia. *Tropical Life Sciences Research*, *33*(3), 33–46.

Appalasamy, S., Rathamanalan, S., Arsogah, S., Harni, S., Sam, K. K., Amaludin, N. A., Boon, J. G., Kumaran, J. V., Arumugam, N., & Subramaniam, S. (2020). Gingers Species Diversity and Distribution along a Natural Trail of Lojing Highlands, Kelantan. *IOP Conference Series*, *549*(1), 012036.

Appalasamy, S., Arumugam, N., Rao, P. V. M., Subramaniam, S., Kumaran, J. V., Nor, M. M., & Salam, M. A. (2023). An overview of the pollination interactions between the ginger family (Zingiberaceae) and insects in Asia. *European Journal of Entomology*, *120*, 170–181.

Basu, S., Bose, C., Ojha, N., Das, N., Das, J., Pal, M., & Khurana, S. (2015, April 30). Evolution of bacterial and fungal growth media. *Bioinformation*, 11(4), 182–184.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of pharmaceutical analysis*, 6(2), 71-79.

Beristain-Bauza, S. D. C., Hernández-Carranza, P., Cid-Pérez, T. S., Ávila-Sosa, R., Ruiz-López, I. I., & Ochoa-Velasco, C. E. (2019). Antimicrobial activity of ginger (*Zingiber officinale*) and its application in food products. *Food Reviews International*, 35(5), 407-426.

Bhargav, H., Shastri, S. D., Poornav, S., Darshan, K., & Nayak, M. M. (2016, February). Measurement of the Zone of Inhibition of an Antibiotic. *2016 IEEE 6th International Conference on Advanced Computing (IACC)*.

Blagodatskaya, E., & Kuzyakov, Y. (2013). Active microorganisms in soil: Critical review of estimation criteria and approaches. *Soil Biology & Biochemistry*, 67, 192–211.

Bokhari, A., Chuah, L. F., Yusup, S., Ahmad, J., & Aziz, H. (2015). Kapok seed oil extraction using soxhlet extraction method: optimization and parametric study. *Australian Journal of Basic and Applied Sciences*, 9(37), 429-431.

Chan, E.WC., Limb, Y.Y., Wong, S.K., (2013). Botany uses phytochemistry, and pharmacology of selected *Etlingera* gingers: A review. *Pharmacognosy Communications.*

Chandana, A. R., Swetha, L., Shetty, V. R., Ravi, V. M., Ramesh Babu, D. R., & Sunanda. (2024). Inhibition Zones: A Window into Antibiotic Resistance. *International Journal for Multidisciplinary Research (IJFMR)*

Chavarria, G., & dos Santos, H. P. (2012). Plant water relations: absorption, transport and control mechanisms.

Chongkraijak, W., Ngamnriabsakul, C., & Poulsen, A. D. (2013). Morphological Diversity and Distribution of *Etlingera littoralis* (König) Giseke (Zingiberaceae) in Southern Thailand. *Walailak Journal of Science and Technology*, *10*(6), 643–656.

Chew, Y. L., Mahadi, A. M., Wong, K. M., & Goh, J. K. (2018). Anti-methicillin-resistance *Staphylococcus aureus* (MRSA) compounds from Bauhinia kockiana Korth. And their mechanism of antibacterial activity. *BMC Complementary and Alternative Medicine*, 18(1).

Daniel-Jambun, D., Dwiyanto, J., Lim, Y. Y., Tan, J. B. L., Muhamad, A., Yap, S. W., & Lee, S. M. (2017). Investigation on the antimicrobial activities of gingers (*Etlingera coccinea* (Blume) S. Sakai & Nagam and *Etlingera sessilanthera* RM Sm.) endemic to Borneo. *Journal of applied microbiology*, 123(4), 810-818.

Darus, N.F.M., (2020). Distribution and anatomy of *Etlingera* spp. Jeli, kelatan. *University Malaysia Kelatan, campus jeli.*

De Luna, S. L. R., Ramírez-Garza, R. E., & Saldívar, S. O. S. (2020). Environmentally friendly methods for flavonoid extraction from plant material: Impact of their operating conditions on yield and antioxidant properties. *The Scientific World Journal*.

Dhawan, D., & Gupta, J. (2017). Comparison of Different Solvents for Phytochemical Extraction Potential from Datura metel Plant Leaves. *International Journal of Biological Chemistry*, 11(1), 17-22.

Elshikh, M., Ahmed, S., Funston, S., Dunlop, P., McGaw, M., Marchant, R., & Banat, I. M. (2016). Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. *Biotechnology letters*, 38, 1015-1019.

Faner, R., Sibila, O., Agustí, A., Bernasconi, E., Chalmers, J. D., Huffnagle, G. B., ... & Monsó, E. (2017). The microbiome in respiratory medicine: current challenges and future perspectives. *European Respiratory Journal*, 49(4).

Girisa, S., Shabnam, B., Monisha, J., Fan, L., Halim, C. E., Arfuso, F., Ahn, K. S., Sethi, G., & Kunnumakkara, A. B. (2019). Potential of Zerumbone as an Anti-Cancer agent. *Molecules/Molecules Online/Molecules Annual*, 24(4), 734.

Hamma, I. I., Tafinta, I. Y., Abdulmalik, A., Theophilus, J., & Abubakar, M. (2020). Phytochemical Screening and Antibacterial Activity of the Crude Extract of Scent Leaf (Ocimum gratissimum) on Escherichia coli and Staphylococcus aureus. *Asian Plant Research Journal*, 5(2), 1-7.

Ivanović, M., Makoter, K., & Razboršek, M. I. (2021). Comparative study of chemical composition and antioxidant activity of essential oils and crude extracts of four characteristic Zingiberaceae herbs. *Plants*, *10*(3), 501.

Jang, J., Hur, H. G., Sadowsky, M. J., Byappanahalli, M. N., Yan, T., & Ishii, S. (2017). Environmental *Escherichia coli*: ecology and public health implications—a review. *Journal of applied microbiology*, 123(3), 570-581.

Jiang, C. Y., Dong, L., Zhao, J. K., Hu, X., Shen, C., Qiao, Y., Zhang, X., Wang, Y., Ismagilov, R. F., Liu, S. J., & Du, W. (2016, April). High-Throughput Single-Cell Cultivation on Microfluidic Streak Plates. *Applied and Environmental Microbiology*, 82(7), 2210–2218.

Joshi, L. R., Tiwari, A., Devkota, S. P., Khatiwada, S., Paudyal, S., & Pande, K. R. (2014). Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in dairy farms of Pokhara, Nepal. *International Journal of Veterinary Science*, 3(2), 87-90.

Kachhawa, D. (2017). Microorganisms as a biopesticides. *Journal of Entomology and Zoology Studies*, 5(3), 468-473.

Kim, M., Bae, J., & Kim, T. (2017). Long-Term and programmable bacterial subculture in completely automated microchemostats. *Analytical Chemistry*, *89*(18), 9676–9684.

Khaleghi, F., Yaacob, W., Din, L. B., & Khalilzadeh, M. A. (2012, January). Volatile Oil Compositions of Several Parts of *Etlingera fulgens* from Malaysia. *Journal of Essential Oil Bearing Plants*, 15(2), 180–185.

Kowalska-Krochmal, B., & Dudek-Wicher, R. (2021). The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. *Pathogens*, 10(2), 165.

Kress, W. J. (2013). The phylogeny and classification of the Zingiberales. *Annals of the Missouri Botanical Garden*, 698-721.

Laxmi, & Begum, T. (2022). Phytochemical analysis of leaf extract of the medicinal plants. *Journal of Medicinal Plants Studies*, 10(6), 34-36

Lee, A. S., De Lencastre, H., Garau, J., Kluytmans, J., Malhotra-Kumar, S., Peschel, A., & Harbarth, S. (2018). Methicillin-resistant *Staphylococcus aureus*. *Nature reviews Disease primers*, 4(1), 1-23.

Li, J., Pettinato, M., Campardelli, R., De Marco, I., & Perego, P. (2022). High-Pressure Technologies for the Recovery of Bioactive Molecules from Agro-Industrial Waste. Applied Sciences, 12(7), 3642.

Lim, T. K. (2012). Edible Medicinal and Non-Medicinal Plants: Volume 3, Fruits. *Springer Science & Business Media.*

Mahdavi, B., WA, Y., & Din, L. B. (2017). Antioxidant and Antimicrobial Activity of the Extracts from Different Parts of *Etlingera sayapensis* (Zingiberaceae). *Sains Malaysiana*, 46(9), 1565–1571.

Mostafa, A. A., Al-Askar, A. A., Almaary, K. S., Dawoud, T. M., Sholkamy, E. N., & Bakri, M. M. (2018, February). Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. *Saudi Journal of Biological Sciences*, 25(2), 361–366.

Mouton, J. W., Meletiadis, J., Voss, A., & Turnidge, J. (2018). Variation of MIC measurements: the contribution of strain and laboratory variability to measurement precision. *Journal of Antimicrobial Chemotherapy*, 73(9), 2374-2379.

Myles, I. A., & Datta, S. K. (2012). *Staphylococcus aureus*: an introduction. *Seminars in Immunopathology*, 34(2), 181–184.

Nagappan, T., Tatin, Y., & Skornickova, J. L. (2019). Investigation on bioactive potential of selected wild ginger, genus *Etlingera* from Tasik Kenyir, Terengganu. *Greater Kenyir Landscapes: Social Development and Environmental Sustainability: From Ridge to Reef*, 75-82.

Naveed, R., Hussain, I., Tawab, A., Tariq, M., Rahman, M., Hameed, S., Mahmood, M. S., Siddique, A. B., & Iqbal, M. (2013, October 14). Antimicrobial activity of the bioactive components of essential oils from Pakistani spices against *Salmonella* and other multi-drug resistant bacteria. *BMC Complementary and Alternative Medicine*, 13(1).

Nicolas, I., Bordeau, V., Bondon, A., Baudy-Floc'h, M., & Felden, B. (2019). Novel antibiotics effective against gram-positive and -negative multi-resistant bacteria with limited resistance. *PLoS Biology*, 17(7), e3000337.

Niissalo, M. A., Leong-Škorničková, J., Khew, G., & Webb, E. L. (2016). Very small relict populations suggest high extinction debt of gingers in primary forest fragments of a tropical city. *American Journal of Botany*, *104*(1), 182–189.

Ndenge, M. J. (2021). *Antimicrobial activity of the phytexponent against methicilin resistant staphylococcus aureus*. [Degree, A Research Project, Mount Kenya University].

Ngamwonglumlert, L., Devahastin, S., & Chiewchan, N. (2017). Natural colorants: Pigment stability and extraction yield enhancement via utilization of appropriate pretreatment and extraction methods. *Critical reviews in food science and nutrition*, 57(15), 3243-3259.

Otto, M. (2014). *Staphylococcus aureus* toxins. *Current opinion in microbiology*, 17, 32-37.

Okorie, N., Fasogbon, S. A., Mohammed, U., Abubakar, U., Avwioro, O. G., Tsamiya, R. I., & Tangaza, A. T. (2020). Study of phytochemical compositions and separation of colours using thin layer column chromatography for Bixa orellana (annatto) seed extracts. *Yen Medical Journal*, 2(1), 43-49

Park, J. Y., & Seo, K. S. (2019). *Staphylococcus aureus*. *Food microbiology: fundamentals and frontiers*, 555-584.

Parvekar, P., Palaskar, J., Metgud, S., Maria, R., & Dutta, S. (2020). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver nanoparticles against Staphylococcus aureus. *Biomaterial investigations in dentistry*, 7(1), 105-109.

Payne, T. D., Dixon, L. R., Schmidt, F. C., Blakeslee, J. J., Bennett, A. E., & Schultz, Z. D. (2024). Identification and quantification of pigments in plant leaves using thin layer chromatography-Raman spectroscopy (TLC-Raman). *Analytical Methods*, 16(16), 2449-2455.

Pitopang, R., Udayana, R. A. D. S., Pratiwi, A. D., Ananda, M., Harso, W., & Ramawangsa, P. A. (2021, May). Antibacterial activities of *Etlingera flexuosa* AD Poulsen (Zingiberaceae) from Central Sulawesi on *Staphylococcus aureus* and *Escherichia coli*. *In IOP Conference Series: Earth and Environmental Science* (Vol. 743, No. 1, p. 012065). IOP Publishing.

Rama, R. S., Milind, R. G., & Kunchiraman, B. N. (2012). Comparative study of antimicrobial activity and phytochemical screening of aqueous and alcoholic leaf
Journal of Environmental Research and development, 7(1A).

Redfern, J., Kinninmonth, M., Burdass, D., & Verran, J. (2014, May). Using Soxhlet Ethanol Extraction to Produce and Test Plant Material (Essential Oils) for Their Antimicrobial Properties. *Journal of Microbiology & Biology Education*, 15(1), 45–46.

Rouhan, G., & Gaudeul, M. (2014). Plant taxonomy: a historical perspective, current challenges, and perspectives. *Molecular plant taxonomy: Methods and protocols*, 1-37.

Sahri, Z., Nordin, S., & Haron, H. (2012). Malaysia Indigenous Herbs Knowledge Representation. *ResearchGate*.

Sass, C., Iles, W. J., Barrett, C. F., Smith, S. Y., & Specht, C. D. (2016, January 21). Revisiting the Zingiberales: using multiplexed exon capture to resolve ancient and recent phylogenetic splits in a charismatic plant lineage. *PeerJ*, 4, e1584.

Savoia, D. (2012). Plant-derived antimicrobial compounds: alternatives to antibiotics. *Future microbiology*, 7(8), 979-990.

Saeed, N., Khan, M. R., & Shabbir, M. (2012). Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. *BMC Complementary and Alternative Medicine*, 12(1).

Selvaraj, D., Sarma, R. K., & Ramalingam, S. (2008). Phylogenetic analysis of chloroplast matK gene from Zingiberaceae for plant DNA barcoding. *Bioinformation*, 3(1), 24–27.

Sen, A., & Batra, A. (2012). Evaluation of antimicrobial activity of different solvent extracts of medicinal plant: Melia azedarach L. *International Journal of Current Pharmaceutical Research*, 4(2), 67-73.

Simpson, M. G. (2019). Diversity and classification of flowering plants: *amborellales, nymphaeales, austrobaileyales, magnoliids, monocots,* and *ceratophyllales*. *In Elsevier eBooks* (pp. 187–284).

Stéphane, F. F. Y., Jules, B. K. J., Batiha, G. E., Ali, I., & Bruno, L. N. (2021). Extraction of bioactive compounds from medicinal plants and herbs. *Nat Med Plants*.

Subramaniam, R,. Neethirajan, K., Jayakumararaj, R. (2012). Profile of bioactive compounds in Syzygium cumini. *Journal of Pharmacy Research*. 5(8): 4548-4553.

Syazwan, M. A. (2023) Determination of bioactive compounds of *Etlingera punicea* (Family Zingiberaceae) using Liquid Chromatography. *[Unpublished Thesis University Malaysia Kelanatan (umk)]*

Tankeshwar, A. (2022, October 28). Streak Plate Method: Principle, Procedure, Uses. Microbe Online. https://microbeonline.com/streak-plate-method-principle-purpose-procedure-results/

Tintino, S. R., Souza, C. E., Guedes, G. M., Costa, J. I., Duarte, F. M., Chaves, M. C. O., ... & Coutinho, H. D. (2014). Modulatory antimicrobial activity of *Piper arboretum* extracts (Zingiberaceae). *Acta Botanica Croatica*, 73(1), 281-289.

Ud-Daula, A. S., & Basher, M. A. (2019). Genus *Etlingera* - A review on chemical composition and antimicrobial activity of essential oils. *Journal of Medicinal Plants Research*, 13(7), 135-156.

Ud-Daula, A. S., Demirci, F., Salim, K. A., Demirci, B., Lim, L. B., Baser, K. H. C., & Ahmad, N. (2016). Chemical composition, antioxidant and antimicrobial activities of essential oils from leaves, aerial stems, basal stems, and rhizomes of *Etlingera fimbriobracteata* (K. Schum.) RM Sm. *Industrial Crops and products*, 84, 189-198.

Uma, E., & Muthukumar, T. (2014). Comparative root morphological anatomy of Zingiberaceae. *Systematics and Biodiversity*, *12*(2), 195–209.

Valle Jr, D. L., Cabrera, E. C., Puzon, J. J. M., & Rivera, W. L. (2016). Antimicrobial activities of methanol, ethanol and supercritical CO2 extracts of Philippine Piper betle L. on clinical isolates of gram positive and gram negative bacteria with transferable multiple drug resistance. *PloS one*, 11(1), e0146349.

Van Hal, S. J., Lodise, T. P., & Paterson, D. L. (2012). The Clinical Significance of Vancomycin Minimum Inhibitory Concentration in *Staphylococcus aureus* Infections: A Systematic Review and Meta-analysis. *Clinical Infectious Diseases/Clinical Infectious Diseases (Online. University of Chicago. Press*), 54(6), 755–771.

Voukeng, I. K., Kuete, V., Dzoyem, J. P., Fankam, A. G., Noumedem, J. A., Kuiate, J. R., & Pages, J. M. (2012). Antibacterial and antibiotic-potentiation activities of the methanol extract of some cameroonian spices against Gram-negative multi-drug resistant phenotypes. *BMC Research Notes*, 5, 1-10.

Wulandari, L., Retnaningtyas, Y., Nuri, & Lukman, H. (2016). Analysis of flavonoid in medicinal plant extract using infrared spectroscopy and chemometrics. *Journal of Analytical Methods in Chemistry*, 2016, 1–6.

Xu, Z., & Chang, L. (2017). Zingiberaceae. *Identification and Control of Common Weeds*: Volume 3, 909-911.

Yassen, D., & Ibrahim, A. E. (2016). Antibacterial activity of crude extracts of ginger (*Zingiber officinale Roscoe*) on *Escherichia coli* and *Staphylococcus aureus*: A Study in vitro. *Indo American Journal of Pharmaceutical Research*, 6(06), 5830-35.

Zahara, M. (2020, January 1). Identification of morphological and stomatal characteristics of Zingiberaceae as medicinal plants in Banda Aceh, Indonesia. *IOP Conference Series: Earth and Environmental Science*, 425(1), 012046.

Zhao, S., & Zhang, D. (2014, September). Supercritical CO2 extraction of Eucalyptus leaves oil and comparison with Soxhlet extraction and hydro-distillation methods. *Separation and Purification Technology*, *133*, 443–451.

Zygler, A., Słomińska, M., & Namieśnik, J. (2012). Soxhlet Extraction and New Developments Such as Soxtec. *Comprehensive Sampling and Sample Preparation*, 65–82