ANTIBACTERIAL ACTIVITY AND MECHANISMS OF ACTION OF THE SEMI-PURIFIED FRACTIONS FROM *Melaleuca cajuputi* LEAVES AGAINST SELECTED BACTERIAL STRAINS

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by

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LIST OF SYMBOLS

<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
±	Plus-minus sign
%	Percentage
α	Alpha
β	Beta
γ	Gamma
°C	Degree Celcius
g	Gram
L	Litre
µ	Micro
µg	Microgram
µg/mL	Microgram per millilitre
µL	Microlitre
mg	Milligram
mg/mL	Milligram per millilitre
mL	Millilitre
mM	Millimolar
mol	Mole

LIST OF ABBREVIATIONS

Abs	Absorbance
ATCC	American-type culture collection
AU	Arbitrary unit
BSLT	Brine shrimp lethality test
CFU	Colony forming units
FTIR	Fourier Transform Infra-Red
GCMS	Gas chromatography Mass spectrometry
h	Hour (s)
ICP-MS	Inductively coupled plasma mass spectrometry
ID	Identity
kcal	kilocalorie
MBC	Minimum bactericidal concentration
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
min	Minute (s)
MS	Mass spectrometry
NIST	National Institute of Standards and Technology
nm	Nanometer
PBS	Phosphate buffer saline
PDB	Protein Data Bank
PDBQT	Protein Data Bank, Partial Charge, and Atom Type
pINT	*p*-Iodonitrotetrazolium violet
pH	Potential hydrogen
ppm	Parts per million
RT	Retention time
rpm	Revolution per minute
SD	Standard deviation
SEM	Scanning electron microscope
SPSS	Statistical software for social sciences
USA	United States of America

USM	Universiti Sains Malaysia
UV	Ultraviolet
v/v	Volume per volume
WHO	World Health Organization
w/v	Weight per volume
w/w	Weight per weight

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AKTIVITI ANTIBAKTERIA DAN MEKANISME TINDAK BALAS FRAKSI SEPARA TULEN DARIPADA DAUN *MELALEUCA CAJUPUTI* TERHADAP BAKTERIA TERPILIH

ABSTRAK

Kejadian jangkitan bakteria yang semakin meningkat dan penyebaran rintangan antimikrob yang pantas menggariskan keperluan untuk mencari ubat alternatif baru daripada sumber semula jadi, terutamanya tumbuhan ubatan. Oleh itu, kajian ini bertujuan untuk menyiasat aktiviti antibakteria ekstrak daun *Melaleuca cajuputi* dan mendedahkan mekanisme antibakteria bagi fraksi separuh tulen yang paling aktif terhadap strain bakteria terpilih. Kandungan mineral dalam daun *M. cajuputi* dianalisis menggunakan spektometri jisim – plasma berpasangan secara induktif (ICP-MS). Ekstrak metanol, etanol, dan akueus diperoleh melalui rendaman sejuk. Selepas itu, ekstrak mentah yang paling aktif telah diperingkatkan untuk mendapatkan fraksi separa tulen dengan teknik pemeringkatan berpandukan biocerakinan aktiviti antibakteria ekstrak mentah dan fraksi *Melaleuca* separa tulen (MFs) dinilai menggunakan ujian mikropencairan. Spektroskopi ultralembayung nampak (UV-Vis), inframerah transformasi Fourier (FTIR), dan spektrometri jisim kromatografi gas (GC-MS) digunakan untuk mengenal pasti komposisi kimia ekstrak paling aktif dan MF separa tulen. Mekanisme tindakan MF yang paling aktif telah disiasat menggunakan ujian masa-maut, pemeriksaan morfologi sel, dan kajian dok molekul dalam siliko. Profil ketoksikan telah dinilai menggunakan ujian maut anak udang (BSLT). Makronutrien seperti kalium (7182.042 mg/kg), natrium (3895.795 mg/kg), kalsium (3730.259 mg/kg), dan unsur surih termasuk besi (89.394 mg/kg), mangan (57.070 mg/kg), dan zink (51.626 mg/kg) telah dikesan dalam

ekstrak daun *M. cajuputi*. Ujian antibakteria menunjukkan bahawa MF2c dan MF2d adalah yang paling aktif, dengan nilai kepekatan perencatan minimum (MIC) masing-masing berjulat daripada 0.13 mg/mL hingga 0.25 mg/mL dan 0.063 mg/mL hingga 0.25 mg/mL. Sebatian bioaktif yang dikenal pasti dalam MF2c ialah β-eudesmol (71.96%), α-eudesmol (18.83%), dan γ-eudesmol (9.21%). Sementara itu, 2-isopropil-10-metilfenanthrena (83.09%), 10-metilantrasena-9-karbokaldehid (10.95%), asid trimetil galik (2.60%), metillatodoratin (2.10%), dan metoksiamina (0.28%) dikenal pasti dalam MF2d. Ujian masa-maut mendedahkan bahawa MF2c dan MF2d mempamerkan kesan bakteria yang bergantung kepada kepekatan terhadap strain bakteria yang diuji. Mikrograf elektron pengimbasan bakteria yang dirawat menunjukkan kerosakan membran sel yang jelas dicirikan oleh pemanjangan sel yang tidak normal, pengecutan, dan serpihan organik pada permukaan sel. Tambahan pula, analisis dok molekul dalam siliko mendedahkan bahawa 2-isopropil-10-metilfenantrena mempunyai kecenderungan pengikatan tertinggi terhadap polimerase RNA yang bergantung kepada DNA, D-alanil transferase, DNA girase, dan sithase dihidropteroat, dengan skor tenaga dok 8.4, -6.9, -6.5, dan -6.1 kcal/mol masing-masing. Berdasarkan hasil kajian ketoksikan, ekstrak metanol *M. cajuputi* (LC_{50} 781 μg/mL menunjukkan ketoksikan ringan, manakala MF2c (LC_{50} 6621 μg/mL) dan MF2d (LC_{50} 1165 μg/mL) adalah tidak toksik. Kesimpulannya, semi-MF yang telah dibuktikan menunjukkan kesan antibakteria yang luar biasa dan tidak toksik. Penemuan ini menjanjikan pembangunan strategi terapi alternatif untuk memerangi jangkitan bakteria.

ANTIBACTERIAL ACTIVITY AND MECHANISMS OF ACTION OF THE SEMI-PURIFIED FRACTIONS FROM *MELALEUCA CAJUPUTI* LEAVES AGAINST SELECTED BACTERIAL STRAINS

ABSTRACT

The increasing incidence of bacterial infections and the rapid spread of antimicrobial resistance underscores the need to find novel alternative medications from natural sources, especially medicinal plants. Thus, this study aimed to investigate the antibacterial activities of *Melaleuca cajuputi* leaf extract and unveil the possible antibacterial mechanisms of the most potent semi-purified fractions against selected bacterial strains. The mineral content in *M. cajuputi* leaf was analyzed using inductively coupled plasma-mass-spectrometry (ICP-MS). Methanolic, ethanolic, and aqueous extracts were obtained by cold maceration. Subsequently, the most potent crude extract was fractionated to obtain semi-purified fractions by bioassay-guided fractionation technique. The antibacterial activity of the crude extracts and semi-purified *Melaleuca* fractions (MFs) was evaluated using a broth microdilution assay. Ultraviolet-visible (UV-Vis), Fourier transform infrared (FTIR) spectroscopies, and gas chromatography-mass spectrometry (GC-MS) were employed to identify the chemical compositions of the most potent extract and semi-purified MFs. Mechanisms of action of the most potent MFs were investigated using time-kill assay, cell morphology examination, and *in-silico* molecular docking studies. The toxicity profile was evaluated using the brine shrimp lethality test (BSLT). Macronutrients such as potassium (7182.042 mg/kg), sodium (3895.795 mg/kg), calcium (3730.259 mg/kg), and trace elements including iron (89.394 mg/kg), manganese (57.070 mg/kg), and zinc (51.626 mg/kg) were detected in *M.*

cajuputi leaf extract. The antibacterial assays demonstrated that MF2c and MF2d were the most potent, with minimum inhibitory concentration (MIC) values ranging from 0.13 mg/mL to 0.25 mg/mL and 0.063 mg/mL to 0.25 mg/mL, respectively. The bioactive compounds identified in MF2c were β-eudesmol (71.96%), α-eudesmol (18.83%), and γ-eudesmol (9.21%). Meanwhile, 2-isopropyl-10-methylphenanthrene (83.09%), 10-methylanthracene-9-carboxaldehyde (10.95%), trimethyl gallic acid (2.60%), methyl-lathodoratin (2.10%), and methoxyamine (0.28%) were identified in MF2d. Time-kill assay revealed that MF2c and MF2d exhibited concentration-dependent bactericidal effects against the tested bacterial strains. The scanning electron micrographs of the treated bacteria showed apparent cell membrane damage characterized by abnormal cell elongation, shrinkage, and organic debris on the cell surfaces. Furthermore, the *in-silico* molecular docking analysis revealed that 2-isopropyl-10-methylphenanthrene had the highest binding propensity against DNA-dependent RNA polymerase, D-alanyl transferase, DNA gyrase, and dihydropteroate synthase, with docking energy scores of -8.4, -6.9, -6.5, and -6.1 kcal/mol respectively. Based on the toxicity results, *M. cajuputi* methanolic extract (LC_{50} 781 μg/mL showed mild toxicity, whereas MF2c (LC_{50} 6621 μg/mL) and MF2d (LC_{50} 1165 μg/mL) were non-toxic. In conclusion, the semi-purified MFs showed remarkable antibacterial effects and were non-toxic. The findings hold promise for developing alternative therapeutic strategies to combat bacterial infection

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

In light of growing challenges about antibiotic resistance and the diminishing efficacy of synthetic antibiotics, antibacterial research has emerged as a vital field in modern medicine (Chinemerem *et al.*, 2022). Antibacterial research is a multidisciplinary strategy that aims to understand the biology of bacterial pathogens, elucidate antibiotic resistance mechanisms, and uncover novel therapeutic targets. This interdisciplinary discipline uses microbiology, biochemistry, pharmacology, and computational biology knowledge to address the alarming issues of bacterial infections (Pisano *et al.*, 2019; Mogana *et al.*, 2020; Álvarez-Martínez *et al.*, 2021). Alongside pursuing new therapeutic agents, antibacterial research involves improving existing antibiotics through chemical modification, combination therapy, and developing antibiotic adjuvants that increase bacterial susceptibility to antimicrobial agents (Vaou *et al.*, 2021). Historically, humans encountered several diseases instigated by microbes, primarily bacteria, resulting in significant escalations in global morbidity and mortality rates. In the early 1940s, penicillin demonstrated significant efficacy as an antibacterial agent against bacterial pathogens. However, the effectiveness of penicillin has been diminished due to the evolution of various resistance mechanisms in bacteria ((Abushaheen *et al.*, 2020), hence rendering the treatment of bacterial infections more challenging. Antimicrobial resistance (AMR) is characterized by the ability of microorganisms, such as bacteria, fungi, and viruses, to adapt and proliferate in the presence of drugs that previously were effective against them (Dadgostar, 2019).

Antimicrobial resistance is a significant challenge to public health systems in both developed and developing countries (Chinemerem *et al.*, 2022). For instance, in the European Union, almost 3 million individuals are affected by nosocomial infections annually, with roughly 50,000 cases resulting in death. One of the primary causes of these infections is multi-drug resistant bacteria, particularly staphylococci (Miklasińska-Majdanik *et al.*, 2018). Furthermore, there is also a significant economic burden associated with AMR. In Europe alone, it has been estimated that antimicrobial resistance is linked to a cost of over nine billion euros annually. Similarly, the Centers for Disease Control and Prevention (CDC) has reported that antimicrobial resistance significantly increases 20 billion dollars in direct healthcare costs in the United States (Dadgostar, 2019). Consequently, the rise of antibiotic resistance, caused by the persistent presence of resistant bacteria, poses a significant global health concern. The situation is worsened by the decrease in drug production since the late 1960s, irrational use of antibiotics, and immigration (Alsheikh *et al.*, 2020). Given these circumstances, there is a growing interest in exploring alternatives to antibiotics to prevent and treat bacterial infections. Thus, naturally occurring compounds from medicinal plants could offer a promising alternative to antibiotics (Banerjee *et al.*, 2017; Zengin *et al.*, 2022). Plants have been a good source of diverse herbal remedies to treat various diseases since ancient times (Shubham *et al.*, 2018) and have continued as promising therapeutic agents in primary healthcare delivery (Acharya *et al.*, 2021). However, only a limited proportion of these plants have undergone scrutiny for their bioactive components and pharmacological properties (Zahra *et al.*, 2021). Notably, the pharmaceutical industry progressively targets plant-derived bioactive compounds and aromatic

herbs, with complexity and diverse mechanisms of action (Boukhatem and Setzer, 2020). The plant's secondary metabolites of pharmacological importance include polyphenols, essential oils, terpenoids, alkaloids, glycosides, and flavonoids. These bioactive compounds have been successfully isolated and studied to unveil their biological activities (Boukhatem and Setzer, 2020). However, the scientific evaluation of the ethnopharmacological claims of these plants still needs to be adequately resolved (Siddiqui, 2021). The investigation of the therapeutic potential of medicinal plants encompasses the validation of their ethnopharmacological usage, as well as the identification, isolation, and characterisation of the active component responsible for their biological activities (Kouakou *et al.,* 2019). Bioactive compounds can be obtained from plants either as pure compounds or as mixtures of chemically related compounds (semi-purified fractions) that confer synergistic effects (Ullah *et al.*, 2022).

Melaleuca cajuputi is a fascinating evergreen plant indigenous to Malaysia, Thailand, Indonesia, the Philippines, and Australia. It is also known as the cajuput tree, white tea in English, or Gelam in Malaysia. Traditionally, the leaves and essential oils of the plant are used to treat various diseases, including sepsis, digestive and respiratory issues, urinary tract infections, anxiety, and stress (Awam, 2023). Moreover, Cajuput oil from *M. cajuputi* is used in aromatherapy (Plant *et al.*, 2019). In recent times, there has been a growing interest in investigating the medicinal properties of *M. cajuputi* due to its traditional significance and pharmacological properties, rendering it a subject of continuous investigation within the field of ethnopharmacology (Toan *et al.*, 2020; Chaudhari *et al.*, 2022). The essential oils of *M. cajuputi* have been the subject of many investigations on the plant's bioactivity (My *et al.*, 2020; Toan *et al.*, 2020; Chaudhari *et al.*, 2022). Even

though leaves from various plants are medicinally important, there have been few studies on the antibacterial properties of *M. cajuputi* leaf extracts. Furthermore, the potential antibacterial mechanisms of fractionated extract or isolated compounds from the plant remained relatively unexplored. Therefore, it is imperative to investigate the therapeutic potential of this plant.

Bioassay-guided fractionation and structural elucidation are crucial in natural product research for drug discovery and development (Bakun *et al.,* 2021). These techniques are used to separate bioactive compounds from plant materials and identify the active components responsible for the bioactivity (Kouakou *et al.*, 2019). This approach employed the use of chromatographic techniques, including vacuum liquid chromatography (VLC), column chromatography (CC), and thin-layer chromatography (TLC) (Mroczek *et al.*, 2020). Subsequently, the isolated compounds can be analyzed using techniques such as molecular spectroscopic analyses (UV-visible, FTIR), mass spectrometry (MS), X-ray crystallography, and nuclear magnetic resonance (NMR) (Van Puyvelde *et al.*, 2021). These techniques have been highly successful in discovering novel drugs and several bioactive lead molecules throughout the history of pharmacological research (Mogana *et al.*, 2020). However, evaluating the safety profile of plant extracts and their fractions is essential when considering their potential uses in pharmaceuticals, cosmetics, and various other industries (Sintayehu *et al.*, 2022). Moreover, the toxicity evaluation must confirm that these extracts and fractions do not cause harmful effects whether applied topically or administered systemically (Manzo *et al.*, 2019).

1.2 Problem Statement and Rationale of the Study

Bacterial infections continue to pose a significant threat to public health, with the rise of antibiotic-resistant strains exacerbating the challenge of effective treatment (Murray *et al.*, 2022). Conventional antibiotics are becoming less effective, leading to an urgent need to discover novel antibacterial agents from natural products (Tan *et al.*, 2022). The issue of antimicrobial resistance is a matter of great concern in the context of global public health (Bamoro *et al.*, 2021). It is worth mentioning that certain bacterial species, such as *Escherichia coli, Acinetobacter species, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Klebsiella pneumoniae*, have shown a concerning level of resistance to multiple drugs. (Noor *et al.*, 2019). Despite the widespread spread of severe bacterial infections, developing new antimicrobials has slowed since the 1990s, mainly due to regulatory constraints, technical problems, and low profitability compared to long-term treatments for chronic illnesses. Therefore, if nothing is done, infectious diseases that were previously eradicated may re-emerge (Tan *et al.*, 2022). Likewise, prolonged use of synthetic antibiotics has been linked to significant host side effects, including immunological suppression, hypersensitivity reaction, loss of symbiotic intestinal bacteria, diarrhea, and allergies (Bilal *et al.*, 2020). Therefore, natural sources of therapeutic agents like plants could offer promising alternatives for infectious disease control (Wińska *et al.*, 2019). In drug discovery and development, molecular docking simulation is crucial in identifying potential therapeutic agents from natural products (Roney *et al.*, 2023). *In-silico* molecular docking analysis predicts the pharmacokinetics, pharmacology, and toxicology of plant extracts, fractions, and isolated compounds (Konappa *et al.*,

2020). This technique produces data on the interaction between ligands (compounds) and proteins (enzymes), which can forecast the outcome of potential drugs based on the binding affinities towards the target microbial enzyme (Mir *et al.*, 2022).

Melaleuca cajuputi, a widely recognized medicinal plant, presents an intriguing avenue for investigation due to its historical use and potential bioactive properties (Awam, 2023). Despite the growing interest in *the M. cajuputi plant*, there remains a gap in understanding the antibacterial activities of semi-purified fractions derived from its leaf extracts and their specific mechanisms of action against bacterial strains. To address these knowledge gaps, this research aims to investigate the antibacterial activities of semi-purified fractions of *M. cajuputi* leaf extract against selected bacterial strains. The selected bacterial strains represent clinically relevant pathogens for resisting conventional antibiotics (Noor *et al.*, 2019). In the same vein, examining the antibacterial mechanism of the semi-purified fractions by time-kill assay, cell morphology studies, and molecular docking analysis will provide valuable insights into the potential modes of action. Furthermore, the toxicity effect of both the extract and semi-purified fractions was evaluated to ascertain the potency and safety of the plant.

1.3 Research Questions.

1. What are the mineral compositions of *M. cajuputi* leaves extract and their benefit in the context of disease prevention?
2. Which extract exhibits the highest antibacterial activity, and what are the minimum inhibitory concentration (MIC) values against specific bacterial strains?
3. What semi-purified fractions are obtained from the most potent leaf extract, and what are their antibacterial activities against selected bacterial strains?
4. What are the bioactive compounds in the most potent semi-purified fractions using UV-Vis, FTIR spectroscopies, and GC-MS, and how do they contribute to the observed antibacterial effects?
5. What are the potential antibacterial mechanisms of the identified bioactive compounds in semi-purified fractions, as revealed by time-kill assays, cell morphology studies, and molecular docking analysis?
6. How do the methanolic extract and the most potent semi-purified fractions impact brine shrimp lethality, and what are their respective LC_{50} values?

1.4 Objectives of the Study

1.4.1 General Objective

To investigate the antibacterial activity and mechanisms of action of semi-purified fractions from *M. cajuputi* leaf extracts against selected bacterial strains.

1.4.2 Specific Objectives

1. To screen the mineral composition and heavy metal content of *M. cajuputi* leaf extract using inductively coupled plasma mass spectrometry (ICP-MS).
2. To evaluate the antibacterial activities of methanolic, ethanolic, and aqueous extracts from *M. cajuputi* leaves against selected bacterial strains using the broth microdilution assay.
3. To isolate the bioactive compound(s) from the most potent crude extract via bioassay-guided fractionation using vacuum liquid chromatography (VLC) and column chromatography (CC).
4. To analyze the chemical compositions of the most potent semi-purified fractions obtained from bioassay-guided fractionation using ultraviolet-visible (UV-Vis), Fourier transform infrared (FTIR) spectroscopies, and gas chromatography-mass spectrometry (GC-MS).
5. To investigate the potential antibacterial mechanism of the most potent semi-purified fractions on selected bacterial strains through time-kill assays, cell morphology studies, and *In-silico* molecular docking analysis
6. To evaluate the toxicity effects of the crude extract and the most potent semi-purified fractions using a brine shrimp lethality test (BSLT).

CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal Plants

Herbal medicines refer to substances or products obtained from plants that possess medicinal properties or provide various benefits to humans. These products encompass raw and processed ingredients from one or more plant species (Giannenas *et al.*, 2019). Traditional practices for disease remedy, such as those originating from China, Native America, Tibet, and India's Ayurvedic heritage, are highly esteemed within their countries of origin and across other nations worldwide (Li *et al.*, 2022). It has been estimated that 50% of herbal treatments are derived from natural sources, such as plants, microbes, and animals (Boukhatem and Setzer, 2020). Furthermore, medicinal plants have been used in traditional medicine for disease remedies since ancient civilizations (Wińska *et al.*, 2019). The therapeutic characteristics of these plants have been investigated in recent scientific advancements, taking into account their potency, environmental sustainability, minor or absent side effects, and economic feasibility (Isah *et al.*, 2020). Investigating bioactive constituents derived from medicinal plants for their potential therapeutic applications has become a crucial field of inquiry within biomedical and natural product research (Shakya, 2016). These agents from plants hold immense potential in combating the escalating challenge of drug resistance and the prevalence of severe microbial infections (Yan *et al.*, 2021).

2.2 Phytochemical Compositions of Medicinal Plants

Secondary metabolites (phytoconstituents or bioactive compounds) are products of secondary metabolism. They are used as valuable resources for developing medications, food additives, flavors, and various industrial materials (Chiocchio *et al.*, 2021). The mechanism by which plant secondary metabolites affect microorganisms is based on their chemical structure and complexity. Microbial cells can be influenced through various mechanisms, including the alteration of cell rigidity and integrity, inhibition of membrane proteins, interference with nucleic acid synthesis, inhibition of cell wall synthesis, induction of cytoplasmic constituents coagulation, and modulation of quorum sensing (Gorlenko *et al.*, 2020). Generally, plant bioactive compounds are considered a valuable source of novel antimicrobial agents due to their notable pharmacological properties. Therefore, it is imperative to present an overview of these bioactive compounds. Phytochemical compounds are commonly categorized into four (4) classes based on their metabolic pathways: polyphenols, terpenoids, alkaloids, and flavonoids (Mera *et al.*, 2019).

2.2.1 Polyphenols

Polyphenols represent a substantial and intricate group of phytochemicals synthesized by plants. These group compounds are formed via the shikimate biosynthesis pathway, which serves as a source of precursors for aromatic molecules. The classification of polyphenols within the biosynthetic pathway is based on various characteristics, including the number of aromatic rings, carbon atoms, and hydroxyl groups. These compounds are further categorized into sub-classes, which include simple phenols (such as resorcinol, orcinol, catechol, guaiacol, hydroquinone, and phloroglucinol), phenolic acids (such as gallic acid, vanillic acid, and syringic acid), flavonoids (including flavones, flavonols, flavanones, anthocyanidin, and

isoflavonoid), and tannins (Shrinet *et al.*, 2020). The efficacy of polyphenols as antibacterial agents has been demonstrated by their ability to disrupt bacterial membranes, hinder the activity of virulence factors such as enzymes and toxins and impede the production of bacterial biofilms (Mera *et al.*, 2019).

2.2.2 Terpenes and Terpenoids

Terpenes comprise many naturally occurring compounds with significant structural and functional properties. Terpenes are categorized according to the number of isoprene units in their molecular structure, typically showing a condensed head-to-tail arrangement (Calabrò, 2015). Numerous terpenes possess pharmacological attributes and are used in treating ailments in both human and animal populations. Terpenes are divided into monoterpenes ($C_{10}H_{16}$), diterpene ($C_{20}H_{32}$), triterpenes ($C_{30}H_{48}$), tetraterpenes ($C_{40}H_{64}$), and sesquiterpenes ($C_{15}H_{25}$) (Cappiello *et al.*, 2020). Terpenes are widely used in various industrial sectors, including pharmaceuticals, food production, cosmetics, perfumery, and agriculture. (Guimarães *et al.*, 2019). Numerous research has documented the antibacterial effectiveness of terpenes and terpenoids by cellular membrane disruption (Guimarães *et al.*, 2019; Cappiello *et al.*, 2020).

2.2.3 Alkaloids

Alkaloids are a distinct group of chemical compounds with significant medicinal properties due to their nitrogen-containing structure. Alkaloids can be categorized into different groups based on their chemical structures, including isoquinolines, pyrroles, quinolines, and indoles. Multiple *in vivo* and *in vitro* studies have documented alkaloids' antibacterial, anticancer, antiviral, and anti-inflammatory properties (Yan *et al.*, 2021). Morphine, a narcotic analgesic; codeine, a cough medicine; vinblastine, an anticancer drug; and berberine hydrochloride, an

antibacterial drug, are therapeutic agents from alkaloids. These drugs have gained widespread acceptance in clinical practice (Miklasińska-Majdanik *et al.,* 2018). Previous studies on the antibacterial mechanism of natural alkaloids have demonstrated their ability to disrupt the integrity of bacterial cell membranes, interfere with DNA functionality, and inhibit protein synthesis, making them valuable for developing novel antimicrobial agents (Othman *et al.*, 2019; Yan *et al.*, 2021).

2.2.4 Flavonoids

Flavonoids are phenolic compounds widely used as biomolecules with significant therapeutic advantages for human health. Phenolics are characterized by a phenolic ring, exemplified by caffeic acid, trimethyl gallic acid, and coumaric acid. These compounds contain carboxylic acid and hydroxyl functional groups. Furthermore, flavonoids are a type of polyphenols characterized by the presence of at least two phenolic rings. These compounds can be classified into sub-categories: isoflavonoids, flavanols, flavonols, flavanones, and flavones**.** Previous research has established the pharmacological properties of flavonoids, encompassing antibacterial, free radical scavenging, anticancer, and antidiabetic properties (Patle *et al.*, 2020).

2.2.4(a) Isoflavonoids

In isoflavonoids, the B-ring is connected to the third position of the central ring-C, in contrast to other flavonoids, where it is often attached to the second position. Based on the carbon chain, isoflavonoids can be classified into 14 and 23 sub-classes. Isoflavonoids can be found in Leguminosae plants, particularly soybeans. Due to their estrogen activity and therapeutic benefits, soybean isoflavonoids are included in the category of phytoestrogens (Panche *et al.*, 2016).

2.2.4(b) Flavanols

Flavanols are compounds that can be classified as the 3-hydroxy derivatives of flavanones. These compounds are alternatively referred to as dihydroflavonols. This subgroup of flavonoids exhibits a wide range of diversity and versatility. The hydroxyl group in flavanols is consistently bonded to the third position of the C ring. Therefore, flavanols are alternatively referred to as flavan-3-ols. In contrast to the double bond between positions 2 and 3 in other flavonoid structures, this compound lacks such a double bond. Flavanols are abundant in plants, fruits, and vegetables and possess promising therapeutic potential (Shrinet *et al.*, 2020).

2.2.4(c) Flavonols

Flavonols are a subclass of flavonoids characterized by a ketone functional group. These substances can be considered as fundamental components in the formation of proanthocyanins. Flavonols are abundantly present in various plant species. The flavonols that have garnered significant scholarly interest are myricetin, quercetin, and kaempferol. The ingestion of flavonols has been associated with various therapeutic benefits, including antibacterial properties, antioxidant effects, and a reduced risk of cardiovascular disease. In contrast to flavones, flavonols possess a hydroxyl group at position 3 on the C ring, similarly susceptible to glycosylation (Miklasińska-Majdanik *et al.*, 2018).

2.2.4(d) Flavanones

Flavanones are a notable compound in citrus fruits and other botanical species. Eriodictyol, hesperitin, and naringenin represent flavonoids in this category. The health benefits of flavanones are ascribed to their ability to eliminate free radicals. The presence of these chemical compounds is responsible for the characteristic bitter taste observed in both the juice and peel of citrus fruits. Citrus

flavonoids demonstrate noteworthy pharmacological properties, functioning as antioxidants, antimicrobials, and anti-inflammatory agents. Flavanones, which are alternatively referred to as dihydroflavones, possess a saturated C ring (Panche *et al.*, 2016).

2.2.4(e) Flavones

Flavones are subgroups within the broader classification of flavonoids and are found in different parts of plants, including leaves, flowers, and fruits, where they exist in the glucoside configuration. Luteolin, tangeritin, and apigenin belong to the subclass of flavonoids. These compounds display a chemical structure with a double bond between positions 2 and 3 and a ketone group at position 4 on the C ring. Many flavones in plants, vegetables, and fruits have a hydroxyl group at position 5 on the A ring. Nevertheless, the hydroxylation occurring at alternative sites, precisely position 7 of the A rings or 3′ and 4′ of the B ring, may exhibit variations based on the taxonomic classification of the individual botanical specimen. It has been empirically established that these compounds exhibit antibacterial, anticancer, and antioxidant properties (Pagare *et al.*, 2015).

2.3 The Selection of Medicinal Plants for Drug Development

The principal objective of using ethnomedicinal plants as a source of therapeutic agents is to isolate physiologically active constituents that can be used for pharmaceutical purposes. Plant-derived drugs include morphine from *Papaver somniferum,* vincristine from *Catharanthus roseus,* taxol from *Taxus brevifolia,* and berberine from *Berberis vulgaris* (Shrinet *et al.*, 2020). Some plant-based molecules are subsequently subjected to modifications, resulting in the development of semi-synthetic drugs. These newly redesigned molecular moieties produced compounds

with highly desirable pharmacological properties. A good example include oxycodone, verapamil, and metformin (Zhang *et al.*, 2020). The use of ethnomedicinal plant extracts extends to their consumption as herbal remedies. For instance, cranberry is commonly used to treat urinary tract infections (UTIs), feverfew to alleviate malaise, and ginkgo to address dementia, respiratory issues, and kidney disorders (Shrinet *et al.*, 2020).

Plant selection for drug discovery may use randomization, followed by phytochemical screening, which is critical in drug discovery and development. The investigation determines the plant extract's significant phytoconstituents like polyphenols, terpenes, flavonoids, and alkaloids and their medicinal effect. These compounds are then linked to the observed biological activity (Anand *et al.*, 2019; Gorlenko *et al.*, 2020). The second approach entails plant selection based on ethnobotanical surveys and bioactivity testing. This method evaluates plant extracts and isolated compounds for antibacterial, antifungal, anticancer, anti-inflammatory, cytotoxicity, and antioxidant properties to validate the ethnopharmacological claims (Van Puyvelde *et al.*, 2021).

Previous data on medicinal plants' biological activities are also considered when selecting plants for drug discovery and development. The investigation focused on analyzing plant extracts with notable pharmacological properties. The primary objective is to identify the specific bioactive component responsible for the observed bioactivity to assess their potential as therapeutic agents and industrial applications (Giannenas *et al.*, 2019). Plants could also be selected based on the history of traditional usage. This approach offers substantial ethnomedicinal knowledge, encompassing the use of plants in diverse traditional medicinal practices like Ayurveda, Kampo, and Chinese traditional medicine. These conventional systems

have a rich historical background and have been used in disease remedies for millennia. However, it is necessary to establish greater credibility for these traditional practices as there is insufficient documentation regarding the adverse effects associated with the plants (Lin *et al.*, 2021). Folklore, herbalism, and shamanism are traditional medical systems in which knowledge is transmitted from one generation to the next through the guidance of experienced herbalists or shamans. Within this system, the healers often maintain secrecy regarding trivial details about the plant, which are recorded. The herbalist or shaman, who assumes the duties of health practitioners, incorporates other spiritual and cultural beliefs to cure illness. These treatments are frequently regarded as being associated with mysticism or the supernatural. This medicinal system has gained significant recognition in both Africa and South America. Thus, this approach can also be used in plant selection to discover novel anti-infective agents for infectious disease control (Giannenas *et al.*, 2019; Obakiro *et al.*, 2020).

2.4 Antimicrobial Drug Discovery

The search for innovative therapeutic agents in medical science has prompted researchers to investigate various natural products. Among these options, medicinal plants have emerged as a prominent and outstanding source of pharmacological compounds with a rich historical legacy (Arifullah *et al.*, 2014). The use of plant-based treatments has a long history, spanning thousands of years, as traditional healers from various cultures on different continents have tapped into the innate healing powers of plants. In contemporary society, investigating these natural resources to discover novel anti-infective agents has confirmed the efficacy of a vast collection of unique compounds with notable pharmacological properties (Süntar,

2020). Medicinal plants are a significant repository of bioactive constituents that have undergone extensive investigation to establish interactions with many biological systems. These bioactive compounds have several roles within the plant system, including but not limited to defense mechanisms against predators, the attraction of pollinating agents, and adaptation to environmental adversities (Mendoza and Silva, 2018). When tested for medicinal properties, these bioactive compounds demonstrate diverse pharmacological activity, encompassing anti-inflammatory, antibacterial, antifungal, antiviral, and anticancer properties (Anand *et al.*, 2019). Some examples of plant antimicrobial agents include berberine, tea tree oil, allicin, carvacrol, thymol, cinnamaldehyde, and quinine (Gorlenko *et al.*, 2020).

2.5 Isolation and Characterization of Bioactive Compounds from the Plant Extracts

The elucidation of bioactive components from plant extracts is crucial in various fields, including pharmaceuticals, nutraceuticals, and natural product chemistry. This process involves several steps to extract, purify, and identify the active components present in plant materials (Ullah *et al.*, 2022). The initial step in extracting active compounds from natural products, particularly medicinal plants, typically involves the identification of a plant that is recognized for its potential therapeutic properties, collection, and preparation of plant material by removal of impurities and moisture followed by extraction using solvent systems of varying polarity (Bezerra *et al.*, 2019). The solvent extraction methods include maceration, ultrasound-assisted extraction, liquid-liquid extraction, and extraction by Soxhlet (Buszewski *et al.*, 2019). Moreover, the maceration method involves immersing plant material in a solvent for a specific time, facilitating the diffusion of compounds into the solvent. It is frequently used to produce herbal tinctures and is suitable for

heat-sensitive samples (Saqallah *et al.*, 2022). Extraction procedures are commonly used as preliminary purification techniques to eliminate interfering components from plant material. The pre-purification protocols encompass a range of steps, such as filtration, precipitation, concentration, and desiccation (Mendoza and Silva, 2018). The crude extracts are subsequently partitioned into distinct fractions following the extraction process, using successive liquid-liquid extraction based on solvent polarity (Mendoza and Silva, 2018). Fractionation techniques are extensively used for separation purposes in liquid chromatography. This approach encompasses column chromatography and planar chromatography. Thin-layer chromatography (TLC) and preparative thin-layer chromatography (PTLC) are the basic procedures used in planar chromatography. The mobile phase is passed through a stationary phase, often silica gel, as an adsorbent to facilitate elution, and Sephadex LH-20 is used to achieve purity (Malviya and Malviya, 2017). This technique eliminates any binders or fluorescent indicators that may co-extract alongside the scraped-off compounds (Malviya and Malviya, 2017; Van Puyvelde *et al.*, 2021). The isolated semi-purified or pure compounds undergo chemical structure elucidation through molecular spectroscopic techniques (Mabasa *et al.*, 2021). Bioassay-guided fractionation is used in natural product chemistry and drug development to isolate bioactive compounds from complex mixtures like plant extracts (Abdallah *et al.*, 2021). Plant extracts are separated using chromatographic techniques, and bioactive components are identified using molecular spectroscopy (Malviya and Malviya, 2017). Therefore, bioassay-guided fractionation is a valuable tool in the early stages of drug discovery and development from natural products such as medicinal plants, facilitating the identification of lead compounds with therapeutic potential and paving the way for developing novel drugs to combat microbial infection (Mani *et al.*, 2022).

2.6 Antimicrobial Agents and their Target Sites on Bacterial Cells

Antimicrobial agents are substances or compounds that can inhibit or kill microorganisms and are used in medicine, agriculture, and food industries. Antimicrobials are grouped into various classes according to their mechanism of action on the target sites of bacterial cells (Abushaheen *et al.*, 2020). Figure 2.1 illustrates multiple target sites of antimicrobials on the bacterial cell.

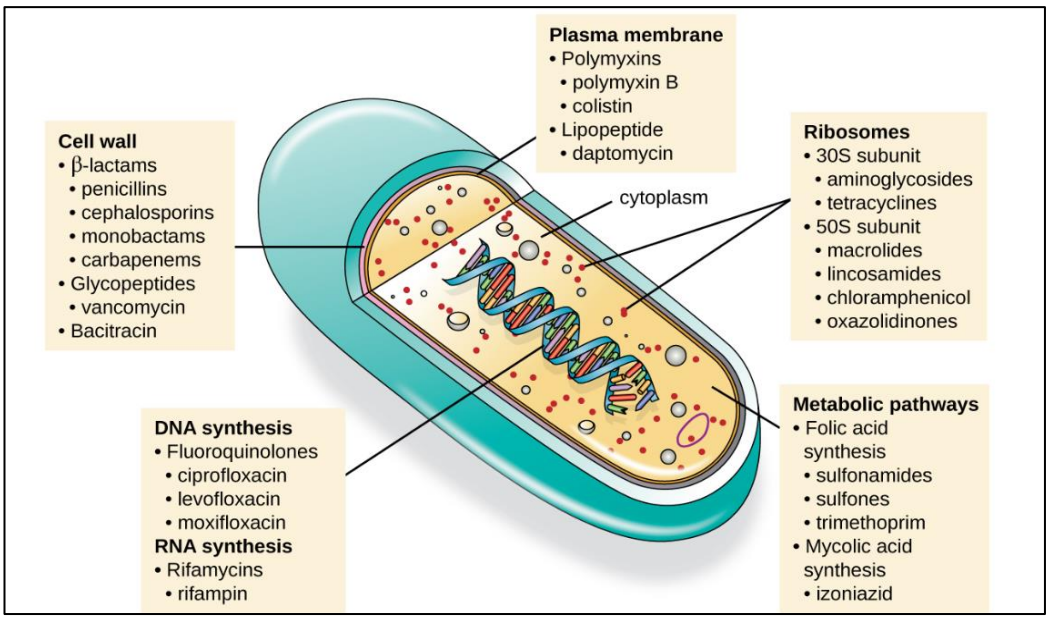

Figure 2.1: A schematic diagram of a typical bacterial cell shows the sites of action of the major antimicrobials. (Adopted from James and Elston, (2021))

2.6.1 Cell Wall Synthesis Inhibitors

The bacterial cell wall is an important organelle that maintains bacterial cells' structural integrity and protects them from lysis due to increased intracellular osmotic pressure. Peptidoglycan functions as the principal component in the bacterial cell wall. The murein consists of elongated glycan chains composed of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). The interconnection of these chains is facilitated by transpeptidase and carboxypeptidase enzymes, commonly referred to as penicillin-binding proteins (PBPs) (Lima *et al.*, 2020). The antibiotic families that selectively impede the synthesis of bacterial cell walls include β-lactams and glycopeptides. β-lactam antibiotics comprise a diverse range of classes that exhibit selectivity towards different bacterial strains, particularly those that possess penicillin-binding proteins (PBPs).

β-lactam antibiotics have a structural resemblance to the D-Ala-D-Ala dipeptide found in the nascent murein. As a result, a covalent connection is established between them and the serine active site of penicillin-binding proteins (PBPs), hindering the cross-linking process of peptidoglycan molecules during bacterial cell wall formation (Hutchings *et al.*, 2020). In clinical practice, two main categories of glycopeptide antibiotics, vancomycin, and teicoplanin, are frequently used for disease treatment. Both compounds exhibit bactericidal effects against gram-positive bacteria. The antibacterial mechanisms of these antibiotics involve targeting the transpeptidase enzyme and D-alanyl transferase, leading to the inhibition of cross-link between the layers of peptidoglycan, resulting in cellular damage and eventual death of the bacteria (Lima *et al.*, 2020).

2.6.2 Cytoplasmic Membrane Inhibitors

Specific antimicrobial agents could disrupt the integrity and functionality of cell membranes. Polymyxins are a category of naturally occurring polypeptide antibiotics first discovered in 1947 from *Bacillus polymyxa*. In clinical settings, polymyxin B and polymyxin E (colistin) are the only polymyxins acceptable for bacterial infections. The antibiotics possess lipophilic properties and detergent-like characteristics, which makes them interact with the lipopolysaccharide component in the outer membrane of gram-negative bacteria. Consequently, these antibiotics disrupt membrane integrity, resulting in cell lysis and escape of cellular contents. Unfortunately, the specific mechanism by which these drugs target the microbial membrane lacks selective toxicity. As a result, when administered systemically, these drugs have the potential to cause severe side effects on the host. Polymyxin B is frequently used as a topical ointment, such as Neosporin, due to its notable adverse effects and limited gastrointestinal absorption. In the past, oral colistin was used mainly for bowel decontamination to prevent infections from bowel microorganisms in immunocompromised patients/individuals (Noor *et al.*, 2019; Abushaheen *et al.*, 2020). Colistin drug is often used as a last resort for treating severe bacterial infections. Daptomycin is an antibacterial agent classified as a cyclic lipopeptide derived from *Streptomyces roseosporus* and shares a mechanism of action like polymyxins. However, it operates by incorporating into the bacterial cell membrane and causes cell damage. In contrast to polymyxin B and colistin, which selectively target gram-negative bacteria, daptomycin specifically targets gram-positive bacteria. The administration of these drugs is typically done intravenously and is generally well tolerated, with observed reversible toxicity in skeletal muscles (Noor *et al.*, 2019).

2.6.3 Protein Synthesis Inhibitors

Protein synthesis is a fundamental biological process in which living cells undergo transcription and translation stages for the biosynthesis of functional proteins. This process consists of four main steps: initiation, elongation, termination, and recycling. Typically, antibiotics hinder bacterial protein synthesis by targeting either the 30S subunit or the 50S component of the 70S bacterial ribosome (Rourke *et al.,* 2020). The inhibition of protein synthesis can occur by binding some antibiotics, such as streptomycin and tetracyclines, to the 30S subunit. Carbohydrate groups exhibit a positive charge that enters the bacterial cell by binding to the negatively charged plasma membrane and diffuses into the cell. Upon infiltration into the bacterial cell, these drugs adhere to the A-site of the 30S subunit of the ribosome, inducing a transition in translation from an intra-helical to an extra-helical conformation. This modification results in an incorrect matching between mRNA and tRNA, resulting in a misinterpretation of protein synthesis and the synthesis of nonfunctional proteins (Hutchings *et al.*, 2020). Moreover, the inhibition of the 50S subunit interferes with synthesizing a polypeptide chain within the peptidyl transferase center (PTC) located in the 50S subunit of the bacterial ribosome, achieved by the amino acids' linkage. In addition, the 50S subunit possesses a nascent peptide exit tunnel (NPET) that aids in releasing the polypeptide chain from the ribosome. The interaction between chloramphenicol and erythromycin with the 50S subunit occurs within the NPET and PTC regions. This interaction hinders the passage of freshly produced polypeptides through the tunnel. Consequently, the inhibition of protein synthesis occurs when the elongation step is interrupted (Abushaheen *et al.*, 2020).

2.6.4 Nucleic Acid Synthesis Inhibitors

The process of bacterial DNA synthesis requires enzymes known as topoisomerases. These enzymes can be categorized into two primary types: type IA, which can be further subdivided into Topo I and Topo III, and type IIA, which can be further subdivided into DNA 5 Gyrase and Topo IV. The presence or absence of these enzymes can affect the supercoiling conformational structure, resulting in the biosynthesis of atypical DNA structures (Khameneh *et al.*, 2019). Fluoroquinolones are a category of broad-spectrum antibiotics that are bactericidal against a wide range of bacteria. They function as inhibitors of the DNA gyrase in gram-negative bacteria, playing a crucial role in DNA replication. Additionally, they serve as inhibitors of the topoisomerase IV enzyme in gram-positive bacteria crucial for segregating daughter cells (Hutchings *et al.*, 2020). The antibacterial actions of quinolone antibiotics are the induction of changes in DNA supercoiling, leading to the deterioration of DNA structure and bacterial death (George *et al.*, 2020).

2.6.5 Folate Antagonists

The folate biosynthetic pathway is a crucial target for certain antibiotics to inhibit bacterial growth. Simultaneously, bacteria obtain their folate through a de novo biosynthesis pathway. The enzyme dihydropteroate synthase (DHPS) requires para-aminobenzoic acid (PABA) as a precursor molecule for folate synthesis. It has been shown that sulphonamides effectively compete with PABA in the bacterial folate synthesis pathway. Sulphonamides demonstrate a structural resemblance to PABA and function as competitive inhibitors by acting as an alternative substrate to hinder bacterial growth by reducing the folate pool. Diaminopyrimidine antibiotics, like trimethoprim, have been used as inhibitors of dihydrofolate reductase (DHFR),

which is the ultimate enzyme involved in the synthesis of folate in bacteria (Abushaheen *et al.*, 2020; Mir *et al.*, 2022).

2.7 *Melaleuca cajuputi* Powell

Melaleuca cajuputi Powell is commonly called "Gelam" or "Kayu Putih" in Malaysia, and the Cajuput tree is in English. This species is a member of the Myrtaceae family and is predominantly found in Malaysia, Thailand, Vietnam, Indonesia, and Australia (Awam, 2023). The plant collection consists of more than 200 species, predominantly indigenous to Australia, with a small number also present in Southeast Asia and neighboring islands. These plants are called "paperbarks" due to their distinctive bark that exfoliates in thin, papery layers from the trunk. The *Melaleuca* species are renowned for their aromatic leaves; many have been used for various traditional and medicinal purposes. Some important species within the *Melaleuca* genus include *Melaleuca cajuputi (*also called *Melaleuca leucadendra or Melaleuca leucadendron), Melaleuca citrina, Melaleuca viridiflora, Melaleuca alternifolia, Melaleuca quinquenervia* and *Melaleuca linariifolia* (Oliveira *et al.*, 2020).

2.7.1 Biogeography and Ecology of *M. cajuputi*

The *M. cajuputi* plant is primarily found in the lowland and coastal areas of Southeast Asia and Western Australia. It shows adaptation to this geographical area's tropical and subtropical climates. The Cajuput tree is distributed within its native range, encompassing diverse habitats such as lowland rainforests, peat swamps, and coastal areas. This plant is particularly prevalent in regions characterized by waterlogged or swampy soils and commonly thrives near riverbanks, coastal areas, and peat swamps. The Cajuput tree is a crucial habitat and food source for diverse