

***IN-VITRO* ANTIBACTERIAL ACTIVITY OF CERTAIN  
MEDICINAL PLANTS USED IN SAUDI ARABIA**

BY

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**LIFE SCIENCES/ BIOENGINEERING**

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*To the unsung heroes who sacrificed everything, to the martyrs...*

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## LIST OF ABBREVIATIONS

|                           |   |                                                 |
|---------------------------|---|-------------------------------------------------|
| <b>%I</b>                 | : | Percent Inhibition                              |
| <b><i>A. etbaica</i></b>  | : | <i>Acacia etbaica</i>                           |
| <b>Ae</b>                 | : | <i>A. etbaica</i>                               |
| <b>AMR</b>                | : | Antimicrobial Resistance                        |
| <b><i>C. procera</i></b>  | : | <i>Calotropis procera</i>                       |
| <b>CDC</b>                | : | Centers for Disease Control                     |
| <b>CHCl<sub>3</sub></b>   | : | Chloroform                                      |
| <b>CIN</b>                | : | Cinoxacin                                       |
| <b>CIP</b>                | : | Ciprofloxacin                                   |
| <b>CP</b>                 | : | <i>C. procera</i>                               |
| <b>DCM</b>                | : | Dichloromethane                                 |
| <b>DEE</b>                | : | Diethyl Ether                                   |
| <b>dH<sub>2</sub>O</b>    | : | Distilled Water                                 |
| <b>DMSO</b>               | : | Dimethyl Sulfoxide                              |
| <b><i>E. faecalis</i></b> | : | <i>Enterococcus faecalis</i>                    |
| <b><i>E. coli</i></b>     | : | <i>Escherichia coli</i>                         |
| <b>EthOH</b>              | : | Ethanol                                         |
| <b>I</b>                  | : | Intermediate                                    |
| <b>KFUPM</b>              | : | King Fahad University of Petroleum and Minerals |
| <b>KSA</b>                | : | Kingdom of Saudi Arabia                         |
| <b>MBC</b>                | : | Minimum Bactericidal Concentration              |
| <b>MDR</b>                | : | Multidrug-Resistant                             |

|                             |   |                                                    |
|-----------------------------|---|----------------------------------------------------|
| <b>MIC</b>                  | : | Minimum Inhibitory Concentration                   |
| <b>min</b>                  | : | Minutes                                            |
| <b>mm</b>                   | : | Millimeters                                        |
| <b>MRSA</b>                 | : | Methicillin-Resistant <i>Staphylococcus aureus</i> |
| <b>MSSA</b>                 | : | Methicillin-Sensitive <i>Staphylococcus aureus</i> |
| <b>NA</b>                   | : | No Activity                                        |
| <b>NA</b>                   | : | Nutrient Agar                                      |
| <b>NB</b>                   | : | Nutrient Broth                                     |
| <b><i>P. aeruginosa</i></b> | : | <i>Pseudomonas aeruginosa</i>                      |
| <b><i>P. granatum</i></b>   | : | <i>Punica granatum</i>                             |
| <b>PG</b>                   | : | <i>P. granatum</i>                                 |
| <b>PVL</b>                  | : | Panton-Valentine Leucocidin                        |
| <b>R</b>                    | : | Resistance                                         |
| <b>R.</b>                   | : | <i>Rhamnus</i>                                     |
| <b>Rh</b>                   | : | <i>Rhamnus sp.</i>                                 |
| <b>rpm</b>                  | : | Revolutions per Minute                             |
| <b>S</b>                    | : | Sensitivity                                        |
| <b><i>S. aureus</i></b>     | : | <i>Staphylococcus aureus</i>                       |
| <b>S1</b>                   | : | <i>E. coli</i>                                     |
| <b>S2</b>                   | : | <i>E. faecalis</i>                                 |
| <b>S3</b>                   | : | <i>P. aeruginosa</i>                               |
| <b>S4</b>                   | : | <i>S. aureus</i>                                   |
| <b>SM</b>                   | : | Secondary Metabolites                              |
| <b>UD</b>                   | : | Undefined                                          |
| <b>UN</b>                   | : | United Nations                                     |

|            |   |                           |
|------------|---|---------------------------|
| <b>-ve</b> | : | Pure DMSO                 |
| <b>WDA</b> | : | Well Diffusion Assay      |
| <b>WHO</b> | : | World Health Organization |

## ABSTRACT

Full Name : Ikrimah Ahmad Al-Hanbali

Thesis Title : *In-vitro antibacterial activity of certain medicinal plants used in Saudi Arabia*

Major Field : Life Sciences

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This study explores the antibacterial properties of four medicinal plants against four model bacterial species. The selection of these plants was based on their widespread use in various ethnic groups for common disorders. This research represents the first biological screening of these plants in the eastern region of the Kingdom of Saudi Arabia. The selected medicinal plants, *Calotropis procera*, *Rhamnus sp.*, *Acacia etbaica*, and *Punica granatum* - underwent solvent extraction using different solvents of increasing polarity, resulting in two fractions: Chloroform and 95% ethanol. After the solvent evaporated, the highest yield was obtained from the ethanol fraction of *Punica granatum* (38.2%), while the lowest was from the chloroform fraction of *Punica granatum* (1.2%).

All four plant extracts showed activity against bacteria, dependent on dosage and the solvent used for extraction. Notably, the ethanol extract of *Punica granatum* exhibited activity against all tested bacteria at an exceptionally low concentration of 0.25 mg. However, certain extracts showed no activity against most bacteria, particularly the chloroform extract of *Punica granatum* at 30 mg and the ethanol extract of *Acacia etbaica* at 30 mg, as evaluated by the well diffusion assay (WDA).

Nevertheless, all plant extracts exhibited inhibitory activity based on minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), with concentrations ranging from 3.125 to 200 mg/mL using the dilution method. The lowest MIC concentrations were recorded against *E. coli* (3.125 mg/mL) for the chloroform extract of *Calotropis procera*, *P. aeruginosa* (3.125 mg/mL) for the ethanol extract of *Punica granatum*, and *E. faecalis* (3.125 mg/mL) for the ethanol extract of *Rhamnus sp.* Conversely, the highest MIC concentration reached 50 mg/mL for different extracts against various bacteria. Likewise, MBC values varied, with the lowest concentrations exhibited by ethanolic extract of *Punica granatum* (25 mg/mL) against *P. aeruginosa* and *E. coli*. However, higher concentrations, up to 200 mg/mL, were recorded across different plant extracts against several bacterial strains.

The observed antibacterial activity in both dilution and diffusion assays is attributed to the presence of different bioactive secondary metabolites, as ascertained through thin layer chromatography (TLC) and gas chromatography–mass spectrometry (GC-MS) analysis of these four plant species. This encompasses a diverse spectrum of bioactive compounds, prominently featuring fatty acids, sterols, triterpenes, phenolic compounds, and others. These findings portend promising avenues for subsequent research endeavors aimed at elucidating the pharmacological potential of these botanical entities and their constituent bioactive compounds. Future studies may delve into the specific mechanistic underpinnings and potential therapeutic applications proffered by these bioactive agents.

## ملخص الرسالة

الاسم الكامل: عكرمة بن أحمد الحنبلي

عنوان الرسالة: النشاط المخبري المضاد للبكتيريا لبعض النباتات الطبية المستخدمة في المملكة العربية السعودية

التخصص: علوم الحياة/ الهندسة الحيوية

تاريخ الدرجة العلمية: كانون الأول، 2023

يُعتبر التنوع البيولوجي للنباتات الطبية ذو أهمية اقتصادية كبيرة ويعمل على تحسين صحة الإنسان. حيث تم عزل العديد من المركبات النشطة بيولوجيا من نباتات ذات استخدام في الطب الشعبي. هذه المركبات قد تُعتبر كدواء جديد أو أساس لتكوين دواء جديد. في هذه الدراسة تم تحديد النشاط المضاد للبكتيريا لعدد من النباتات الطبية في المنطقة الشرقية من المملكة العربية السعودية للمرة الأولى. هذه النباتات معروفة بالأسماء الشعبية التالية: العشار والسدر أو ما يعرف بالنبق والعراد وكذلك نبات الرمان.

تم تحديد النشاط البكتري بطريقتين، الأولى باستخدام طريقة الإنتشار في الآجار، والثانية التركيز المخفف. تم جمع النباتات، وتعريفها ثم استخلاص المواد الفعالة منها واستخراجها باستخدام المذيبات العضوية مثل الإيثانول والكلوروفورم، وهما مختلفان في القطبية بشكل كبير. وكانت البكتيريا المختبرة (*E. coli*, *P. aeruginosa*, *S. aureus*, and *E. faecalis*) حيث تم تسجيل أقصى نشاط للمستخلص الإيثانولي من نبات الرمان تجاه جميع البكتيريا حتى مع تراكيز قليلة. ولكن بعض النبات لم تظهر أي نشاط حتى مع تراكيز كبيرة، هذه البيانات ظهرت بشكل أكبر فيما يخص نبات الرمان المستخلص بالكلوروفورم أو حتى عند الحديث عن المستخلص الكحولي لنبات العراد (أو العراد الرعوي). فيما يتعلق بهذه التحاليل فقد أظهرت جميع المستخلصات من النباتات المذكورة نشاطا تثبيطيا لنمو البكتيريا بشكل ملحوظ.

يعزى النشاط المضاد للميكروبات الملاحظ في الاختبارات المستخدمة إلى وجود مركبات ثانوية حيوية نشطة، كما تم تحديدها من خلال تحليل الغاز الكروماتوغرافي-الطيف الكتلي لهذه النباتات الأربعة. ويتضمن ذلك مجموعة متنوعة من المركبات الثانوية الحيوية، حيث يبرز من بينها بشكل بارز الأحماض الدهنية والستيرولات والترايترينيات والمركبات الفينولية وغيرها. تحت هذه النتائج البحوث اللاحقة التي تهدف إلى توضيح الإمكانيات الدوائية لهذه المستخلصات النباتية والمركبات الثانوية التي تتضمنها. وقد تناولت الدراسات المستقبلية أيضا التطبيقات العلاجية المحتملة التي يمكن تقديمها من قبل هذه النباتات. وكذلك تحديد آلية عملها في تثبيط النمو البكتيري.

# CHAPTER 1

## INTRODUCTION

### 1.1 General Overview

There is a significant global problem of antibiotic resistance, and it is becoming more complicated by the year. Currently, antimicrobial resistance (AMR) is considered a hidden pandemic with a high prevalence among human beings. As soon as penicillin was discovered and developed in the 1900s, medical science was hopeful. There is a natural resistance to antibiotics in bacteria, which explains why penicillin was not effective against the majority of bacterial species. AMR has been found in different strains including *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* (*P. aeruginosa*), *Streptococcus pneumonia*, *Escherichia coli* (*E. coli*), and more. Rapid genetic changes in resistant bacteria, suggested by Bush et al. (2004), may result in antibiotics losing their potency in the near future.

There are about fifty thousand people in the world who die of infectious diseases every day, according to statistics released by the World Health Organization (WHO). In the World Health Report, out of 52 million deaths per year, more than 17 million deaths were due to infectious diseases (WHO, 1997). To counter the threat of resistant (or multidrug-resistant) strains of bacteria and fungi, it is crucial to create new antibacterial substances that are safe, economical, non or less toxic than synthetic drugs, and affordable to the general public. With the rise of AMR, the WHO and CDC (Centers for Disease Control)

have begun conducting research that will lead to new, and more effective therapies to combat this dilemma (WHO, 2014). It is possible to use recently developed antibacterial alone or in combination with conventional antibiotics as synergetic drugs. There is a possibility of combining these new agents with conventional antibiotics as a synergetic drug or using them alone.

There have been cultures around the world that use plants for their preservation properties in human health and to treat diseases like infectious diseases and for the benefits of plant-based medicines. Over the past decade, more detailed research has been done on natural medicines. Even though, it was in the late 19<sup>th</sup> century that plants and their components were studied for the first time for their antibacterial properties. It is estimated that over 20,000 medicinal plant species are known to exist, and the WHO has described medicinal plants as potential drug sources (Yadav, R. et al., 2011). More than 100 countries have established regulations regarding medicinal plants. According to Tajkarimi, M. et al. (2010), more than 30,000 antimicrobial compounds that have specific antibacterial activity have been identified from more than 1340 plants. In fact, approximately twenty five percent of prescription drugs worldwide are derived from wild or cultivated plants based on industry estimates (Newman, D.J. et al., 2003). A recent work in synthetic chemistry identified natural compounds as having potential in treating and preventing several diseases better than other synthetic compounds (El-Shahaby, O.A et al., 2019). Plants still have the potential to be a promising source of new drugs, therefore, it is important to recognize their activity against bacteria and to know more their phytochemical composition.

Kingdom of Saudi Arabia (KSA) has a high rich variety of natural medicinal plants due to its wide area and geological differences. The climate in Saudi Arabia is desert climate

conditions. The atmosphere is dry and hot throughout the year, with little rain, and the soil is high in salt and sand. There are many effects of these local harsh climatic conditions on the flora in this area such as the production of secondary metabolites (SM), the physiological performance of plants, their defense mechanisms, and medicinal and biological activities as well. 2300 different plant species are thought to make up Saudi Arabia's flora. Despite the diversity of flora in the local area, antibacterial properties of some medicinal plants are not well explored. The reports on the activities of some plants are not well documented, although some have been tested. The antibacterial properties of medicinal plants grown in Saudi Arabia's eastern region have, to author knowledge, not been studied. On these bases, four medicinal plants grown locally in the eastern part of Saudi Arabia will be studied *in-vitro* to determine their antibacterial properties, including *Calotropis procera* (*C. procera*), *Rhamnus sp.*, *Acacia etbaica* (*A. etbaica*), and *Punica granatum* (*P. granatum*).

## **1.2 Problem Statement**

The introduction of new classes of antimicrobials to drug stores has declined significantly since the 1970s, with only five new antimicrobial agents approved for clinical use between 2003 and 2007 in the United States. CDC predictions indicate that antimicrobial resistance will cause millions of deaths over the coming decades. For instance, around 36% of deaths in Saudi Arabia in 2019 resulted from infectious diseases, based on a sample of 200,000 (WHO et al., 2019). Furthermore, the failure to address drug-resistant infections could result in an additional 10 million deaths per year worldwide by 2050. As part of the fight

against AMR, the United Nations (UN) recently established a global task force to combat the threat of antimicrobial resistance.

### **1.3 Study Significance**

It has become necessary to develop novel antibacterial agents as a result of bacterial resistance to currently available antibiotics. According to the WHO, traditional medicines made from medicinal plants are still helpful to most of the population in countries (Khuda, F. et al., 2022). Medicinal plants are contained chemicals compounds are thought to inhibit viruses, fungi, protozoa, and bacteria through multilabel processes than those now employed by antimicrobials and can be useful in treating resistant microbial strains (Shankar, S.R et al., 2010). Accordingly, this research will include four locally medicinal plants, which are available, cheap, and safe or with limited toxicity, and they are a promising target to identify new source of antibacterial agents and decrease the cost of the manufacturing of antibiotics.

### **1.4 Research Aim and Objectives**

The aim of this work is to investigate the *in-vitro* antibacterial activity of four locally medicinal plants against several model bacterial strains by determining the following specific objectives:

1. To collect and identify medicinal plants from the eastern region of Saudi Arabia.
2. To extract the selected medicinal plants using different organic solvents.
3. Identify of the isolated extracts by standard gas chromatography–mass spectrometry (GC-MS).

4. *In-vitro* evaluation of the antibacterial activity of these plant extracts against various known bacteria and determined their Minimum Inhibitory Concentration (MICs) and Minimum Bactericidal Concentration (MBCs).

## **1.5 Thesis Organization**

The proposed thesis is structured into five main chapters. Chapter 1 comprises an introduction that provides an overview of the research, a clearly defined problem statement, a discussion on the significance of the study, and the research objectives. Chapter 2 conducts a thorough literature review, focusing on selected medicinal plants, their pharmacological activities, and the associated phytochemical compounds. In Chapter 3, the research methodology is outlined, detailing the selected tests and the process of data analysis. Chapter 4 presents the study's findings, discussing results in comparison with other relevant studies and offering explanations for the obtained results are outlined in Chapter 5. Finally, Chapter 6 concludes the study and outlines future prospects for research. This structured approach ensures a logical and clear progression throughout the thesis.

## **CHAPTER 2**

### **LITERATURE REVIEW**

#### **2.1 Introduction**

Infections caused by AMR are increasing worldwide, adding to the growing threat. Multidrug-resistant (MDR) bacterial and fungal pathogens have been developed as a result of overdosing and unrestricted antibiotic use, preserving food as well. As a result of the excessive risk associated with antibiotic development, as well as the possibility of diminishing returns, antibiotics are not considered a priority for the pharmaceutical industry (Boucher HW, et al., 2009). Therefore, to combat these diseases regularly, we must have plenty of new antibiotics available that are both safe and affordable. In response, intensive research efforts are now being directed toward discovering new anti-infective treatments. In many ways, antimicrobial research aims to combat harmful microbes, especially those resistant to conventional antibiotics, by the creation of novel antimicrobials. In addition, to use conventional antimicrobial agents, these new antimicrobial substances can be used as a new class or in combination with old conventional antimicrobial agents to increase their effectiveness.

Human diseases have been treated with plants and their products since the beginning of civilization. With time, synthetic drugs have been developed using several of those phytochemical compounds. Therefore, Newer drugs can still be derived from plants and their compounds (Veeresham et al., 2012). It is based on these principles that a large

number of plant products possessing high therapeutic potential remain unexplored and tested (Al-Ghanayem et al., 2017) even though wild or cultivated plants make up more than 25% of prescription drugs worldwide. Furthermore, medicinal plants are a promising source of natural antimicrobial agents because they produce natural therapeutic agents through secondary metabolic pathways. To fully utilize the benefits of folk medicine, it is crucial to examine the medicinal plants that are commonly used in it and are known to have antimicrobial properties. This examination will help identify their antimicrobial properties and their chemical compositions. By doing so, we can create medicines that are not only effective but also less toxic, affordable, and potent. This will increase the efficacy rate of traditional medicines and make them more accessible to people in need.

Physiological behaviors, climate changes, and geographical location influence SM in medicinal plants, as well as extraction techniques. However, it has been observed that essential and heavy oils in the same plant can vary according to the part of the plant they are found (Formisano et al., 2013). These chemical substances include diverse range of SMs such as alkaloids, flavones, flavonoids, phenolics, phenylpropanoids, glycosides, sesquiterpene, lactones, diterpenes, triterpenes, naphthoquinones, anthocyanin, coumarin, catechins, iso-catechins, and others that show various pharmacological activities. Moreover, among essential oils, terpenoids are the most common. Plant oils and other phytochemical components are considered plant SMs. Plant's oils are described by modern pharmacists as a source of aromatherapy because they have therapeutic benefits such as preventing microbes from multiplying, fighting free radicals, reducing inflammation, relieving pain, curing inflammation, and analgesic potentials (Park et al., 2008).

Accordingly, Saudi Arabia has a harsh weather condition which could lead to a change in concentration of SMs or add new compounds for its medicinal plants. In this context, four of the available plants, in Saudi Arabia's eastern region, are chosen to discover new antimicrobial agents, these plants are *C. procera*, *Rhamnus sp.*, *A. etbaica*, and *P. granatum*.

## **2.2 *Acacia etbaica***

*Acacia* plants are described as trees or shrubs with white or yellow flowers and branched or unbranched trunks. *Acacia* is a genus belonging to the *Fabaceae* family (*Leguminosae*). A stipular spine and corporate pollen grains adorn its bipinnate compound leaves. *Acacia* species have various uses such as firewood, perfumes, tannins, Arabic gum, fuel, proteins, ink, paint, and disinfectants, and play an important role in soil stabilization and industries. The plant often lives in drylands, subtropical, temperate, tropical grasslands, and desert climates, as well as woodlands, and savannas as well. Le Houerou et al. (1980) stated that the *Acacia* genus includes about 1300 species. These species are found in tropical and warm temperate regions, as well as in India, America, and Asia. Australia alone contains 960 species of these, while the other species can be found in Egypt, America, Europe, tropical Africa, southern Asia, and Saudi Arabia (Alajmi et al., 2017).

Multiple species of *Acacia* are utilized in folk and traditional medicine all over the world. *Acacia* species are utilized to treat diarrhea, fever, hemoptysis, leukorrhea, and throat infections traditionally, as well as for a variety of other health problems. Additionally, Ethiopia's Kiltie Awulaelo area used *Acacia* species to cure ringworm infection, scabies, hemorrhoids, anthrax, fire burns, edema, moreover cattle eye infections. Traditional Indian

medicine also uses a variety of fresh parts of various *Acacia* species for a variety of health issues such as anthelmintics, abortifacients, astringents, demulcents, and spasmolytics. *A. etbaica* is one of these species. It is commonly known as Qrad. It is traditionally taken orally in Yemen and the western region of Saudi Arabia which is done by crushing the leaves of *A. etbaica* and mixing them with hot water, to reduce stomach pain.

*Acacia* species have been found to possess significant pharmacological properties. For instance, Lin and colleagues (2013) reported that its extracts showed cytotoxic activity against prostate cancer. Davis et al. (2000) found that *Acacia* extracts have antidiabetic properties and can be used to treat type 2 diabetes. Additionally, it is also used as an antifungal agent and in pharmaceutical production for skin whitening purposes (Lee et al., 2018). Moreover, *A. etbaica*'s ethanolic leaves extract reduced inflammatory cytokines in lipopolysaccharide-stimulated RAW 264.7 cells (Tekleweyni et al., 2020).

It has been established that several *Acacia* species are capable of preparing microorganism-killing handwashes and disinfectants (Cock et al., 2011). According to the literature, different *Acacia* species, including *Acacia catechu*, *Acacia salicina*, *Acacia aulacocarpa*, and *Acacia complates*, have been discovered they possess antimicrobial properties against non-pathogenic and pathogenic microorganisms. As well as antimicrobial activity, many species of *Acacia* exhibited foaming properties, for example, the *Acacia concinna*'s bark contains significant levels of saponins. According to traditional Egyptian medicine, *A. etbaica* leaves, stems, flowers, seeds, and pods possess strong antibacterial and antifungal properties (Mahmoud, M. F et al., 2016). From a different perspective, the effects of *A. etbaica* n-butanol extracts against several cell lines used to study human tumors, including, Mammary gland breast cancer (MCF-7), Hepatocellular carcinoma (HEPG-2), and

Colorectal carcinoma (HCT-116) shown high to extremely strong cytotoxic activity (Kayed et al., 2020).

Furthermore, it has been demonstrated that *Acacia* is commercially important due to the abundance of polyphenols which are the most prominent compounds found in this genus (Ziani et al., 2020). Numerous flavonoids, including flavan-3-ols, flavan-3, 4-diols, aglycones, and (epi)-catechins bearing gallolyl moieties and hydroxyl functional groups, have been found in a variety of *Acacia* species (Ziani et al., 2020). The leaves of *A. etbaica* contain significant levels of condensed tannins (Alajmi et al., 2017). Research on the fatty acid content of *Acacia* sp. seed oil from the northwest region of India revealed that these acids efficiently lower blood cholesterol. Additionally, three novel simple phenolic compounds named resorcinol, resorcinol-O-glucoside, and resacetophenone-6-methyl were discovered in aerial portions of *A. etbaica* (Kayed et al., 2021). Additionally, analysis of dry bark extracts and fresh leaves of *A. etbaica* collected from Jazan in the southern region of KSA revealed variances in the amounts of vitamin folic acid, B12, B2, and B1.

Generally, *A. etbaica* is one of the plants that has not been studied enough in Saudi Arabia, although that it is widely spread in most regains of the Kingdom.

### **2.3 *Rhamnus* sp.**

*Rhamnus* (R) is a genus of about 110 species, belonging to the *Rhamnaceae* family, commonly known as buckthorns. This shrub is perennial and dioecious. The *Rhamnaceae* family consists of trees, shrubs, small-to medium-sized deciduous, and evergreen vines in flowering trees, including Rhamnales. Mostly found in tropical, subtropical, and temperate

regions, the family has a worldwide distribution (Ahmad, J. et al., 2021). Among the flora species of *Rhamnus* in Egypt, East Asia, and North America, it is also native to the Arabian Peninsula, such as Lebanon, Syria, Palestine and Saudi Arabia, and the northern Middle East.

This plant has been extensively used in traditional medicine to treat cancer, wound and throat infections, inflammation, and other health issues as well. For example, in traditional medicine, the leaves, stems, roots, and fruits of *R. prinoides* have been utilized throughout eastern Africa to treat different types of illnesses including pneumonia, throat infections, scabies, rheumatism, dandruff, tonsillitis, and nose and ear infections. It is effective against both eukaryotes and prokaryotes in research conducted on it (Ahmad, J. et al., 2021). Also, the bark and fruit of *R. catharticus* are utilized as a mild laxative in traditional medicine (Hänsel et al., 2007). Furthermore, native Americans relied on *R. californica* for treating poison oak dermatitis, infection, and burns, and for treating grippe. Leaf decoctions, crushed leaves, and berries were used to treat these conditions. Moreover, skin, respiratory, and gastrointestinal infections are generally treated with it in Native American traditional medicine.

Many phytochemicals have been reported in the genus *Rhamnus* as the major SMs, including anthraquinones, flavonoids, alkaloids, phenolic constituents (Khuda, F. et al., 2022), glycosides, benzyl glucoside (Mohammed, H. A et al., 2021), chalcones, naphthalene derivatives, and anthocyanins have been reported in different phytochemical studies. *Rhamnus* species are considered to be a source of anthraquinones and other phenols (Zeouk, I et al., 2021). Whereas anthraquinones display pharmacological activities on a variety of levels they are considered useful and promising SMs. Several phenolic

substances are also found in *Rhamnus* species, but the most interesting ones are extracts derived from anthrhopoids, which are found in *R. purshianus* DC and *R. catharticus* L. Both of those substances are listed in PhEur 5 and DAB 1999, respectively, as a drug (Hänsel R et al., 2007). *Rhamnus* is also a source of flavanols such as rhamnocitrin, kaempferol, and rhamnetin, as presented in Cuoco, G et al. study's (2014). The biological use of these guns has already been investigated by numerous studies due to its abundance of phytochemical compounds. Many pharmacological properties are highlighted in these studies, such as antihyperlipidemic and antigenotoxicity activity. In addition, certain *Rhamnus* species have been shown to act as free radical scavenging agents, antioxidants, anticancer agents, antibacterial agents, and insecticides. Furthermore, studies have been done on *R. alaternus*'s crude extracts have demonstrated potent cytotoxic, antimutagenic properties, and antiproliferative activity (Ben Ammar et al., 2007).

Otherwise, a recent study found that the n-hexane and chloroform extracts of *R. purpurea* have considerable effects contra several tumor cells, including colon cell line (Caco-2), breast cell line (MDA-MB-321), lung cell line (A549), and hepatocellular line (HepG2) with differing IC50 values, and the HPAEpiC and HRPTEpiC cell lines, which are normal epithelial cells. Additionally, the same fractions showed strong selectivity against the aforementioned cell lines, indicating that they are safe for use with normal human cells (Khuda, F. et al., 2022). In the same line, a study done in 2021 by Mohammed, H on *R. disperma* showed that phytochemical compounds have anticancer characteristics against human ovary adenocarcinoma (A2780), normal human fetal lung fibroblasts (MRC5), human breast adenocarcinoma (MCF 7), and human colon adenocarcinoma (HT 29) cell lines.

Meanwhile, *R. alaternus* leaves can inhibit *Staphylococcus aureus* (*S. aureus*), methicillin-resistant *S. aureus* (MRSA), and MSSA-PVL (methicillin-sensitive *S. aureus*- panton-valentine leucocidin) *in-vitro*, and emodin has been identified as the active ingredient responsible for this action (Zeouk, I et al., 2021). Emodin has antibacterial properties and exhibits various biological characteristics both *in-vitro* and *in-vivo*, including antiviral, antiallergic, anti-inflammatory, anticancer, antioxidant, and antibacterial properties. The results of another study that used the bark of *R. virgata* suggested that all extracts have significant antimicrobial activity against *Candida albicans*, *Salmonella typhi*, *P. aeruginosa*, *S. aureus*, *E. coli*, and *Shigella sp.* as a result of the presence of different bioactive compounds (Ahmad, J. et al., 2021). In addition, stem extracts of *R. prinoides* inhibited 97 percent of the formation of *Streptococcus mutans* and *Candida albicans* biofilms co-culture (Campbell, M et al., 2020).

## **2.4 *Punica granatum***

*P. granatum* (Pomegranate) has gained popularity since ancient times due to its nutritional benefit in the human diet and multifunctionality. *P. granatum* is a small tree of the *Punicaceae* family, that produces fruit-bearing branches between three and seven meters in height. The pomegranate is characterized by obtuse and opposite leaves, and flowers with wrinkled yellow, orange, or white petals. In the fruit, the seeds are enclosed in a yellow-red peel, and the fleshy arils are consumed. Pomegranates are now cultivated in enormous parts of the world as an extremely valuable commercial fruit crop. Pomegranates originate in Europe, the Mediterranean, Asia and, and they are also grown in warm climate zones of the Americas. They are common in our region as well, including Palestine, Iraq,

Jordan, Egypt, Lebanon, and Saudi Arabia. Yet it is believed to have roots in northern India and Iran (Hussein, E. I et al., 2021).

*P. granatum* is widely used in traditional medicine to treat various ailments, and its fruits are also used in diets. Different cultures across the globe utilize the different parts of the pomegranate tree to prepare herbal remedies for a wide range of common diseases. The peel of pomegranate, for example, has been traditionally used to treat diarrhea and dysentery, while Ayurvedic medicine recommends it for treating parasitic diseases, ulcers, and aphthae. Furthermore, it is believed to have anti-diabetic properties according to Unani systems practiced in India and the Middle East.

Previous research has demonstrated that pomegranates possess a variety of biological activities. Pomegranate extract has been found to inhibit bacterial growth, including both Gram-negative and Gram-positive bacteria (Kadi et al., 2011), as well as some food-borne and enteric pathogens (Pai et al., 2011). Additionally, pomegranate extract has broad-spectrum antibacterial activity against oral pathogens (Abdollahzadeh et., 2011). Additionally, it is also active against several fungal strains and yeast (Eldiasty, J. G et al., 2014). In 2016, a study conducted by Gulube, Z et al. discovered that *P. granatum* crude extract has the ability to inhibit the growth of various cancer-related bacteria at higher concentrations. However, at sub-bactericidal concentrations, the extract can prevent the growth of biofilms, as well as the production of extracellular polysaccharides and acid by *Streptococcus mutans*. Furthermore, a study done in 2015 showed that swarm motility regulatory genes of *Salmonella* (flagella synthesis genes), *fliF*, *fliM*, *fliT*, *fliZ*, *fliC*, and *fliA* regulators were downregulated upon exposure to pomegranate peel extract (Mahadwar, G et al., 2015). On the other hand, the extract from the peel of pomegranate fruits stimulates

transcription of the *ydiV* gene, which has been implicated in suppressing motility and flagella regulons (Wada et al., 2012).

According to a recent study, pomegranate extracts, including oil, juice, and peel, have demonstrated cytotoxicity activity. This includes inhibition of angiogenesis formation, invasion, and tumor cell proliferation. It is believed that their anti-inflammatory properties may explain these activities (Hussein, E. I et al., 2021). In other words, a study done in 2016 evaluated the efficiency of Turkish pomegranate juice as an antiproliferative agent against human breast epithelial adenocarcinoma (MCF-7, metastatic) and the result showed that it was strong against cancer cells (Ismail, T, et al., 2016). Recent research by Yassin et al. concludes that after 48 hours of incubation, the compounds have an antiproliferative effect against the MCF-7 cell line (Yassin, M. T et al., 2021). Additionally, the peel extract of the Pomegranate also inhibits the proliferation of HCT-116 and Caco-2 cancer cell lines (Moghazy, A. et al., 2019). Moreover, ethanol extracts of pomegranate peels demonstrated high potency against several tumor types, including WI-38 (normal human fibroblasts), HeLa (cervical cancer), SW620 (colorectal adenocarcinoma, metastatic), MIA PaCa-2 (pancreatic carcinoma), and CFPAC-1 (pancreatic adenocarcinoma derived from metastatic liver). Furthermore, it has also been shown by many studies that pomegranate constituents can effectively inhibit some signaling pathways related to cancer initiation, transformation, inflammation, angiogenesis, cellular hyperproliferation, metastasis, and the final stages of tumorigenesis (Khan et al., 2007; Faria and Calhau, 2011).

In a more holistic view, toothpaste containing hydroalcoholic extracts of pomegranate peel was found to significantly reduce the number of open dentin tubules compared to a control group in experimental testing conducted by Peyro Mousavi, S. F et al. in 2022. Also,

pomegranate fruit consists of most parts that contain antioxidants agent. Amazingly, an *in-vitro* study conducted in 2016 revealed that the pomegranate fruit peel extracts not only have antioxidant and anti-diabetic properties but also can lengthen the lifespan of *Caenorhabditis elegans* (Barathikannan, K et al., 2016). Supplementing *Caenorhabditis elegans* worms with ethanol acetate extract also inhibited lipid accumulation. Furthermore, a variety of pomegranate peel extracts did not exhibit hemolytic activity *in-vivo* studies, confirming their biosafety (Yassin, M.T et al., 2021).

From a chemical point of view, previous studies conducted on pomegranates showed that its juice, peels, flowers, and other parts contained different types of primary and secondary chemical components including polyphenol compounds such as ellagic tannins, hydrolyzable tannins (including ellagitannins and gallotannins), condensed tannins (e.g., pro-anthocyanidins), flavonoids (e.g., anthocyanin and flavanols, catechin, epigallocatechin-3-gallate, 5-hydroxymethylfurfural, 4-fluorobenzyl alcohol, gallic acid, procyanidins, and ellagic acid. The constituents of Pomegranate include vitamins, antioxidants, fatty acids (catechin), quercetin, caffeic acid, punicalic acid, piperidine alkaloids, Ellagitannins (e.g., punicalagin and punicalin), protocatechuic acid, p-hydroxybenzoic acid and D-Limonene (Moghazy, A. M et al., 2019; El-Beltagi et al., 2022). To sum up, it has been proven that *P. granatum* peels, seeds, and fruits have anti-inflammatory, antiradical, antidiabetic, strong antioxidant, hypolipidemic, and antiviral efficiency activity. These properties are directly attributed to the primary and secondary chemical components which are constituents of pomegranate parts.

Although pomegranates are known for their antibacterial properties, this research aims to explore antibacterial by using various parts of the pomegranate plant together, including

its aerial part, fruit, fruit peels, and flower obtained from Dhahran, Saudi Arabia. Currently, there is no information available on the bioactivity of pomegranate aerial parts grown in the agroclimatic conditions of Saudi Arabia's eastern region. The chemical compositions and concentrations of the plant can vary widely and depend on various factors (Heber, D. et al., 2006).

## **2.5 *Calotropis procera***

The genus *Calotropis* (persisting plant) belongs to the *Apocynaceae* family. *C. procera* is a species in this genus. It is commonly known as Ushar. The *Calotropis* grows naturally in a variety level of climates and lands, but it prefers sandy and dry soils. It is salt resistant. Whenever it is cut into pieces, it gives off a milky white latex. Spongy texture and green color are the main characteristics of its fruits. The *Calotropis* can be found in various natural locations such as India, Pakistan, Australia, Thailand, South and Central America, California, and many Pacific Islands including Hawaii. Additionally, it is native to several countries including Algeria, Iran, Iraq, Palestine, Yemen, United Arab Emirates, Oman, Kuwait, and Saudi Arabia.

Different parts of the plant *C. procera* have been utilized in traditional medicine for a long time to treat various ailments, including eczema, pain, mouth bleeding, asthma, diarrhea, jaundice, and skin disease, superficial burns as well. In addition, the leaves of the plant are used to treat snakebite, sinusitis, rheumatism, mumps, etc., while root bark is used to treat high fever, leprosy, and menorrhagia. Further, aboveground portions are used as a decoction in Saudi Arabia to treat fever, joint pain, constipation, and muscle spasms. *C. procera* latex has also been helpful in wound healing, antifungal treatments, skin

infections, and antimicrobials. Due to these reasons, various reports have been published on the pharmacological properties of the plant, with investigations into its toxicological aspects using *in-vivo* and *in-vitro* experimental models.

Different authors have therefore claimed that *C. procera*'s latex has healing, anti-cancer, antipyretic, and analgesic properties, as well as anti-inflammatory activities. The extracts of the aerial parts of *C. procera* have been found to possess antipyretic, anti-depressive, analgesic, and neuromuscular blocking properties in previous studies (Garabadu et al., 2019). A significant reduction in blood glucose levels has also been demonstrated for leaf extracts of *C. procera*, demonstrating its antihyperglycemic potential. In addition, Arya and Kumar et al. (2005) found that it possesses similar anti-inflammatory properties to Phenylbutazone in both chronic and acute inflammation models. Further, it has been recorded in 2010 that the laticifer proteins of the *C. procera*'s latex protect against *Salmonella enterica* serovar Typhimurium infection in adult male Swiss mice (Lima-Filho, J. V et al., 2010). Moreover, latex's laticifer proteins prevented 70% of animals from dying in a recent study. It also reduced inflammation and restored blood-coagulation homeostasis. (Sousa, B. F et al., 2020) Furthermore, several studies have shown that different parts of *C. procera* possess antimicrobial properties (Saddiq, A. et al., 2022). In contrast, in animals treated with whole latex, adverse effects such as toxicity, hyperalgesia, and lethality have also been reported (Kumar and Sehgal, 2007). Also, Abd Alrheam et al. (2015) have found that the *C. procera* causes acute hepatotoxicity and cardiotoxicity.

Various plant parts contain tannins, cardiac glycosides, saponins, terpenoids, alkaloids, flavonoids, steroids, and cardenolides (Pattnaik, P.K et al., 2017; Falana, M.B et al., 2022). For instance, leaf extracts of *C. procera* contain linoleic acids, amino acids, palmitic acid

esters, and fatty acid ethyl esters (Pattnaik, P.K et al., 2017). Also, a recent study found that the main components in *C. procera* leaves are  $\alpha$ -myrin, lupeol acetate, phytol, hexadecanoic acid, stigmasterol, and gombasterol A (Doleib, N.M et al., 2022). As active constituents, four flavonoid glycosides were isolated, the crude flavonoid fractions were identified as (1) the flavonoid 5-hydroxy-3,7- dimethoxyflavone-40-O-b-glucopyranoside (2) 3-O-rutinosides of quercetin (3) isorhamnetin besides (4) kaempferol (Nenaah, G. et al., 2013). Moreover, this plant's essential oils were studied for Indian species, Nigerian species, Egyptian species, and Saudi species. Results showed that environmental and climatic conditions affected the chemical composition of this tree either in quality or quantity. In addition to hydrocarbons, aromatics, and carotenoids, terpenes were the most predominant components. The sesquiterpenes accounted for most of the essential oils in the Saudi sample, whereas the diterpenes accounted for the majority in the Egyptian sample. *C. procera* is reported to have many therapeutic properties in the literature, but its biological and ecological characteristics (especially in golf countries or as a whole plant) have received only limited attention. Except for their activities against a small range of microorganisms (Gomah E et al., 2015).

## CHAPTER 3

### Materials and Methods

This chapter is devoted to providing a comprehensive description of the research methodology. Figure 1 illustrates the study's methodological framework, which is explained in the following sections.

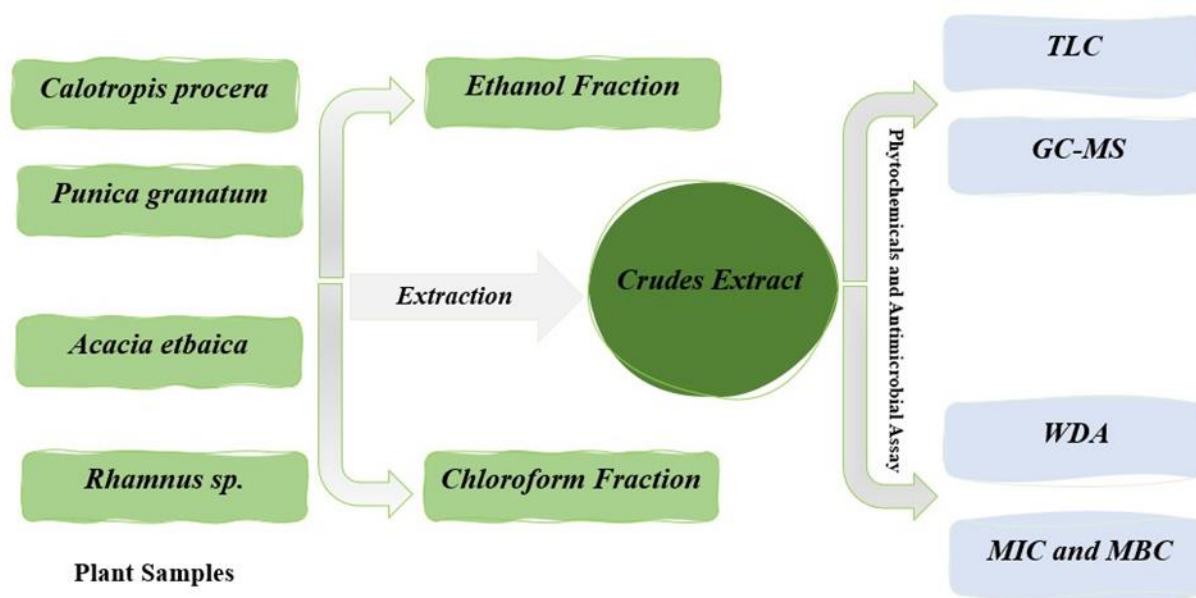


Figure 1 The Experimental Procedures Framework

#### 3.1 Materials

The materials employed in this study encompass a diverse array of components, including medicinal plants, tested bacteria, commercially available antibiotics, and various chemical reagents. Each of these elements is meticulously detailed in the subsequent subsections.

### **3.1.1 Medicinal Plants**

Four medicinal herbs, namely *Calotropis procera* (*C. procera*), *Punica granatum* (*P. granatum*), *Acacia etbaica* (*A. etbaica*), and *Rhamnus* *sp.* native to Saudi Arabia were utilized. Folk medicine and earlier investigations suggest that these herbs have significant pharmacological properties. These plants were freshly collected from Dhahran, in the Eastern Province of Saudi Arabia (17 meters elevation). These plants were collected from September 2022 to mid-October 2022. The collected plant specimens were identified and confirmed by plant taxonomists for their authenticity (Table 1).

### **3.1.2 Bacterial Strains**

In this investigation, a variety of bacterial strains were used, including *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853, *Escherichia coli* (*E. coli*) ATCC 25922, *Staphylococcus aureus* (*S. aureus*) ATCC 25923, and *Enterococcus faecalis* (*E. faecalis*) ATCC 29212. These organisms are commonly employed as models for antibiotic susceptibility testing. The bacterial strains studied were obtained from the Microbiology Laboratory, Faculty of Chemical and Materials, King Fahad University of Petroleum and Minerals (KFUPM), Kingdom of Saudi Arabia.

### **3.1.3 Reference Antibiotics**

As positive controls for various tests, Ciprofloxacin and Cinoxacin were employed at concentrations of 15 µg and 50 µg, respectively, specifically for the WDA.

### 3.1.4 Chemical Substances

For the extraction and analysis processes, a range of solvents was employed, including ethanol (EthOH), chloroform (CHCl<sub>3</sub>), diethyl ether (DEE), dichloromethane (DCM), methanol, hexane, dimethyl sulfoxide (DMSO), water, and distilled water (dH<sub>2</sub>O). In addition, the culturing process utilized nutrient agar (NA) and nutrient broth (NB).

## 3.2 Methodology

This section encompasses methodologies, providing a comprehensive overview of the experimental procedures. Each of these procedures is meticulously detailed in the subsequent subsections.

### 3.2.1 Plants Extraction Procedure

Plants were air-dried for 30 days at room temperature (25±2 °C). Afterward, if needed, samples were further dried at 40 °C for 2 hours in an oven before undergoing grinding. Using an electrical blender, well-dried plant samples were ground into fine powders. After homogenization into a fine powder, the samples were labeled and stored at 4 °C, away from sunlight, in air-tight plastic containers until the extraction process was carried out.

**Preparation of Plant Extracts.** Each powdered sample (100 g) was soaked in 500 mL of two different absolute solvents (95% EthOH and CHCl<sub>3</sub>). Subsequently, the mixtures were placed in a shaking incubator at 27 °C, with a gradual speed of 100 revolutions per minute (rpm; min), for up to seven days. Following this, the extracts were filtered separately. The next step involved using a centrifuge for the mixture (10000 rpm for 10 min). The debris-

free organic supernatant was then concentrated by subjecting it to a rotary evaporator between 35 °C and 42 °C for 3 hours. The final yields were weighed and stored separately and then refrigerated at -4 °C until used. The plant yield percentages were calculated using the following formula: Extract yield% =  $D/A \times 100$ , where D represents the weight of plant residues, whereas A represents the weight of raw samples.

### **3.2.2 Preparation of the Standard Concentrations**

A 0.6 g of pre-prepared organic crude extracts will be dissolved in 1 mL of pure DMSO, a biochemically inert substance. As a result, extracts with a concentration of 600 mg/mL will be utilized as the standard concentration for testing their antibacterial properties. The stock solution will be diluted fivefold using autoclaved dH<sub>2</sub>O to prepare various concentrations (500 mg/mL, 250 mg/mL, 125 mg/mL, 50 mg/mL, and 5 mg/mL).

### **3.2.3 Preparation of Bacterial Inoculums**

NA medium was prepared under sterile conditions, based on the manufacturer's instructions. After that, the medium was poured into the sterile petri dishes (temperature of about 65 °C). Then, the plates were stored at laboratory temperature for solidification and were refrigerated at 4 °C until use. The bacterial inoculums containing  $1-1.5 \times 10^6$  bacterial cells/mL were sub-cultured onto prepared NA medium using plates spread technique and then were incubated at 37 °C. Antibacterial tests of plant extracts will be then subjected to three different tests, to assess their potential activity.

### **3.2.4 *In-Vitro* Antibiotics Activity Assays**

Antibiotics were applied to the NA medium inoculated with test bacterial cells using WDA. After 18 hours of incubation under standard conditions, the zone width of inhibition was measured in millimeters (mm) to assess the sensitivity (S), intermediate (I), or resistance (R) of the bacteria to the employed antibacterial drugs (reference drugs).

### **3.2.5 Antibacterial Susceptibility Testing of Plant Extracts**

Plant extracts were subjected to two different tests, to assess their potential activity as antibacterial compounds. For a quick assessment (preliminary screening) of antibacterial activity, WDA was conducted. However, due to the disadvantages of diffusion assay methods, such as poor diffusion of bio-active compounds through agar, the MIC assay was also performed. The antibacterial activity assays were all conducted after determining the sensitivity of the bacteria themselves to DMSO.

#### **Well Diffusion Assay (WDA)**

The standard agar WDA was employed for the initial screening of the antibacterial activities for both ethanolic and chloroformed plant extracts. Loop inoculated with a single colony of each tested bacterium was separately immersed in a 15 mL tube containing 6 mL of sterile sodium chloride (0.9%) and incubated. The bacterial suspension turbidity was adjusted to  $(1-1.5 \times 10^8)$  bacterial cells/mL. A 100  $\mu$ L of suspension broth was then spread separately onto freshly prepared NA using an L-shaped sterile spreader, and the inoculum was allowed to dry for a suitable period. Wells were then created in the center of each seeded NA medium plate using the reverse side of a sterilized micropipette tip. A 50  $\mu$ L of

each plant's crude extract was piped into the wells at concentrations of 500 mg/mL, 250 mg/mL, 125 mg/mL, 50 mg/mL, and 5 mg/mL per well, resulting in a final concentration of 0.25 mg/mL, 2.5 mg/mL, 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL, and 30 mg/mL. The plates were allowed to stand at room temperature for an hour for proper diffusion and then incubated at 37 °C for up to 24 hours. The success of the inhibitory activity was marked by the absence of bacterial growth in the area surrounding the wells. The experiment was conducted in two replications. The average diameter of inhibitory zones produced by plant extracts was compared to the controls. As a negative control, wells containing pure DMSO were used. Additionally, server wells were loaded with Cinoxacin and Ciprofloxacin to serve as standard antibacterial agents.

The percent inhibition (%I) of growth was determined by measuring the diameter of cleared zones created by both the sample and the Ciprofloxacin. The mean values were subsequently recorded.

### **Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC)**

The inoculum was prepared by transferring a single colony of each tested bacteria, separately, to a sterile NB media, kept overnight at 37 °C on a shaker bed at 100 rpm. Spectroscopy was used to measure the OD<sub>600 nm</sub> of the bacterial suspension, which was adjusted to absorbance 0.05, corresponding to  $1-1.5 \times 10^6$  bacterial cells/mL. The MIC of the ethanolic and chloroformed extract were determined using conventional broth dilution. The dilution technique followed the guidelines of the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards, 2000) with some modifications. Under a laminar flow cabinet, nine tubes were prepared for each tested

bacteria by dispensing NB for bacteria into each tube. The first tube received an injection of an equal volume from the stock solution of the tested plant extracts, which had a concentration of 400 mg/mL. Using a pipette, serial two-fold dilutions were performed horizontally up to tube-labeled 9. The achieved concentration ranged from 200 to 0.78 mg/mL, and 1:25  $\mu$ L of tested suspended bacteria was added to each tube. Blank control for strain viability and medium sterility were used. Ciprofloxacin and cinoxacin were used as reference drugs (positive controls), with concentrations of 60 and 30  $\mu$ g/mL for Ciprofloxacin, and 100 and 50  $\mu$ g/mL for Cinoxacin. Additionally, 50% (v/v) of DMSO was used as a negative control. After that, the tubes were incubated at 37°C overnight (up to 18 hours) after well-mixing.

After incubation at 37 °C, the tubes were visually examined for the presence or absence of visible bacterial growth and compared with growth in blank control tubes. However, obtaining MIC results through visual investigation was difficult due to the presence of color in the plant extracts, which precipitated with the culture medium. Consequently, the MIC was defined as the lowest concentration (highest dilution) of extract that allowed no more than 20% growth of each organism on the agar plate after spreading 100  $\mu$ L of each concentration on the surface of NA plates, followed by incubation at 37 °C for 18 hours. MBC, which were more suitable for determining subinhibitory concentrations for subsequent experiments, represented the lowest concentration leading to a microbicidal effect (where viable bacterial colonies could not be observed on the surface of fresh agar plates). The presence of one or two colonies was regarded as no growth (Mitscher et al., 1987; Kawther, 2007). All assays were performed in duplicate. The results are expressed in mg/mL. Regardless of their low levels of inhibition in antibacterial tests, all tested

bacteria were evaluated for MIC and MBC. Antibacterial activity was also assessed based on the MBC/MIC ratio. If the MBC/MIC ratio was  $\leq 4$ , the effect was considered to be bactericidal induced; however, if the ratio exceeded 4, the effect was defined as bacteriostatic induced (Levison, M. E., 2004).

### **3.2.6 Phytochemical Analysis**

#### **Thin-Layer Chromatography (TLC)**

TLC was employed to determine the appropriate solvents for the plant extracts. Both the ethanolic and chloroformed fractions were utilized. Silica gel plates (20 x 20 cm, Merck) were chosen as the stationary phase for TLC. Spots from each fraction of the plant extract were carefully applied to the silica gel plates. Various mobile phases were tested, including  $\text{CHCl}_3$ , DCM, DEE, methanol, hexane, and a combination of hexane and DCM in equal proportions. The residues of each extract were then detected using UV light ( $\lambda=365/254\text{nm}$  Lamp, Vilber, France), and the corresponding distance values were duly recorded.

#### **Gas chromatography-mass spectrometry (GC-MS)**

The GC-MS (TQD8040, SHIMADZU, Japan) instrument is used to separate, and identify the main components in the sample extract mixture. To detect the composition of the extract mixture, the plants' crude extracts were submitted to phytochemical testing using GC-MS to identify the presence of various chemical compounds, following the selection of appropriate solvents determined by TLC assay. Additionally, control samples were prepared using pure DEE. The samples were then analyzed using Gas Chromatography coupled with a tandem Mass Spectrometer detector.

By comparing the spectrum of the unknown component with known components stored in the library's database, or by directly comparing it with the instrument library, unidentified components were identified. The names and molecular weights of the chemical compounds were determined for the main peaks in the chromatograms. Also, the structure of the phytochemical compounds has antimicrobial features that were generated using the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>).

### **3.2.7 Statistical Analysis**

For statistically valid results, each analysis was carried out independently in duplicate to ensure consistency of all findings, and data were provided as means. All calculations were made in Microsoft Excel 2019.

## **CHAPTER 4**

### **RESULTS**

#### **4.1 Introduction**

After a comprehensive examination of the existing literature, four plant species were identified to be employed in this research. Extraction, as defined in pharmaceutical terms, pertains to the technique utilized for segregating therapeutically active natural compounds through treatment with specific solvents. Evaluating the raw plant extracts for the desired bioactivity stands as a critical operation in medicinal plant investigations, and extraction marks the initial pivotal phase of this process. Diverse methods can be applied to liberate the compounds from the biomass. While water finds application as an extractant in numerous traditional protocols, contemporary extraction methods primarily opt for organic solvents with varying polarities to leverage the diverse solubilities of the plant constituents. This involves the solvents' capacity to enhance cell wall permeability, thereby facilitating the effective extraction of substantial quantities of polar and medium-to-low polarity constituents.

Numerous techniques have been employed in extracting potential antibacterial compounds from natural products. The traditional approach to assessing an antibacterial agent from natural sources involves the preparation of a crude extract, which is subsequently purified, analyzed, and tested against bacterial strains. This study utilized organic solvents extraction procedure to obtain the natural crude from plant sources.

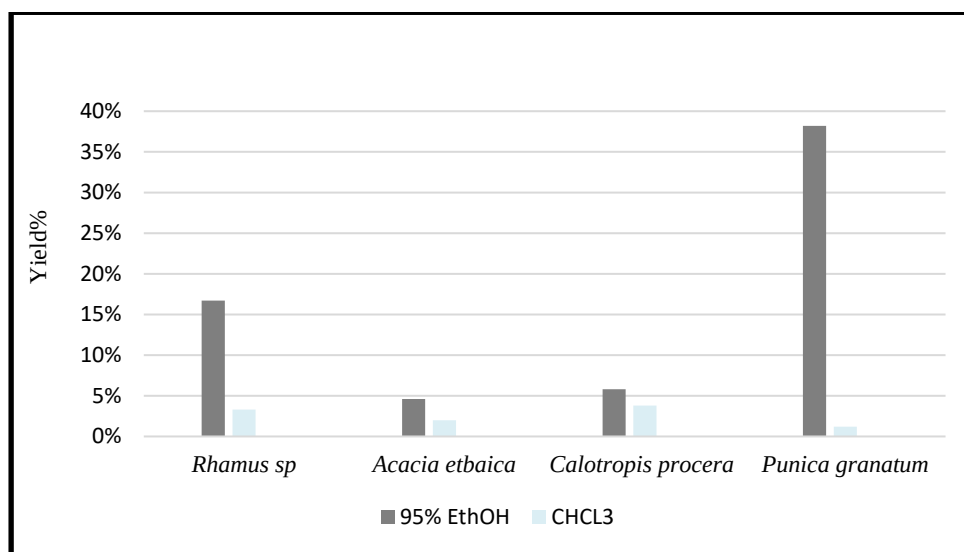
## 4.2 Yield of Crude Extracts of Medicinal Plants

The initial materials from *Rhamnus sp.*, *A. etbaica*, *C. procera*, and *P. granatum* were obtained from air-dried and subsequently ground into a fine powder. The resulting products exhibited physical characteristics of dry, yellowish-brown to pale brown powders. These dried powders of medicinal plants underwent organic solvent extraction procedures using absolute solvents —CHCl<sub>3</sub> and 95% EthOH— in a sequence of varying polarities to obtain crude extracts from each respective plant, as detailed in Chapter 3. The percentage yield of the crude extracts (% w/w) from 100 g of plant material is provided in Table 1. Ethanol proved to be a more efficient extraction solvent compared to chloroform as illustrated in Figure 2, resulting in higher yield percentages. *P. granatum* and *Rhamnus sp.* both extracts demonstrated the highest yields in their ethanolic fractions, with percentages of 38.2% and 16.7%, respectively. Conversely, the lowest yields were obtained from the chloroform fractions of *P. granatum* (1.2%) and *A. etbaica* (2%). The remnants of the plants left after extraction displayed a color like the initial materials. Chloroform extracts exhibited a flaky appearance, whereas ethanolic extracts had a thicker, more oily consistency.

## 4.3 Evaluation of Antibiotics Activity

The antibiotics' efficacy against *P. aeruginosa*, *E. coli*, *S. aureus*, and *E. faecalis* was evaluated using the WDA, as detailed in Table 2, and illustrated in Figure 3. *E. coli* demonstrated resistance to Ciprofloxacin, with no observable inhibition. Conversely, Ciprofloxacin exhibited the highest inhibition zone against *E. faecalis* (35 mm), followed by *P. aeruginosa* (34 mm). A similar pattern was observed for Cinoxacin, with *E. coli*

displaying resistance, while it demonstrated strong activity against *E. faecalis* (25 mm) and moderate activity against *P. aeruginosa* (18 mm). DMSO, on the other hand, showed no inhibitory effects against any of the studied bacterial strains, indicating its inert nature in this context, making it a suitable solvent for crude extracts.



**Figure 2 Comparison of Yield (%) of Different Plants Extracts Using Different Solvents**

**Table 1 Plant Information, Time and Location of Collection, the Used Solvents, and Yield of Extracts**

| Plant Scientific Name | Collected Parts                                      | Location                              | Time of collection | Solvent used for extraction | % Yield (w/w) |
|-----------------------|------------------------------------------------------|---------------------------------------|--------------------|-----------------------------|---------------|
| <i>Rhamnus sp.</i>    | Freshly and woody steam, leaves, flowers, and fruits | From the garden of Saudi Aramco, KSA. | September, 2022    | EthOH<br>CHCl <sub>3</sub>  | 16.7<br>3.3   |
| <i>A. etbaica</i>     | Whole plant including flowers                        | From university garden at KFUPM, KSA. | October, 2022      | EthOH<br>CHCl <sub>3</sub>  | 4.6<br>2      |
| <i>C. procera</i>     | Whole plant including fruits and flowers             | From university garden at KFUPM, KSA. | September, 2022    | EthOH<br>CHCl <sub>3</sub>  | 5.8<br>3.8    |
| <i>P. granatum</i>    | Freshly and woody steam, leaves, flowers, and fruits | From the garden of Saudi Aramco, KSA. | September, 2022    | EthOH<br>CHCl <sub>3</sub>  | 38.2<br>1.2   |

**Table 2 Antibacterial Activity of Controls Against Model Bacterial Strains Analyzed by WDA\***

\*Values are means of 2 replicates, Note: mm: millimeter, NA: No Activity, R: Resistance, S: Sensitive, I: Intermediate.

| Standard      | Well Load    | Diameter of Zone of Inhibition (mm) |                           |                                 |                               |
|---------------|--------------|-------------------------------------|---------------------------|---------------------------------|-------------------------------|
|               |              | Gram-Negative Bacterial Strains     |                           | Gram-Positive Bacterial Strains |                               |
|               |              | <i>P. aeruginosa</i> ATCC 27853     | <i>E. coli</i> ATCC 25922 | <i>S. aureus</i> ATCC 25923     | <i>E. faecalis</i> ATCC 29212 |
| Cinoxacin     | 50 µg        | 18 (I)                              | NA (R)                    | 14 (R)                          | 25 (S)                        |
| Ciprofloxacin | 15 µg        | 34 (S)                              | NA (R)                    | 27 (S)                          | 35 (S)                        |
| DMSO          | Pure (50 µL) | NA                                  | NA                        | NA                              | NA                            |

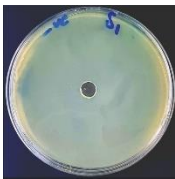
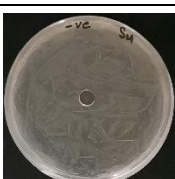
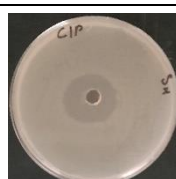
|                                 | Negative and Positive Controls                                                     |                                                                                     |                                                                                      |
|---------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
|                                 | DMSO                                                                               | Cinoxacin                                                                           | Ciprofloxacin                                                                        |
|                                 | Pure                                                                               | 50 µg                                                                               | 15 µg                                                                                |
| <i>E. coli</i> ATCC 25922       |   |   |   |
| <i>E. faecalis</i> ATCC 29212   |   |   |   |
| <i>P. aeruginosa</i> ATCC 27853 |   |   |   |
| <i>S. aureus</i> ATCC 25923     |  |  |  |

Figure 3 Results of Screening of Antibacterial Activities of Negative and Positive Controls Against Model Gram-Negative and Gram-Positive Bacterial Strains. Evaluated Based on Zone of Inhibitions Using Agar WDA. Note: Data are presented as follows, -ve: Pure DMSO, CIN: Cinoxacin, CIP: Ciprofloxacin, S<sub>1</sub>: *E. coli* ATCC 25922, S<sub>2</sub>: *E. faecalis* ATCC 29212, S<sub>3</sub>: *P. aeruginosa* ATCC 27853, and S<sub>4</sub>: *S. aureus* ATCC 25923.

#### 4.4 Evaluation of Plant Extracts Bioactivity (WDA)

This research investigates the antibacterial potential of four medicinal plants (*Rhamnus sp.*, *A. etbaica*, *C. procera*, and *P. granatum*) native to the Eastern Province of Saudi Arabia. These plants have a longstanding history of utilization in traditional medicine, prompting a keen interest in exploring their pharmacological properties. This section is dedicated to screening the activities of ethanolic and chloroformed crude extracts derived from these

medicinal plants against two Gram-negative bacteria, *E. coli* and *P. aeruginosa*, as well as two Gram-positive bacteria, *S. aureus* and *E. faecalis*, using the agar WDA. The diameter readings of the zones of inhibition were measured, and their means were documented for analysis.

The findings presented in Table 3 indicate that the ethanolic extracts of *C. procera* and *A. etbaica* exhibited no zones of inhibition against *E. coli* in the agar WDA, as depicted in Figure 4. Similarly, *A. etbaica* demonstrated no zones of inhibition against *P. aeruginosa* and *S. aureus*, as illustrated in Figures 6 and 7. In contrast, the ethanolic extracts of *C. procera* effectively inhibited the growth of *S. aureus*, *P. aeruginosa*, and *E. faecalis* at a concentration of 30 mg per well, resulting in inhibition diameters of 14 mm, 12 mm, and 11 mm, and inhibition percentages of 52%, 35%, and 31%, respectively, as shown in Figures 7, 6, and 5.

*A. etbaica* also exhibited inhibitory effects against the growth of *E. faecalis* at the same concentration, with an inhibition diameter of 12 mm and an inhibition percentage of 34%, as depicted in Figure 5. On the other hand, the ethanolic extracts of *P. granatum* and *Rhamnus sp.* demonstrated robust zones of inhibition against all tested bacterial strains, as depicted in Figures 4, 5, 6, and 7. The activity of *P. granatum* surpassed that of *Rhamnus sp.*, with inhibition diameters of 33 mm, 28.5 mm, 26.5 mm, and 22.5 mm at 30 mg per well against *E. faecalis*, *P. aeruginosa*, *E. coli*, and *S. aureus*, respectively. This resulted in inhibition percentages of 94%, 84%, and 83% against the growth of *E. faecalis*, *P. aeruginosa*, and *S. aureus*, respectively.

**Table 3 Antibacterial Activity of 95% Ethanolic Extract Against Model Bacterial Strains Analyzed by WDA\***

| Plant Species      | Well Dose (mg) | Diameter of Zone of Inhibition (mm) |    |                           |    |                                 |    |                               |    |
|--------------------|----------------|-------------------------------------|----|---------------------------|----|---------------------------------|----|-------------------------------|----|
|                    |                | Gram-Negative Bacterial Strains     |    |                           |    | Gram-Positive Bacterial Strains |    |                               |    |
|                    |                | <i>P. aeruginosa</i> ATCC 27853     | I% | <i>E. coli</i> ATCC 25922 | I% | <i>S. aureus</i> ATCC 25923     | I% | <i>E. faecalis</i> ATCC 29212 | I% |
| <i>C. procera</i>  | 0.25           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 2.5            | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 6.25           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 12.5           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 25             | 12                                  | 35 | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 30             | 12                                  | 35 | NA                        | UD | 14                              | 52 | 11                            | 31 |
| <i>P. granatum</i> | 0.25           | 7.5                                 | 22 | NA                        | UD | NA                              | UD | 20                            | 57 |
|                    | 2.5            | 20                                  | 59 | 13.5                      | UD | 7.5                             | 28 | 28                            | 80 |
|                    | 6.25           | 22.5                                | 66 | 16                        | UD | 11                              | 41 | 29.5                          | 84 |
|                    | 12.5           | 27.5                                | 81 | 17                        | UD | 19.5                            | 72 | 30                            | 86 |
|                    | 25             | 28.5                                | 84 | 26.5                      | UD | 22.5                            | 83 | 33                            | 94 |
| <i>A. etbaica</i>  | 0.25           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 2.5            | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 6.25           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 12.5           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 25             | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 30             | NA                                  | UD | NA                        | UD | NA                              | UD | 12                            | 34 |
| <i>Rhamnus sp.</i> | 0.25           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 2.5            | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 6.25           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 12.5           | 11                                  | 32 | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 25             | 13.5                                | 40 | NA                        | UD | NA                              | UD | 10.5                          | 30 |
|                    | 30             | 21                                  | 62 | 14                        | UD | 13                              | 48 | 19.5                          | 56 |

Note: \*values are means of 2 replicates, NA: No Activity, UD: Undefined, %I: percent inhibition by using Ciprofloxacin results.

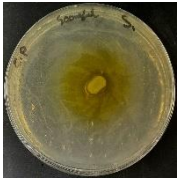
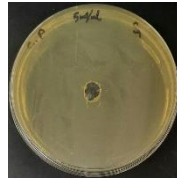
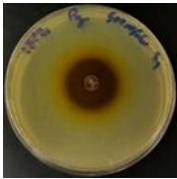
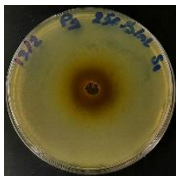
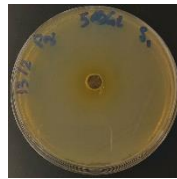
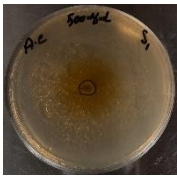
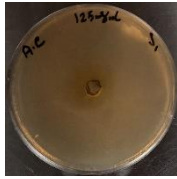
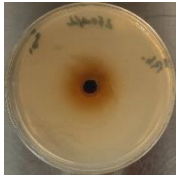
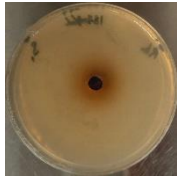
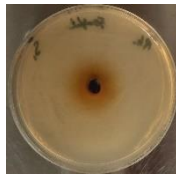
|                    | 30 mg                                                                              | 25 mg                                                                              | 12.5 mg                                                                             | 6.25 mg                                                                              | 2.5 mg                                                                               | 0.25 mg                                                                              |
|--------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>C. procera</i>  |                                                                                    |   |   |   |   |   |
| <i>P. granatum</i> |                                                                                    |   |   |   |   |   |
| <i>A. etbaica</i>  |   |   |   |   |   |   |
| <i>Rhamnus sp.</i> |  |  |  |  |  |  |

Figure 4 Results of Screening of Antibacterial Activities of *C. procera*, *P. granatum*, *A. etbaica* and *Rhamnus sp.* Ethanolic Extract Against Model Gram- Negative (S<sub>1</sub>: *E. coli* ATCC 25922) at 30 mg, 25 mg, 12.5 mg, 6.25 mg, 6.25 mg, 2.5 mg, and 0.25 mg (mg /well). Evaluated Based on Zone of Inhibitions Using Agar WDA. Note: Extract Concentrations (mg mL<sup>-1</sup>) Were 600 mg, 500 mg, 250 mg, 125 mg, 50 mg, 5 mg. Data Are Presented as Follows, CP: *C. procera*, PG: *P. granatum*, Ae: *A. etbaica*, Rh: *Rhamnus sp.*

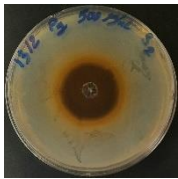
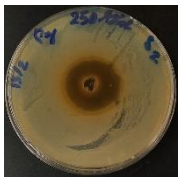
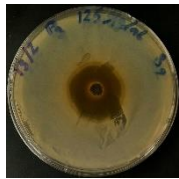
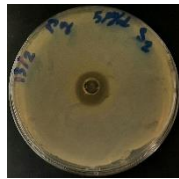
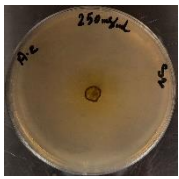
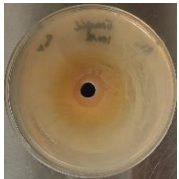
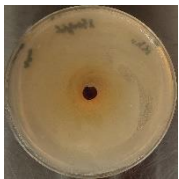
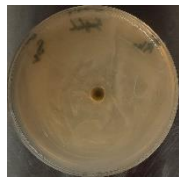
|                    | 30 mg                                                                              | 25 mg                                                                              | 12.5 mg                                                                             | 6.25 mg                                                                              | 2.5 mg                                                                               | 0.25 mg                                                                              |
|--------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>C. procera</i>  |                                                                                    |   |   |   |   |   |
| <i>P. granatum</i> |                                                                                    |   |   |   |   |   |
| <i>A. etbaica</i>  |   |   |   |   |   |   |
| <i>Rhamnus sp.</i> |  |  |  |  |  |  |

Figure 5 Results of Screening of Antibacterial Activities of *C. procera*, *P. granatum*, *A. etbaica* and *Rhamnus sp.* Ethanolic Extract Against Model Gram- Positive (S2: *E. faecalis* ATCC 29212) at 30 mg, 25 mg, 12.5 mg, 6.25 mg, 6.25 mg, 2.5 mg, and 0.25 mg (mg /well). Evaluated Based on Zone of Inhibitions Using Agar WDA.

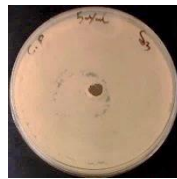
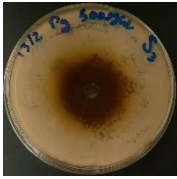
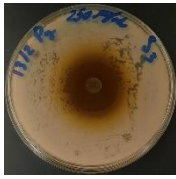
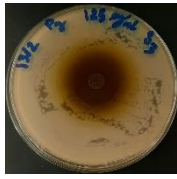
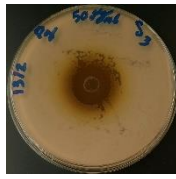
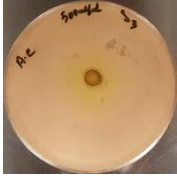
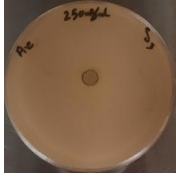
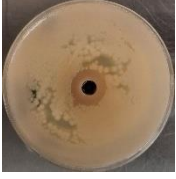
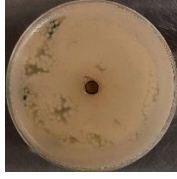
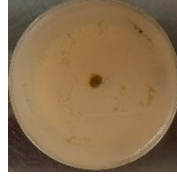
|                    | 30 mg                                                                              | 25 mg                                                                              | 12.5 mg                                                                             | 6.25 mg                                                                              | 2.5 mg                                                                               | 0.25 mg                                                                              |
|--------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>C. procera</i>  |   |   |   |   |   |   |
| <i>P. granatum</i> |                                                                                    |   |   |   |   |   |
| <i>A. etbaica</i>  |   |   |   |   |   |   |
| <i>Rhamnus sp.</i> |  |  |  |  |  |  |

Figure 6 Results of Screening of Antibacterial Activities of *C. procera*, *P. granatum*, *A. etbaica* and *Rhamnus sp.* Ethanolic Extract Against Model Gram- Negative (S3: *P. aeruginosa* ATCC 27853) at 30 mg, 25 mg, 12.5 mg, 6.25 mg, 6.25 mg, 2.5 mg, and 0.25 mg (mg /well). Evaluated Based on Zone of Inhibitions Using Agar WDA.

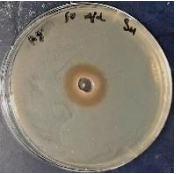
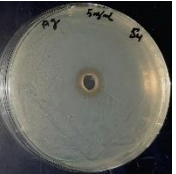
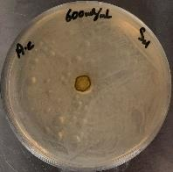
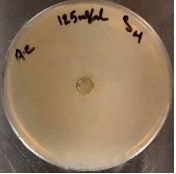
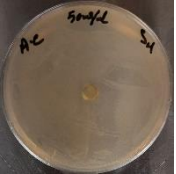
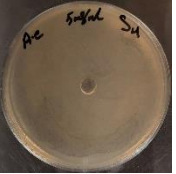
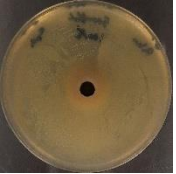
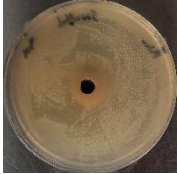
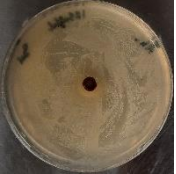
|                    | 30 mg                                                                              | 25 mg                                                                              | 12.5 mg                                                                             | 6.25 mg                                                                              | 2.5 mg                                                                               | 0.25 mg                                                                              |
|--------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>C. procera</i>  |                                                                                    |   |   |   |   |   |
| <i>P. granatum</i> |                                                                                    |   |   |   |   |   |
| <i>A. etbaica</i>  |   |   |   |   |   |   |
| <i>Rhamnus sp.</i> |  |  |  |  |  |  |

Figure 7 Results of Screening of Antibacterial Activities of *C. procera*, *P. granatum*, *A. etbaica* and *Rhamnus sp.* Ethanolic Extract Against Model Gram- Positive (S4: *S. aureus* ATCC 25923) at 30 mg, 25 mg, 12.5 mg, 6.25 mg, 6.25 mg, 2.5 mg, and 0.25 mg (mg /well). Evaluated Based on Zone of Inhibitions Using Agar WDA.

In general, the ethanolic extracts from the selected medicinal plants exhibited higher antibacterial activity against the tested bacteria at varying doses, as outlined in Table 3, owing to their extraction of antibacterial compounds.  $\text{CHCl}_3$  extracts displayed slightly lower activity compared to ethanolic extracts, and in some cases, no activity was observed even at higher concentrations, as indicated in Table 4. However, certain bacteria that showed resistance to ethanolic extracts became susceptible to chloroformed extracts. For instance, *C. procera* exhibited inhibition against *E. coli* with a diameter of 12 mm at 30 mg per well, as shown in Figure 8. Similarly, the chloroformed extracts of *A. etbaica* inhibited the growth of *E. coli* and *S. aureus* at 30 mg per well, with inhibition diameters of 22 mm and 14 mm (inhibition percent 41%), respectively, as depicted in Figures 8 and 11.

The lowest activity recorded in chloroformed extracts was observed in *P. granatum*, with no activity against *E. coli*, *E. faecalis*, and *P. aeruginosa*, but with 14 mm inhibition zones in *S. aureus* (inhibition percent 41%) at 30 mg per well, as shown in Figures 8, 9, 10, and 11. *C. procera* extract demonstrated the ability to inhibit the growth of all tested bacteria, with activity zones as follows: 11 mm, 12 mm, 14 mm, and 19 mm against *P. aeruginosa*, *E. coli*, *S. aureus*, and *E. faecalis*, respectively, as shown in Figures 10, 8, 11, and 9. The activity of *Rhamnus sp.* extract was absent on *P. aeruginosa* compared to its ethanolic extract, as shown in Figures 6 and 10. However, it increased from 13 mm to 14 mm (inhibition percent 41%) in both ethanolic and chloroformed extracts, respectively, when it came to *S. aureus*, as illustrated in Figures 7 and 11. There was a slight or no difference in *E. faecalis* and *E. coli*, with inhibition diameters of 20 mm (59%) and 14 mm, respectively, as shown in Figures 7 and 8.

**Table 4 Antibacterial Activity of Chloroformed Extract Against Model Bacterial Strains Analyzed by WDA\***

| Plant Species      | Well Dose (mg) | Diameter of Zone of Inhibition (mm) |    |                           |    |                                 |    |                               |    |
|--------------------|----------------|-------------------------------------|----|---------------------------|----|---------------------------------|----|-------------------------------|----|
|                    |                | Gram-Negative Bacterial Strains     |    |                           |    | Gram-Positive Bacterial Strains |    |                               |    |
|                    |                | <i>P. aeruginosa</i> ATCC 27853     | I% | <i>E. coli</i> ATCC 25922 | I% | <i>S. aureus</i> ATCC 25923     | I% | <i>E. faecalis</i> ATCC 29212 | I% |
| <i>C. procera</i>  | 0.25           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 2.5            | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 6.25           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 12.5           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 25             | NA                                  | UD | NA                        | UD | 11                              | 32 | NA                            | UD |
|                    | 30             | 11                                  | 32 | 12                        | UD | 14                              | 41 | 19                            | 56 |
| <i>P. granatum</i> | 0.25           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 2.5            | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 6.25           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 12.5           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 25             | NA                                  | UD | NA                        | UD | 14                              | 41 | NA                            | UD |
| <i>A. etbaica</i>  | 0.25           | NA                                  | UD | 13                        | UD | 6.5                             | 19 | NA                            | UD |
|                    | 2.5            | NA                                  | UD | 15                        | UD | 8                               | 23 | NA                            | UD |
|                    | 6.25           | NA                                  | UD | 17                        | UD | 9                               | 26 | NA                            | UD |
|                    | 12.5           | NA                                  | UD | 18                        | UD | 9.5                             | 28 | NA                            | UD |
|                    | 25             | NA                                  | UD | 18                        | UD | 12                              | 35 | NA                            | UD |
|                    | 30             | NA                                  | UD | 22                        | UD | 14                              | 41 | 12                            | 35 |
| <i>Rhamnus sp.</i> | 0.25           | NA                                  | UD | NA                        | UD | NA                              | UD | NA                            | UD |
|                    | 2.5            | NA                                  | UD | NA                        | UD | NA                              | UD | 14.5                          | 42 |
|                    | 6.25           | NA                                  | UD | NA                        | UD | NA                              | UD | 16                            | 47 |
|                    | 12.5           | NA                                  | UD | NA                        | UD | 10                              | 29 | 17.5                          | 51 |
|                    | 25             | NA                                  | UD | 14                        | UD | 12                              | 35 | 19.5                          | 57 |
|                    | 30             | NA                                  | UD | 14                        | UD | 14                              | 41 | 20                            | 59 |

Note: \*values are means of 2 replicates.

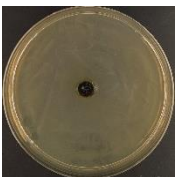
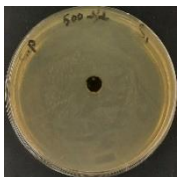
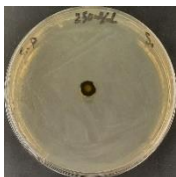
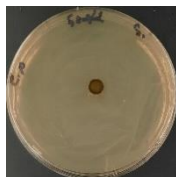
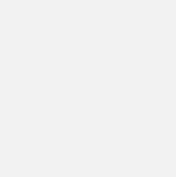
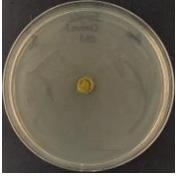
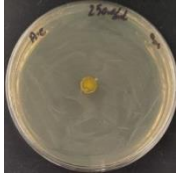
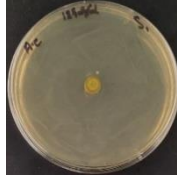
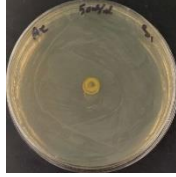
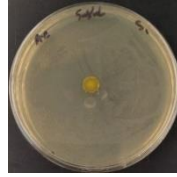
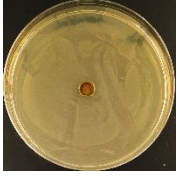
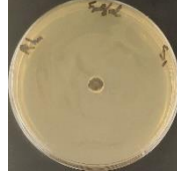
|                    | 30 mg                                                                              | 25 mg                                                                              | 12.5 mg                                                                             | 6.25 mg                                                                              | 2.5 mg                                                                               | 0.25 mg                                                                              |
|--------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>C. procera</i>  |   |   |   |   |   |   |
| <i>P. granatum</i> |   |   |   |   |   |   |
| <i>A. etbaica</i>  |   |   |   |   |   |   |
| <i>Rhamnus sp.</i> |  |  |  |  |  |  |

Figure 8 Results of Screening of Antibacterial Activities of *C. procera*, *P. granatum*, *A. etbaica* and *Rhamnus sp.* Chloroformed Extract Against Model Gram- Negative (S1: *E. coli* ATCC 25922) at 30 mg, 25 mg, 12.5 mg, 6.25 mg, 6.25 mg, 2.5 mg, and 0.25 mg (mg /well). Evaluated Based on Zone of Inhibitions Using Agar WDA.

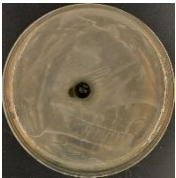
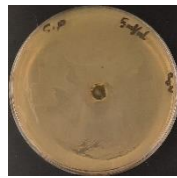
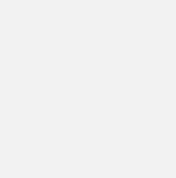
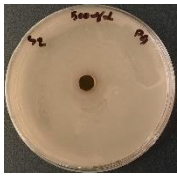
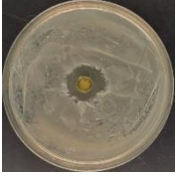
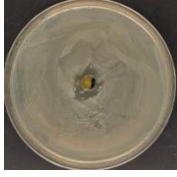
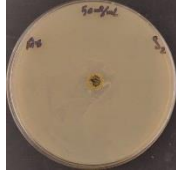
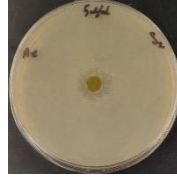
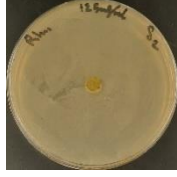
|                    | 30 mg                                                                              | 25 mg                                                                              | 12.5 mg                                                                             | 6.25 mg                                                                              | 2.5 mg                                                                               | 0.25 mg                                                                              |
|--------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>C. procera</i>  |   |   |   |   |   |   |
| <i>P. granatum</i> |   |   |   |   |   |   |
| <i>A. etbaica</i>  |   |   |   |   |   |   |
| <i>Rhamnus sp.</i> |  |  |  |  |  |  |

Figure 9 Results of Screening of Antibacterial Activities of *C. procera*, *P. granatum*, *A. etbaica* and *Rhamnus sp.* Chloroformed Extract Against Model Gram- Positive (S2: *E. faecalis* ATCC 29212) at 30 mg, 25 mg, 12.5 mg, 6.25 mg, 6.25 mg, 2.5 mg, and 0.25 mg (mg /well). Evaluated Based on Zone of Inhibitions Using Agar WDA.

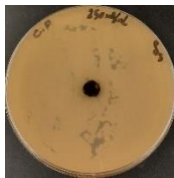
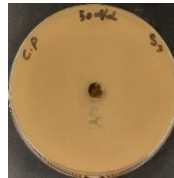
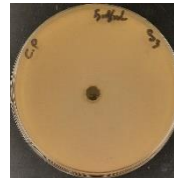
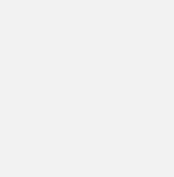
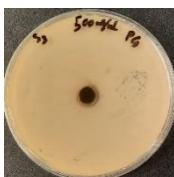
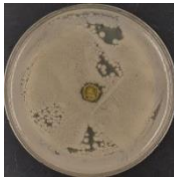
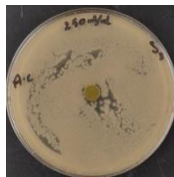
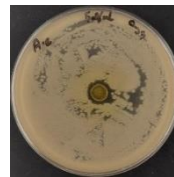
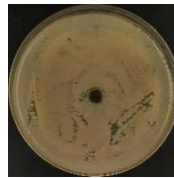
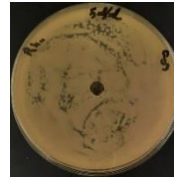
|                    | 30 mg                                                                              | 25 mg                                                                              | 12.5 mg                                                                             | 6.25 mg                                                                              | 2.5 mg                                                                               | 0.25 mg                                                                              |
|--------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>C. procera</i>  |   |   |   |   |   |   |
| <i>P. granatum</i> |   |   |   |   |   |   |
| <i>A. etbaica</i>  |   |   |   |   |   |   |
| <i>Rhamnus sp.</i> |  |  |  |  |  |  |

Figure 10 Results of Screening of Antibacterial Activities of *C. procera*, *P. granatum*, *A. etbaica* and *Rhamnus sp.* Chloroformed Extract Against Model Gram- Negative (S3: *P. aeruginosa* ATCC 27853) at 30 mg, 25 mg, 12.5 mg, 6.25 mg, 6.25 mg, 2.5 mg, and 2.5 mg (mg /well). Evaluated Based on Zone of Inhibitions Using Agar WDA.

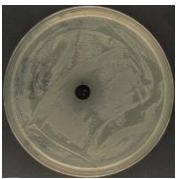
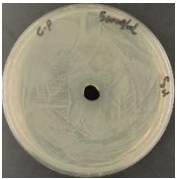
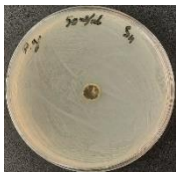
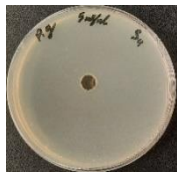
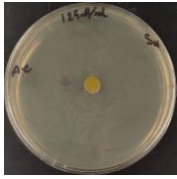
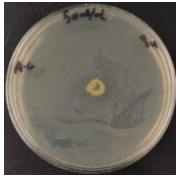
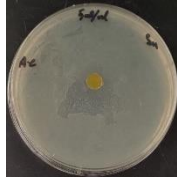
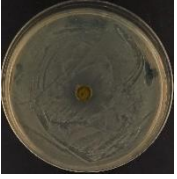
|                    | 30 mg                                                                              | 25 mg                                                                              | 12.5 mg                                                                             | 6.25 mg                                                                              | 2.5 mg                                                                               | 0.25 mg                                                                              |
|--------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>C. procera</i>  |   |   |   |   |   |   |
| <i>P. granatum</i> |                                                                                    |   |   |   |   |   |
| <i>A. etbaica</i>  |   |   |   |   |   |   |
| <i>Rhamnus sp.</i> |  |  |  |  |  |  |

Figure 11 Results of Screening of Antibacterial Activities of *C. procera*, *P. granatum*, *A. etbaica* and *Rhamnus sp.* Chloroformed Extract Against Model Gram- Positive (S4: *S. aureus* ATCC 25923) at 30 mg, 25 mg, 12.5 mg, 6.25 mg, 6.25 mg, 2.5 mg, and 0.25 mg (mg /well). Evaluated Based on Zone of Inhibitions Using Agar WDA.

## 4.5 MIC and MBC

The data obtained from the WDA of the crude extracts suggested that chloroformed extracts had antibacterial activity against Gram-positive and Gram-negative bacteria. Nonetheless, ethanol extract showed antibacterial activity against tested bacteria to various degrees, but the inhibition zones were more eminent in the ethanolic extracts. For this reason, these extracts were tested further on the same bacterial species to determine MIC and MBC. The MIC test tells us about the efficiency of the natural products under investigation, it helps to identify the most effective drug candidate from several natural product extracts. Thus, the results obtained from the MIC test can be used to monitor the preparation of a new antibacterial agent that has greater efficiency. The MBC is complementary to the MIC; whereas the MIC test demonstrates the lowest level of antibacterial agent that inhibits growth, the MBC demonstrates the lowest level of antibacterial agent that results in microbial death.

MIC and MBC values of *C. procera* extracts against tested bacteria are summarized in Table 5 and Figures 12 and 13. MIC of the ethanolic extract derived from *C. procera* exhibited a range between 25 and 50 mg/mL when tested against model bacterial strains. Notably, this extract demonstrated higher potency against *P. aeruginosa* and *E. faecalis* at a concentration of 25 mg/mL. However, its efficacy against *E. coli* and *S. aureus* was slightly lower, requiring a concentration of 50 mg/mL. On the other hand, the chloroform extract displayed a wider range of activity, with MIC values spanning from 3.125 to 25 mg/mL. Notably, this extract exhibited remarkable effectiveness against *E. coli*, showcasing its potential as a potent antibacterial agent. Impressively, all other tested

bacterial strains displayed a similar susceptibility to the chloroform extract, requiring an MIC concentration of 25 mg/mL.

Moving on to the MBC, the ethanolic extract from *C. procera* ranged from 100 to 200 mg/mL against the model bacterial strains. Notably, the 200 mg/mL concentration can be considered relatively high. The ethanolic extract demonstrated an MBC of 100 mg/mL against *E. coli*, *S. aureus*, and *E. faecalis*, but this concentration elevated to 200 mg/mL when targeting *P. aeruginosa*. Similarly, the chloroform extract exhibited MBC values ranging from 50 to 200 mg/mL. Remarkably, a 200 mg/mL concentration was again required for the same bacteria. However, the chloroform extract demonstrated greater potential against the bacteria compared to the ethanolic extract. Specifically, *E. coli* and *E. faecalis* were completely eradicated by a 50 mg/mL concentration, which is notably more effective than the ethanolic extract.

The Ratio of MBC to MIC provides valuable insight into the extract's bactericidal or bacteriostatic nature. In the case of the ethanolic extract, it demonstrated a bacteriostatic effect on *P. aeruginosa* while exerting a bactericidal effect on all other tested bacteria. Similarly, the chloroform extract showed a bacteriostatic effect on *P. aeruginosa* and *E. coli* but proved bactericidal against the other strains.

In conclusion, both extracts of *C. procera* showed promise as potential antibacterial agents. However, the chloroform extract, with its exceptional efficacy against *E. coli*, stands out as a particularly strong candidate.

Table 5 MIC and MBC Measurements (mg mL<sup>-1</sup>) of Organic Extracts of the *C. procera* Against Model Bacterial Strains\*

|                  | Tested Model Organism | Concentrations of <i>C. procera</i> (mg/mL) |      |       |      |      |     |     |     |     | Ratio MBC/MIC  |
|------------------|-----------------------|---------------------------------------------|------|-------|------|------|-----|-----|-----|-----|----------------|
|                  |                       | 0.78                                        | 1.56 | 3.125 | 6.25 | 12.5 | 25  | 50  | 100 | 200 |                |
| Organic Extracts | 95% EthOH             | <i>P. aeruginosa</i> ATCC 27853             |      |       |      |      | MIC |     |     | MBC | Bacteriostatic |
|                  |                       | <i>E. coli</i> ATCC 25922                   |      |       |      |      |     | MIC | MBC | -   | Bactericidal   |
|                  |                       | <i>S. aureus</i> ATCC 25923                 |      |       |      |      |     | MIC | MBC | -   | Bactericidal   |
|                  |                       | <i>E. faecalis</i> ATCC 29212               |      |       |      |      | MIC |     | MBC | -   | Bactericidal   |
|                  | CHCl <sub>3</sub>     | <i>P. aeruginosa</i> ATCC 27853             |      |       |      |      | MIC |     |     | MBC | Bacteriostatic |
|                  |                       | <i>E. coli</i> ATCC 25922                   |      |       | MIC  |      |     | MBC | -   | -   | Bacteriostatic |
|                  |                       | <i>S. aureus</i> ATCC 25923                 |      |       |      |      | MIC |     | MBC | -   | Bactericidal   |
|                  |                       | <i>E. faecalis</i> ATCC 29212               |      |       |      |      | MIC | MBC | -   | -   | Bactericidal   |

Note: \*values represent the result after 2 replications.

Data are presented as follows:

- No noticeable inhibition
- Noticeable inhibition
- The Area Between MIC and MBC

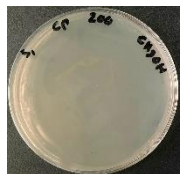
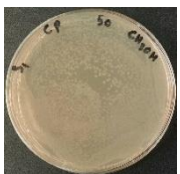
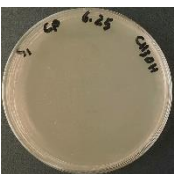
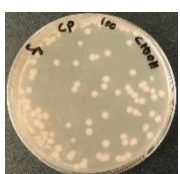
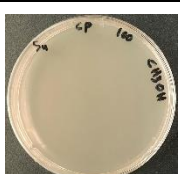
|                      | 200 mg/mL                                                                          | 100 mg/mL                                                                          | 50 mg/mL                                                                           | 25 mg/mL                                                                            | 12.5 mg/mL                                                                           | 6.25 mg/mL                                                                           | 3.125 mg/mL                                                                          | 1.56 mg/mL                                                                           | 0.78 mg/mL                                                                           |
|----------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>E. coli</i>       |   |   |   |   |   |   |   |   |   |
| <i>E. faecalis</i>   |   |   |   |   |   |   |   |   |   |
| <i>P. aeruginosa</i> |   |   |   |   |   |   |   |   |   |
| <i>S. aureus</i>     |  |  |  |  |  |  |  |  |  |

Figure 12 Results of MIC and MBC of Ethanolic Extracts of the *C. procera* Against Model Gram-Negative and Gram-Positive Bacterial Strains, Evaluated Based on Inhibition Bacterial Growth. Note: Extract concentrations ( $\text{mg mL}^{-1}$ ) were 200 mg, 100 mg, 50 mg, 25 mg, 12.5 mg, 6.25 mg, 3.125 mg, 1.56 mg, 0.78 mg. Data Are Presented as Follows, CP: *C. procera*, CH<sub>3</sub>OH: Ethanol, S1: *E. coli* ATCC 25922, S2: *E. faecalis* ATCC 29212, S3: *P. aeruginosa* ATCC 27853, and S4: *S. aureus* ATCC 25923.

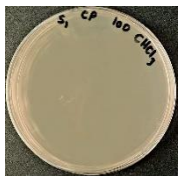
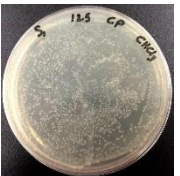
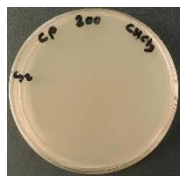
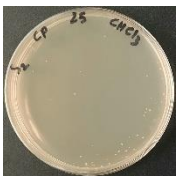
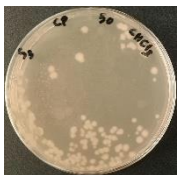
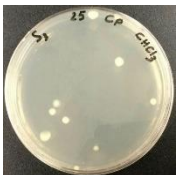
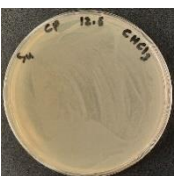
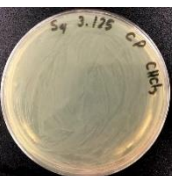
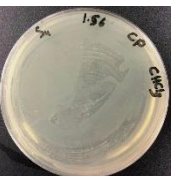
|                      | 200 mg/mL                                                                          | 100 mg/mL                                                                          | 50 mg/mL                                                                           | 25 mg/mL                                                                            | 12.5 mg/mL                                                                           | 6.25 mg/mL                                                                           | 3.125 mg/mL                                                                          | 1.56 mg/mL                                                                           | 0.78 mg/mL                                                                           |
|----------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>E. coli</i>       |   |   |   |   |   |   |   |   |   |
| <i>E. faecalis</i>   |   |   |   |   |   |   |   |   |   |
| <i>P. aeruginosa</i> |   |   |   |   |   |   |   |   |   |
| <i>S. aureus</i>     |  |  |  |  |  |  |  |  |  |

Figure 13 Results of MIC and MBC of Chloroformed Extracts of the *C. procera* Against Model Gram-Negative and Gram-Positive Bacterial Strains, Evaluated Based on Inhibition Bacterial Growth. Data Are Presented as Follows, CHCL<sub>3</sub>: Chloroform.

MIC and MBC values of all *P. granatum* extracts against tested bacteria are summarized in Table 6 and Figures 14 and 15. The MIC and MBC of organic extracts from *P. granatum* were evaluated against model strains. The ethanolic extract displayed a MIC range from 3.125 up to 25 mg/mL. Notably, this extract exhibited high potency against all tested bacteria, particularly demonstrating exceptional inhibition against *E. coli* at 12.5 mg/mL. Furthermore, it was highly effective against *P. aeruginosa* and *E. faecalis*, with MIC concentrations of 3.125 and 6.25 mg/mL, respectively. While the efficacy against *S. aureus* was slightly lower at 25 mg/mL, it still demonstrated considerable potential.

In comparison, the chloroform extract exhibited a broader range of activity, with MIC values spanning from 25 to 50 mg/mL. Intriguingly, this extract demonstrated consistent effectiveness across most tested bacterial strains, requiring an MIC concentration of 50 mg/mL.

Moving on to the MBC measurements, the ethanolic extract presented MBC values ranging from 25 to 50 mg/mL. On the other hand, the chloroform extract exhibited MBC values ranging from 50 to 100 mg/mL. Notably, *E. faecalis* demonstrated the highest sensitivity, requiring an MBC of 50 mg/mL. The remaining bacteria displayed an MBC of 100 mg/mL with 100 mg/mL considered relatively high.

The Ratio of MBC to MIC provides valuable insight into the extract's bactericidal or bacteriostatic nature. For the chloroformed extract, it exerted a bactericidal effect on all tested bacteria, in the same way as ethanolic extract except for *P. aeruginosa* and *E. faecalis*, where it demonstrated a bacteriostatic effect.

In summary, the ethanolic extract of *P. granatum* displayed notable antibacterial potential, particularly against *P. aeruginosa* and *E. faecalis*, albeit at slightly higher concentrations. Meanwhile, the chloroform extract exhibited a broader spectrum of activity, showcasing its efficacy against all tested bacterial strains.

Table 6 MIC and MBC Measurements (mg mL<sup>-1</sup>) of Organic Extracts of the *P. granatum* Against Model Bacterial Strains\*

| Organic Extracts | Tested Model Organism           | Concentrations of <i>P. granatum</i> (mg/mL) |      |       |      |      |     |     |     |     | Ratio MBC/MIC  |
|------------------|---------------------------------|----------------------------------------------|------|-------|------|------|-----|-----|-----|-----|----------------|
|                  |                                 | 0.78                                         | 1.56 | 3.125 | 6.25 | 12.5 | 25  | 50  | 100 | 200 |                |
|                  | 95% EthOH                       |                                              |      |       |      |      |     |     |     |     |                |
| Organic Extracts | <i>P. aeruginosa</i> ATCC 27853 |                                              |      | MIC   |      |      | MBC | -   | -   | -   | Bacteriostatic |
|                  | <i>E. coli</i> ATCC 25922       |                                              |      |       |      | MIC  | MBC | -   | -   | -   | Bactericidal   |
|                  | <i>S. aureus</i> ATCC 25923     |                                              |      |       |      |      | MIC | MBC | -   | -   | Bactericidal   |
|                  | <i>E. faecalis</i> ATCC 29212   |                                              |      |       | MIC  |      |     | MBC | -   | -   | Bacteriostatic |
|                  | <i>P. aeruginosa</i> ATCC 27853 |                                              |      |       |      |      |     | MIC | MBC | -   | Bactericidal   |
|                  | <i>E. coli</i> ATCC 25922       |                                              |      |       |      |      |     | MIC | MBC | -   | Bactericidal   |
|                  | <i>S. aureus</i> ATCC 25923     |                                              |      |       |      |      |     | MIC | MBC | -   | Bactericidal   |
|                  | <i>E. faecalis</i> ATCC 29212   |                                              |      |       |      |      | MIC | MBC | -   | -   | Bactericidal   |

Note: \*values represent the result after 2 replications.

Data are presented as follows:

- No noticeable inhibition
- Noticeable inhibition
- The Area Between MIC and MBC

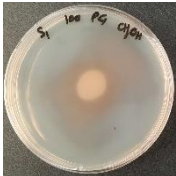
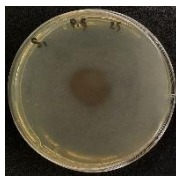
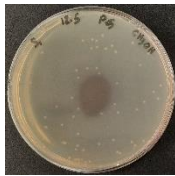
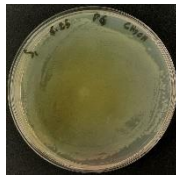
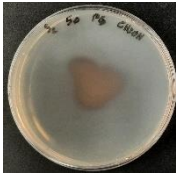
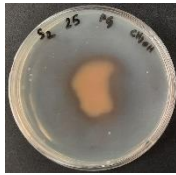
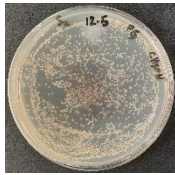
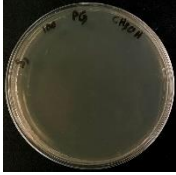
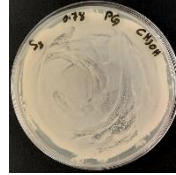
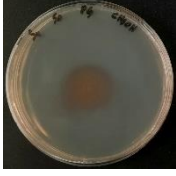
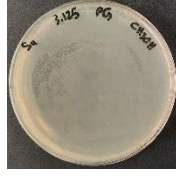
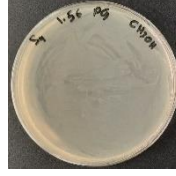
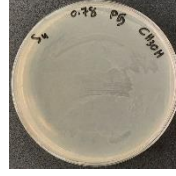
|                      | 200 mg/mL                                                                          | 100 mg/mL                                                                          | 50 mg/mL                                                                           | 25 mg/mL                                                                            | 12.5 mg/mL                                                                           | 6.25 mg/mL                                                                           | 3.125 mg/mL                                                                          | 1.56 mg/mL                                                                           | 0.78 mg/mL                                                                           |
|----------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>E. coli</i>       |   |   |   |   |   |   |   |   |   |
| <i>E. faecalis</i>   |   |   |   |   |   |   |   |   |   |
| <i>P. aeruginosa</i> |   |   |   |   |   |   |   |   |   |
| <i>S. aureus</i>     |  |  |  |  |  |  |  |  |  |

Figure 14 Results of MIC and MBC of Ethanolic Extracts of the *P. granatum* Against Model Gram-Negative and Gram-Positive Bacterial Strains, Evaluated Based on Inhibition Bacterial Growth. Data Are Presented as Follows, PG: *P. granatum*.

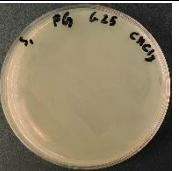
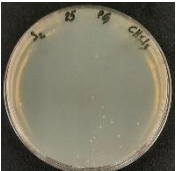
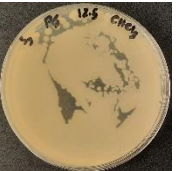
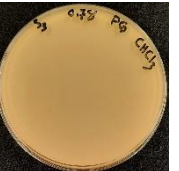
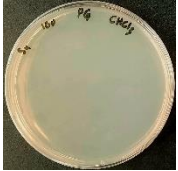
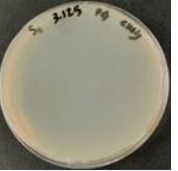
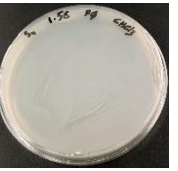
|                      | 200 mg/mL                                                                          | 100 mg/mL                                                                          | 50 mg/mL                                                                           | 25 mg/mL                                                                            | 12.5 mg/mL                                                                           | 6.25 mg/mL                                                                           | 3.125 mg/mL                                                                          | 1.56 mg/mL                                                                           | 0.78 mg/mL                                                                           |
|----------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>E. coli</i>       |   |   |   |   |   |   |   |   |   |
| <i>E. faecalis</i>   |   |   |   |   |   |   |   |   |   |
| <i>P. aeruginosa</i> |   |   |   |   |   |   |   |   |   |
| <i>S. aureus</i>     |  |  |  |  |  |  |  |  |  |

Figure 15 Results of MIC and MBC of Chloroformed Extracts of the *P. granatum* Against Model Gram-Negative and Gram-Positive Bacterial Strains, Evaluated Based on Inhibition Bacterial Growth.

MIC and MBC values of all *A. etbaica* extracts against tested bacteria are summarized in Table 7 and Figures 16 and 17. The MIC values for ethanol and chloroform extracts of *A. etbaica* ranged from 25 to 50 mg/mL. Notably, the chloroform extract exhibited a concentrated effectiveness at 50 mg/mL. The ethanol extract displayed significant activity, particularly against *P. aeruginosa* and *E. faecalis*, requiring only 25 mg/mL for inhibition. Unlike other strains, *P. aeruginosa* showed consistent activity at 25 mg/mL for the chloroformed extract, while higher concentrations (50 mg/mL) were needed for the rest.

The MBC values were concentrated at 100 mg/mL for both extracts, The high MBC values may indicate a lower potency of the extracts against the tested bacterial strains. Furthermore, the chloroform extracts demonstrated reduced activity compared to the ethanol extracts. *S. aureus* and *E. coli* required exceptionally high concentrations (200 mg/mL) for both solvents, indicating their relative resistance. The ratio of MBC to MIC provides valuable insights into the extract's bactericidal nature. In this case, both extracts demonstrated a bactericidal effect against all tested bacteria, with no observed bacteriostatic activity.

In conclusion, both the ethanol and chloroform extracts from *A. etbaica* show antibacterial activity. The observed high MBC values indicate the presence of potent bioactive compounds, possibly at high concentrations, in the plant extracts. Comparatively, the chloroform extracts demonstrated slightly lower activity than the ethanolic extracts.

Table 7 MIC and MBC Measurements (mg mL<sup>-1</sup>) of Organic Extracts of the *A. etbaica* Against Model Bacterial Strains\*

|                  | Tested Model Organism | Concentrations of <i>A. etbaica</i> (mg/mL) |      |       |      |      |     |     |     |     | Ratio MBC/MIC |
|------------------|-----------------------|---------------------------------------------|------|-------|------|------|-----|-----|-----|-----|---------------|
|                  |                       | 0.78                                        | 1.56 | 3.125 | 6.25 | 12.5 | 25  | 50  | 100 | 200 |               |
| Organic Extracts | 95% EthOH             | <i>P. aeruginosa</i> ATCC 27853             |      |       |      |      | MIC |     | MBC | -   | Bactericidal  |
|                  |                       | <i>E. coli</i> ATCC 25922                   |      |       |      |      |     | MIC | MBC | -   | Bactericidal  |
|                  |                       | <i>S. aureus</i> ATCC 25923                 |      |       |      |      |     | MIC |     | MBC | Bactericidal  |
|                  |                       | <i>E. faecalis</i> ATCC 29212               |      |       |      |      | MIC |     | MBC | -   | Bactericidal  |
|                  | CHCl <sub>3</sub>     | <i>P. aeruginosa</i> ATCC 27853             |      |       |      |      | MIC |     | MBC | -   | Bactericidal  |
|                  |                       | <i>E. coli</i> ATCC 25922                   |      |       |      |      |     | MIC |     | MBC | Bactericidal  |
|                  |                       | <i>S. aureus</i> ATCC 25923                 |      |       |      |      |     | MIC |     | MBC | Bactericidal  |
|                  |                       | <i>E. faecalis</i> ATCC 29212               |      |       |      |      |     | MIC | MBC | -   | Bactericidal  |

Note: \*values represent the result after 2 replications.

Data are presented as follows:

- No noticeable inhibition
- Noticeable inhibition
- The Area Between MIC and MBC

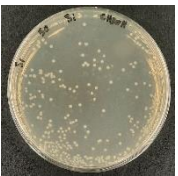
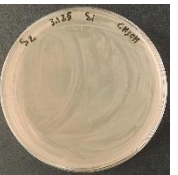
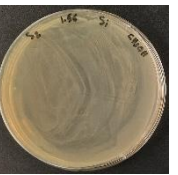
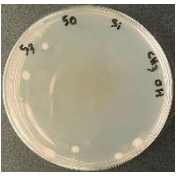
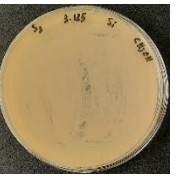
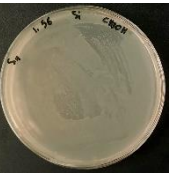
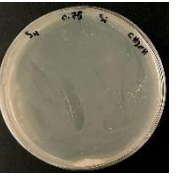
|                      | 200 mg/mL                                                                          | 100 mg/mL                                                                          | 50 mg/mL                                                                           | 25 mg/mL                                                                            | 12.5 mg/mL                                                                           | 6.25 mg/mL                                                                           | 3.125 mg/mL                                                                          | 1.56 mg/mL                                                                           | 0.78 mg/mL                                                                           |
|----------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>E. coli</i>       |   |   |   |   |   |   |   |   |   |
| <i>E. faecalis</i>   |   |   |   |   |   |   |   |   |   |
| <i>P. aeruginosa</i> |   |   |   |   |   |   |   |   |   |
| <i>S. aureus</i>     |  |  |  |  |  |  |  |  |  |

Figure 16 Results of MIC and MBC of Ethanolic Extracts of the *A. etbaica* Against Model Gram-Negative and Gram-Positive Bacterial Strains, Evaluated Based on Inhibition Bacterial Growth. Data Are Presented as Follows, Si or Ae: *A. etbaica*.

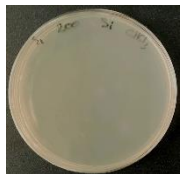
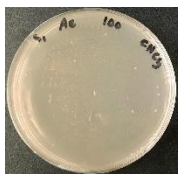
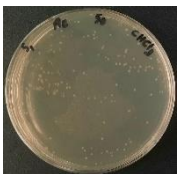
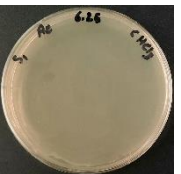
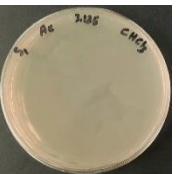
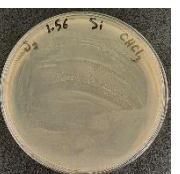
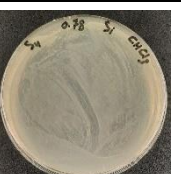
|                      | 200 mg/mL                                                                          | 100 mg/mL                                                                          | 50 mg/mL                                                                           | 25 mg/mL                                                                            | 12.5 mg/mL                                                                           | 6.25 mg/mL                                                                           | 3.125 mg/mL                                                                          | 1.56 mg/mL                                                                           | 0.78 mg/mL                                                                           |
|----------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>E. coli</i>       |   |   |   |   |   |   |   |   |   |
| <i>E. faecalis</i>   |   |   |   |   |   |   |   |   |   |
| <i>P. aeruginosa</i> |   |   |   |   |   |   |   |   |   |
| <i>S. aureus</i>     |  |  |  |  |  |  |  |  |  |

Figure 17 . Results of MIC and MBC of Chloroformed Extracts of the *A. etbaica* Against Model Gram-Negative and Gram-Positive Bacterial Strains, Evaluated Based on Inhibition Bacterial Growth.

MIC and MBC values of all *Rhamnus sp.* extracts against tested bacteria are summarized in Table 8 and Figures 18 and 19. The organic extracts of *Rhamnus sp.* were evaluated for their MIC and MBC against various model bacterial strains. The MIC for the ethanolic extract ranged from 3.125 to 25 mg/mL, while the chloroform extract exhibited MIC values ranging from 12.5 to 50 mg/mL. Notably, the ethanolic extract demonstrated higher potency against most tested bacteria, particularly *E. faecalis* and *P. aeruginosa*, with MIC values of 3.125 and 6.25 mg/mL, respectively. The activity against *E. coli* and *S. aureus* showed susceptibility at 25 mg/mL in both solvents. In contrast, the chloroform extract displayed a lower activity range of MIC values, spanning from 12.5 to 50 mg/mL. It showed reduced efficacy against *E. faecalis* (MIC: 50 mg/mL). However, for *P. aeruginosa* and *E. coli*, the MIC values doubled compared to the ethanolic extract, at 12.5 and 50 mg/mL, respectively.

Moving on to the MBC measurements, the ethanolic extract exhibited a range between 25 and 50 mg/mL. Impressively, it demonstrated bactericidal effects against *E. faecalis* and *P. aeruginosa* at 25 mg/mL, though this concentration elevated to 50 mg/mL for *S. aureus* and *E. coli*. On the other hand, the chloroform extract displayed higher MBC values, ranging from 50 to 200 mg/mL. Notably, for *P. aeruginosa*, *E. faecalis*, and *E. coli*, the MBC values increased by factors of 8, 4, and 2, respectively, compared to the ethanolic extract.

The MBC/MIC ratios provided further insights. The ethanolic extract exhibited a bacteriostatic effect on all tested bacteria except for *P. aeruginosa*, where it displayed a bactericidal effect. In contrast, the chloroform extract demonstrated no bacteriostatic effect on any of the tested bacteria except for *P. aeruginosa*.

In summary, the ethanolic extract demonstrated a broader range of activity with lower MIC values compared to the chloroform extract, especially against *E. faecalis* and *P. aeruginosa*. However, the chloroform extract generally exhibited higher MBC values, indicating a need for higher concentrations to achieve bactericidal effects.

Table 8 MIC and MBC Measurements (mg mL<sup>-1</sup>) of Organic Extracts of the *Rhamnus* sp. Against Model Bacterial Strains\*

| Organic Extracts | Tested Model Organism | Concentrations of <i>Rhamnus</i> sp. (mg/mL) |      |       |      |      |     |     |     |     | Ratio MBC/MIC |                |
|------------------|-----------------------|----------------------------------------------|------|-------|------|------|-----|-----|-----|-----|---------------|----------------|
|                  |                       | 0.78                                         | 1.56 | 3.125 | 6.25 | 12.5 | 25  | 50  | 100 | 200 |               |                |
|                  | 95% EthOH             | <i>P. aeruginosa</i> ATCC 27853              |      |       |      | MIC  |     | MBC | -   | -   | -             | Bactericidal   |
|                  |                       | <i>E. coli</i> ATCC 25922                    |      |       |      |      |     | MIC | MBC | -   | -             | Bacteriostatic |
|                  |                       | <i>S. aureus</i> ATCC 25923                  |      |       |      |      |     | MIC | MBC | -   | -             | Bacteriostatic |
|                  |                       | <i>E. faecalis</i> ATCC 29212                |      |       | MIC  |      |     | MBC | -   | -   | -             | Bacteriostatic |
|                  | CHCl <sub>3</sub>     | <i>P. aeruginosa</i> ATCC 27853              |      |       |      |      | MIC |     |     |     | MBC           | Bacteriostatic |
|                  |                       | <i>E. coli</i> ATCC 25922                    |      |       |      |      |     |     | MIC | MBC | -             | Bactericidal   |
|                  |                       | <i>S. aureus</i> ATCC 25923                  |      |       |      |      |     | MIC | MBC | -   | -             | Bactericidal   |
|                  |                       | <i>E. faecalis</i> ATCC 29212                |      |       |      |      |     |     | MIC | MBC | -             | Bactericidal   |

Note: \*values represent the result after 2 replications.

Data are presented as follows:

- No noticeable inhibition
- Noticeable inhibition
- The Area Between MIC and MBC

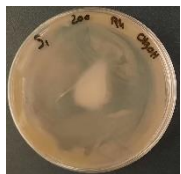
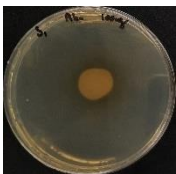
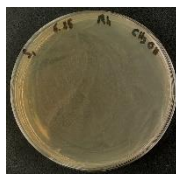
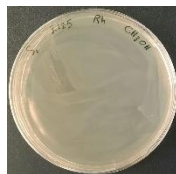
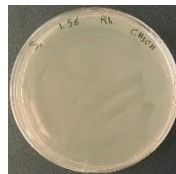
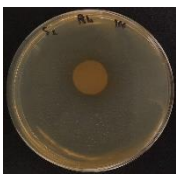
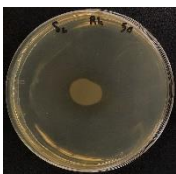
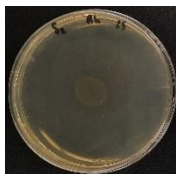
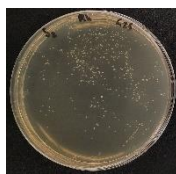
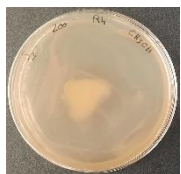
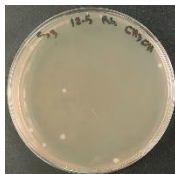
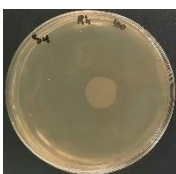
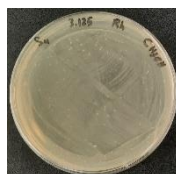
|                      | 200 mg/mL                                                                          | 100 mg/mL                                                                          | 50 mg/mL                                                                           | 25 mg/mL                                                                            | 12.5 mg/mL                                                                           | 6.25 mg/mL                                                                           | 3.125 mg/mL                                                                          | 1.56 mg/mL                                                                           | 0.78 mg/mL                                                                           |
|----------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>E. coli</i>       |   |   |   |   |   |   |   |   |   |
| <i>E. faecalis</i>   |   |   |   |   |   |   |   |   |   |
| <i>P. aeruginosa</i> |   |   |   |   |   |   |   |   |   |
| <i>S. aureus</i>     |  |  |  |  |  |  |  |  |  |

Figure 18 Results of MIC and MBC of Ethanolic Extracts of the *Rhamnus* sp. Against Model Gram-Negative and Gram-Positive Bacterial Strains, Evaluated Based on Inhibition Bacterial Growth. Data Are Presented as Follows, Rh: *Rhamnus* sp.

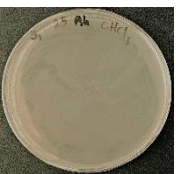
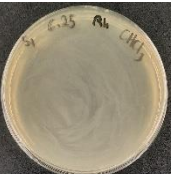
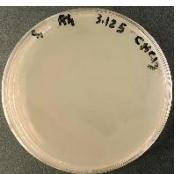
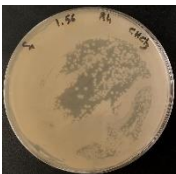
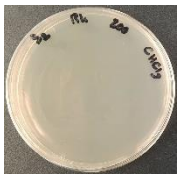
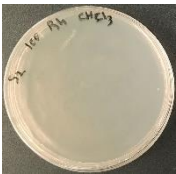
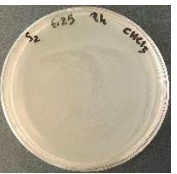
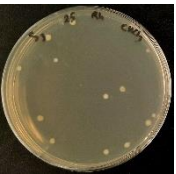
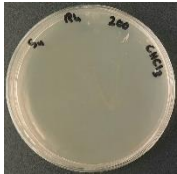
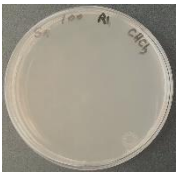
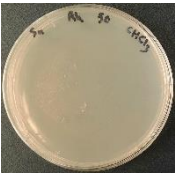
|                      | 200 mg/mL                                                                          | 100 mg/mL                                                                          | 50 mg/mL                                                                           | 25 mg/mL                                                                            | 12.5 mg/mL                                                                           | 6.25 mg/mL                                                                           | 3.125 mg/mL                                                                          | 1.56 mg/mL                                                                           | 0.78 mg/mL                                                                           |
|----------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| <i>E. coli</i>       |   |   |   |   |   |   |   |   |   |
| <i>E. faecalis</i>   |   |   |   |   |   |   |   |   |   |
| <i>P. aeruginosa</i> |   |   |   |   |   |   |   |   |   |
| <i>S. aureus</i>     |  |  |  |  |  |  |  |  |  |

Figure 19 Results of MIC and MBC of Chloroformed Extracts of the *Rhamnus* sp. Against Model Gram- Negative and Gram-Positive Bacterial Strains, Evaluated Based on Inhibition Bacterial Growth.

Also, the study examined the effectiveness of controls against tested bacteria as summarized in Table 9 and Figure 20. The negative control, DMSO, did not affect bacterial growth, corroborating the findings of the well diffusion assay and blank plates. However, Cinoxacin, acting as a positive control, exhibited inhibitory effects on all bacterial strains, except *E. coli*. However, it did not yield any MBC result, even at higher concentrations like 100 µg/mL. Notably, it demonstrated strong inhibition against *P. aeruginosa* and *E. faecalis* at concentrations of 50 and 100 µg/mL, respectively. Similarly, Ciprofloxacin, the second positive control, displayed inhibitory effects on all bacterial strains. It also provided MBC results at 30 µg/mL for *P. aeruginosa* and *E. faecalis*, while requiring a higher concentration, 60 µg/mL, to achieve MIC on *S. aureus*. Interestingly, *E. coli* showed resistance even at 60 µg/mL.

In conclusion, the control antibiotics, potent antibiotics, exhibited potent inhibitory effects against most bacterial strains. However, some degree of resistance was observed in *E. coli* and *S. aureus*, underscoring the importance of exploring alternative antibacterial agents. The investigation into the antibacterial potential of various plant extracts, revealed notable findings regarding their efficacy against these bacterial strains.

**Table 9 MIC and MBC Measurements (µg/mL) of the Controls Against Model Bacterial Strains\***

|                       |                       | Tested Model Organism           | Concentrations of Controls (µg/mL)   |     | Note     |
|-----------------------|-----------------------|---------------------------------|--------------------------------------|-----|----------|
|                       |                       |                                 | 50                                   | 100 |          |
| Positive Controls     | Cinoxacin             | <i>P. aeruginosa</i> ATCC 27853 | MIC                                  | +   | MBC >100 |
|                       |                       | <i>E. coli</i> ATCC 25922       | +++                                  | +++ | MIC >100 |
|                       |                       | <i>S. aureus</i> ATCC 25923     | +++                                  | +   | MIC >100 |
|                       |                       | <i>E. faecalis</i> ATCC 29212   | +                                    | MIC | MBC >100 |
|                       | Tested Model Organism |                                 | Concentrations of Controls (µg/mL)   |     | Note     |
|                       |                       |                                 | 30                                   | 60  |          |
|                       | Ciprofloxacin         | <i>P. aeruginosa</i> ATCC 27853 | MBC                                  | -   | -        |
|                       |                       | <i>E. coli</i> ATCC 25922       | +++                                  | +   | MIC >60  |
|                       |                       | <i>S. aureus</i> ATCC 25923     | ++                                   | MIC | MBC >60  |
|                       |                       | <i>E. faecalis</i> ATCC 29212   | MBC                                  | -   | -        |
| Tested Model Organism |                       |                                 | Concentrations of Controls 50% (v/v) |     |          |
| Negative Control      | DMSO                  | <i>P. aeruginosa</i> ATCC 27853 | +++                                  |     |          |
|                       |                       | <i>E. coli</i> ATCC 25922       | +++                                  |     |          |
|                       |                       | <i>S. aureus</i> ATCC 25923     | +++                                  |     |          |
|                       |                       | <i>E. faecalis</i> ATCC 29212   | +++                                  |     |          |

Note: \*values represent the result after 2 replications. Data are presented as follows, -: No noticeable bacterial growth, +: Slight growth, ++: Moderate growth, +++: Strong growth.

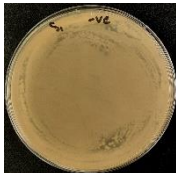
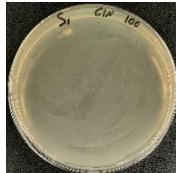
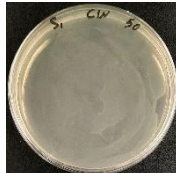
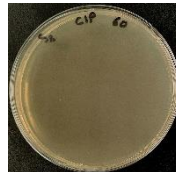
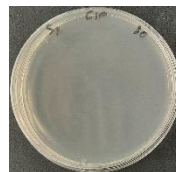
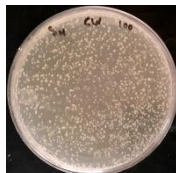
|                      | Blank                                                                               | Negative and Positive Controls                                                      |                                                                                      |                                                                                       |                                                                                       |                                                                                       |
|----------------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
|                      |                                                                                     | DMSO                                                                                | Cinoxacin                                                                            |                                                                                       | Ciprofloxacin                                                                         |                                                                                       |
|                      |                                                                                     | 50% (V/V)                                                                           | 100 µg/mL                                                                            | 50 µg/mL                                                                              | 60 µg/mL                                                                              | 30 µg/mL                                                                              |
| <i>E. coli</i>       |    |    |    |    |    |    |
| <i>E. faecalis</i>   |    |    |    |    |    |    |
| <i>P. aeruginosa</i> |   |   |   |   |   |   |
| <i>S. aureus</i>     |  |  |  |  |  |  |

Figure 20 Results of MIC and MBC of Negative and Positive Controls Against Model Gram-Negative and Gram-Positive Bacterial Strains, Evaluated Based on Inhibition Bacterial Growth. Note: Controls concentrations ( $\mu\text{g mL}^{-1}$ ) 100  $\mu\text{g}$  and 50  $\mu\text{g}$  for Cinoxacin and 60  $\mu\text{g}$  and 30  $\mu\text{g}$  for Ciprofloxacin. Data Are Presented as Follows, -ve: DMSO, CIN: Cinoxacin, and CIP: Ciprofloxacin. Blank Represents Bacterial Growth Without any Treatment.

## 4.6 Phytochemical Analysis

The primary objective of the extraction process is to isolate the soluble metabolites from plant material, leaving behind the insoluble residue. This yields a crude extract that comprises a diverse array of metabolites. Various techniques for the separation of natural products exist. In the present study, chromatographic separation methods were employed, encompassing two principal categories: TLC (a classical method) and GC-MS (a more contemporary approach).

In this investigation, the thoroughly dried plant material underwent a process of pulverization into a fine powder. Subsequently, it was separately immersed in precise quantities of 95% EthOH and pure CHCl<sub>3</sub>. The resulting extracts were then subjected to filtration and subsequently concentrated to dryness utilizing a rotary evaporator. Following this, the individual extracts were amalgamated. Each composite crude extract was subsequently subjected to TLC analysis to ascertain the optimal solvent for subsequent GC-MS analysis. The TLC analysis revealed that DE was the suitable solvent for all the crude extracts from the various plants. DE was utilized as a solvent to dissolve the crude extracts to evaluate phytochemical compounds through GC-MS analysis. The dissolved crude extract was then subjected to GC-MS analysis, providing essential information such as retention time (RT), molecular formula, molecular weight (MWT), and compound name for the identified compounds, all of which were systematically tabulated.

The GC-MS analysis of the DE extract obtained from *C. procera* revealed the presence of various phytochemical constituents, as illustrated in Figure 21. The identified

chemical compounds are detailed in Table 11, predominantly comprising fatty acids, sterols, and triterpenes. Noteworthy bioactive compounds and fatty acids include 9,12-octadecadienoic acid (Z, Z) detected at RT of 12.89 min, followed by 6,9-octadecadienoic acid (RT: 14.157 min) and hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (RT: 15.5 min). Additionally, sterols such as campesterol (RT: 21.07 min) and  $\gamma$ -Sitosterol (RT: 22.4 min) were identified. Triterpenes, namely acorenone B (RT: 14.09 min), lupeol (RT: 25.93 min), and betulin (RT: 26.15 min), were also detected. Furthermore, the analysis revealed the presence of various other natural compounds, encompassing epoxides, aldehydes, anabolic steroids, H<sub>2</sub>-antagonists, progestins, miscellaneous compounds, labdane diterpenoids, and naphthalene propanol derivatives.

Table 11 provides a concise summary of these components, listing their respective RT, molecular formulas, compound classes, and MWT. The chemical structures of the antimicrobial activity compounds present in the DE extract of *C. procera* are depicted in Figure 23. These findings underscore the significant contributions of fatty acids, triterpenoids, sterols, and various other compounds to the demonstrated health benefits of *C. procera*.

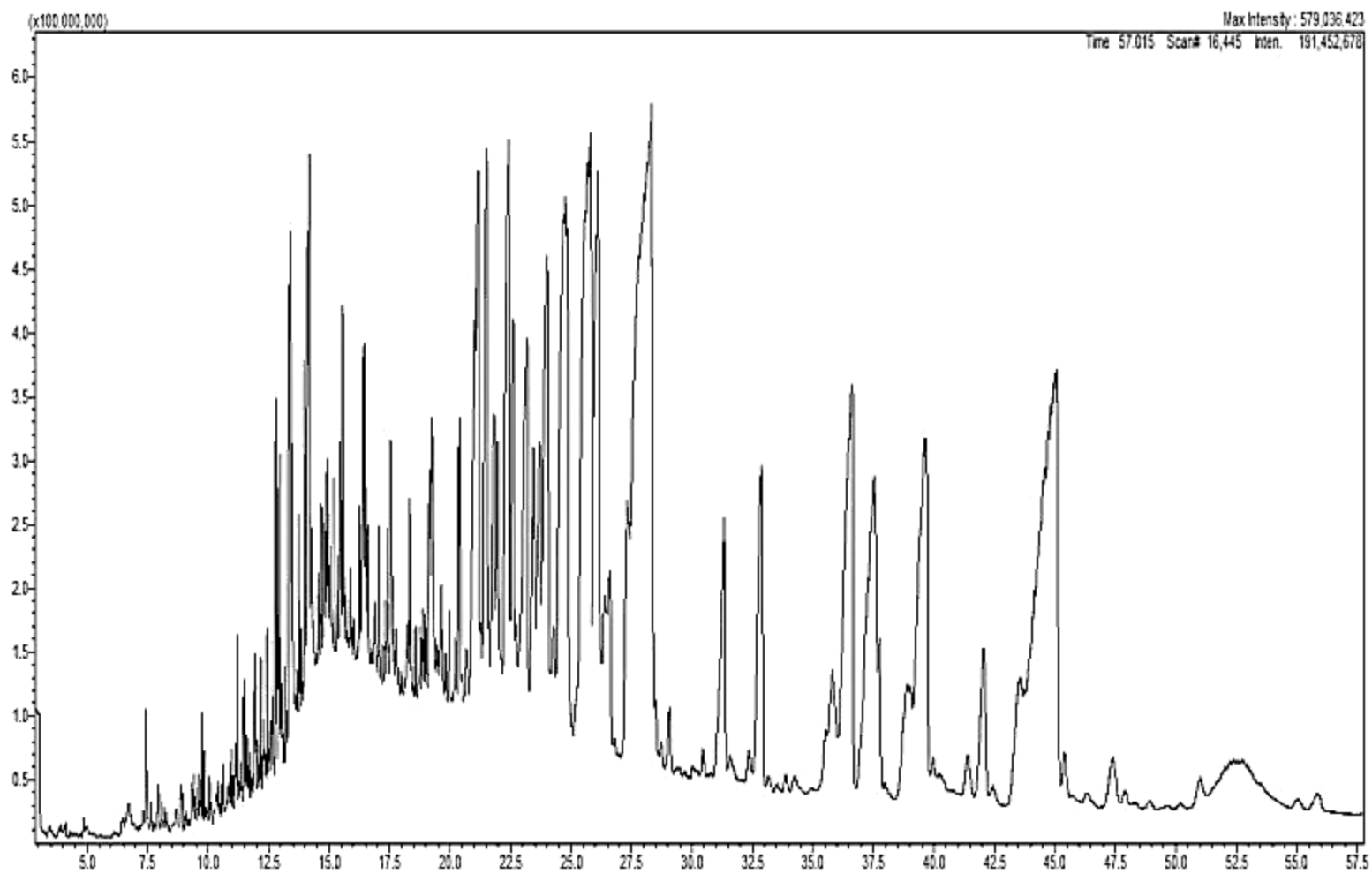
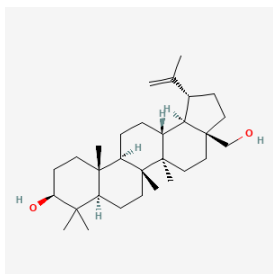


Figure 21 GC-MS Chromatograms of DE fraction of *C. procera*

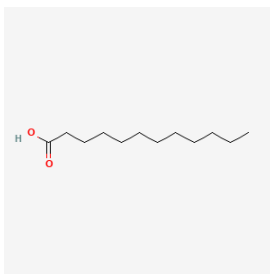
**Table 10 Phytochemical Compounds identified in GC-MS of DE Fraction of *C. procera* and their Antimicrobial Activity**

| Chemical Composition of <i>C. procera</i> |          |                                                                             |                                  |     |                                                  |                        |                           |
|-------------------------------------------|----------|-----------------------------------------------------------------------------|----------------------------------|-----|--------------------------------------------------|------------------------|---------------------------|
| No.                                       | RT (min) | Compound Name                                                               | Compound Class                   | MWT | Formula                                          | Antimicrobial Activity | Ref.                      |
| 1                                         | 12.8     | Oxirane, tetradecyl                                                         | Epoxide                          | 240 | C <sub>16</sub> H <sub>32</sub> O                | ✓                      | Srinivasan, et al., 2022  |
| 2                                         | 12.89    | 9,12-Octadecadienoic acid (Z,Z)                                             | Fatty Acids                      | 280 | C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>   | ✓                      | Tamokou, et al., 2012     |
| 3                                         | 12.98    | Tridecanal                                                                  | Aldehydes                        | 189 | C <sub>13</sub> H <sub>26</sub> O                |                        |                           |
| 4                                         | 13.35    | n-Hexadecanoic acid                                                         | Fatty Acids                      | 256 | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>   | ✓                      | Siswadi and Saragih, 2021 |
| 5                                         | 13.39    | Cimetidine                                                                  | H <sub>2</sub> Antagonists       | 252 | C <sub>10</sub> H <sub>16</sub> N <sub>6</sub> S | ✓                      | Masoud, et al., 2014      |
| 6                                         | 13.45    | Hexadecanoic acid, ethyl ester                                              | Fatty Acids                      | 284 | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>   |                        |                           |
| 7                                         | 13.76    | Pentadecanoic acid                                                          | Fatty Acids                      | 242 | C <sub>15</sub> H <sub>30</sub> O <sub>2</sub>   | ✓                      | Jogaiah, 2020             |
| 8                                         | 13.97    | Oxirane, hexadecyl                                                          | Epoxide                          | 268 | C <sub>18</sub> H <sub>36</sub> O                | ✓                      | Srinivasan, et al., 2022  |
| 9                                         | 14.09    | Acorenone B                                                                 | Triterpenes                      | 220 | C <sub>15</sub> H <sub>24</sub> O                |                        |                           |
| 10                                        | 14.157   | 6,9-Octadecadienoic acid, methyl ester                                      | Fatty Acids                      | 294 | C <sub>19</sub> H <sub>34</sub> O <sub>2</sub>   |                        |                           |
| 11                                        | 15.5     | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester                   | Fatty Acids                      | 330 | C <sub>19</sub> H <sub>38</sub> O <sub>4</sub>   |                        |                           |
| 12                                        | 21.07    | Campesterol                                                                 | Sterols                          | 400 | C <sub>28</sub> H <sub>48</sub> O                | ✓                      | Uttu, et al., 2023        |
| 13                                        | 21.4     | Stigmasterol                                                                | Sterols                          | 412 | C <sub>29</sub> H <sub>48</sub> O                | ✓                      | Arora and Meena, 2018     |
| 14                                        | 22.4     | γ -Sitosterol                                                               | Sterols                          | 414 | C <sub>29</sub> H <sub>50</sub> O                |                        |                           |
| 15                                        | 22.6     | Methandriol                                                                 | Anabolic Steroid                 | 304 | C <sub>20</sub> H <sub>32</sub> O <sub>2</sub>   |                        |                           |
| 16                                        | 22.9     | Ethisterone                                                                 | Progestin                        | 312 | C <sub>21</sub> H <sub>28</sub> O <sub>2</sub>   |                        |                           |
| 17                                        | 23.2     | β -Amyrone                                                                  | Miscellaneous                    | 424 | C <sub>30</sub> H <sub>48</sub> O                |                        |                           |
| 18                                        | 23.85    | Lup-20(29)-en-3-ol, acetate, (3- β)                                         | Triterpenes                      | 468 | C <sub>32</sub> H <sub>52</sub> O <sub>2</sub>   |                        |                           |
| 19                                        | 23.85    | Lupeol                                                                      | Triterpenes                      | 426 | C <sub>30</sub> H <sub>50</sub> O                | ✓                      | Saddiq, et al., 2022      |
| 20                                        | 23.85    | Betulin                                                                     | Triterpenes                      | 442 | C <sub>30</sub> H <sub>50</sub> O <sub>2</sub>   | ✓                      | Lou, et al., 2021         |
| 21                                        | 23.85    | Lup-20(29)-en-3-ol, acetate, (3- β)                                         | Triterpenes                      | 468 | C <sub>32</sub> H <sub>52</sub> O <sub>2</sub>   |                        |                           |
| 27                                        | 25.38    | 15,16-Dinorlabdane, 8,13:13,20-diepoxy-, (13S)                              | Labdane Diterpenoids             | 278 | C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>   |                        |                           |
| 29                                        | 25.53    | 1-Naphthalenepropanol, α.-ethenyldecahydro-2-hydroxy α,2,5,5,8a-pentamethyl | Naphthalene propanol derivatives | 308 | C <sub>20</sub> H <sub>36</sub> O <sub>2</sub>   |                        |                           |
| 37                                        | 26.15    | 4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)    | Miscellaneous                    | 306 | C <sub>20</sub> H <sub>34</sub> O <sub>2</sub>   | ✓                      | Chenniappan, et al., 2013 |

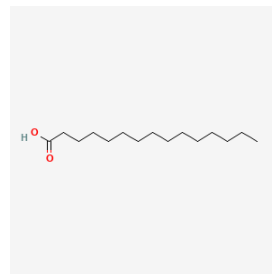
RT: Retention Time, MWT: Molecular Weight.



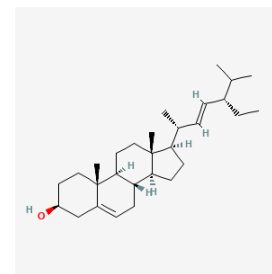
Betulin



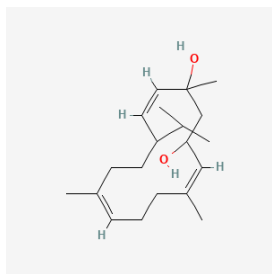
Dodecanoic acid



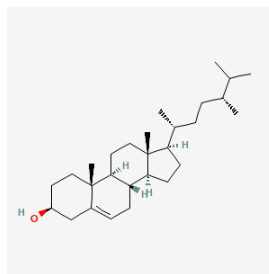
Pentadecanoic acid



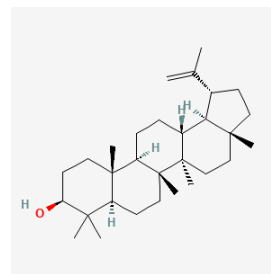
Stigmasterol



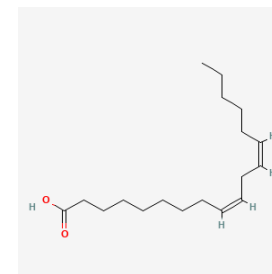
4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)



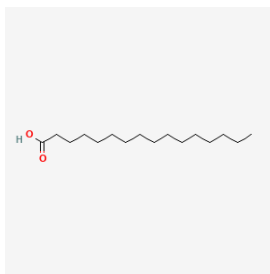
Campesterol



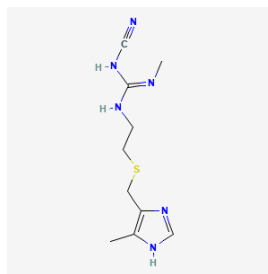
Lupeol



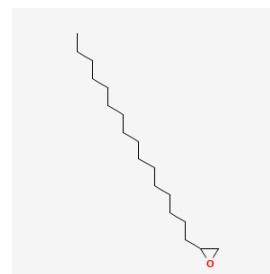
9,12-Octadecadienoic acid (Z,Z)



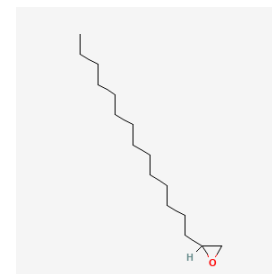
n-Hexadecanoic acid



Cimetidine



Oxirane, hexadecyl



Oxirane, tetradecyl

**Figure 22 Chemical Structures of the Compounds with Antimicrobial Activity Available in *C. procera* (Structures Are Generated by PubChem Database)**

The GC-MS analysis conducted on the DE extract of *P. granatum* revealed a diverse array of phytochemical constituents, as depicted in Figure 23. The identified chemical compounds have been meticulously cataloged in Table 12. Among these compounds, a range of natural entities was observed, encompassing quinones, terpenes, phenolic compounds, sterols, catecholamines, triterpenoids, epoxides, and fatty acids.

Noteworthy findings include the presence of catecholamines, exemplified by, (β)-, 3TMS derivative (RT: 22.39 min), as well as quinones such as 2,5-di-tert-Butyl-1,4-benzoquinone (RT: 11.24 min). Additionally, a phenolic compound, namely phenol, 2,2'-methylenebis[6-(1,1-dimethyl ethyl)-4-methyl], was identified at RT of 15.19 min.

Despite the diversity in chemical classes, a significant portion of the compounds was categorized as fatty acids. This category includes compounds like hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, and 9,12-octadecadienoic acid (Z, Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester, identified at RTs of 15.5 and 16.39 min, respectively. Figure 24 illustrates the chemical structure of the antimicrobial activity compounds present in the DE extract of *P. granatum*.

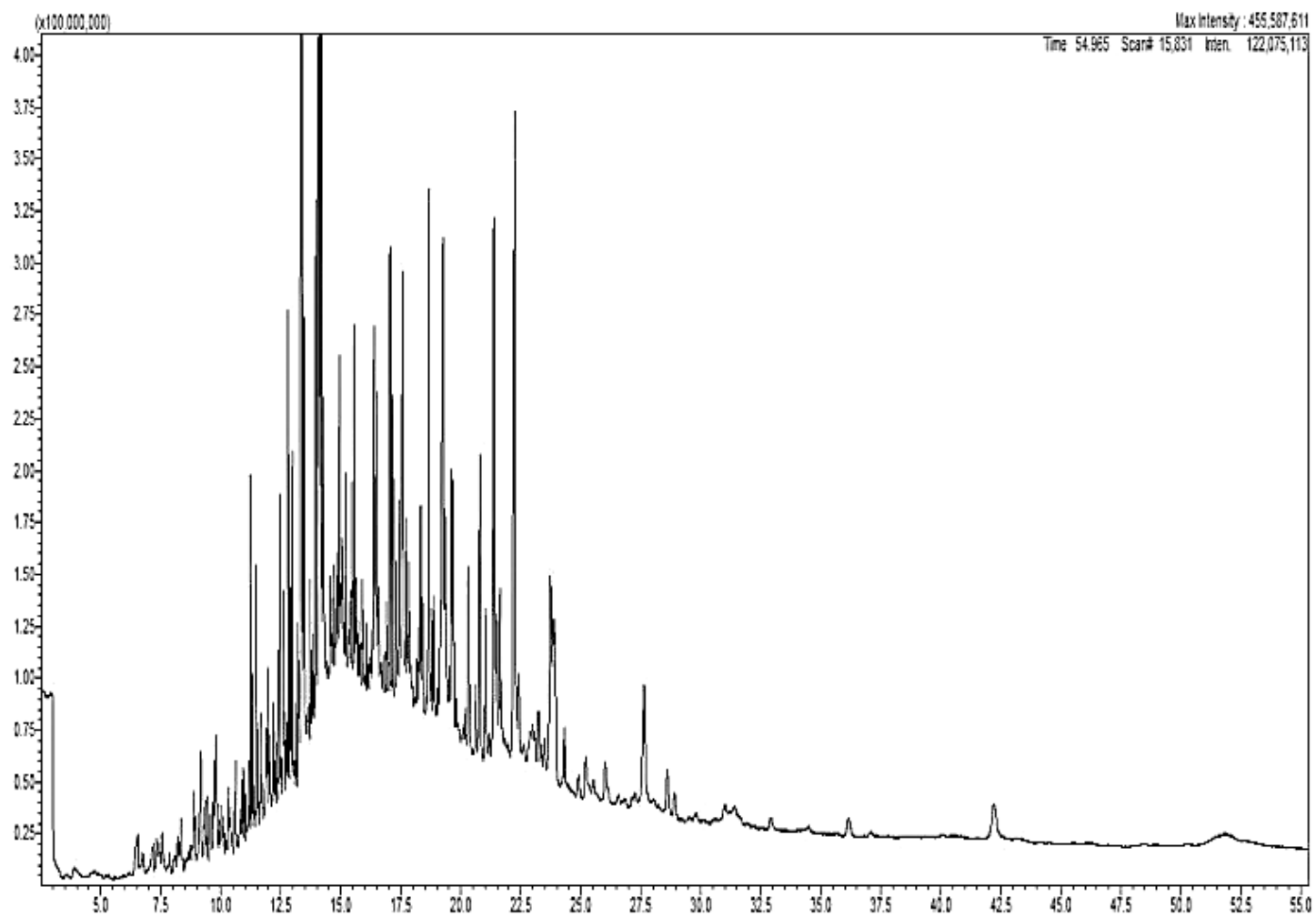
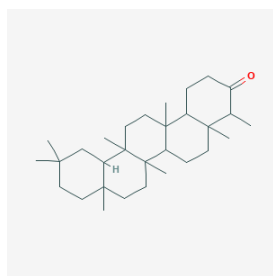


Figure 23 GC-MS Chromatograms of DE fraction of *P. granatum*

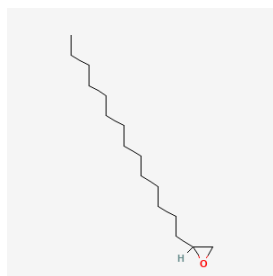
**Table 11 Phytochemical Compounds identified in GC-MS of DE Fraction of *P. granatum* and their Antimicrobial Activity**

| Chemical Composition of <i>P. granatum</i> . |          |                                                                          |                    |     |                                                                 |                        |                           |
|----------------------------------------------|----------|--------------------------------------------------------------------------|--------------------|-----|-----------------------------------------------------------------|------------------------|---------------------------|
| No.                                          | RT (min) | Compound Name                                                            | Compound Class     | MWT | Formula                                                         | Antimicrobial Activity | Ref.                      |
| 1                                            | 11.24    | 2,5-di-tert-Butyl-1,4-benzoquinone                                       | Quinone            | 220 | C <sub>14</sub> H <sub>20</sub> O <sub>2</sub>                  |                        |                           |
| 2                                            | 12.8     | Oxirane, tetradecyl                                                      | Epoxide            | 240 | C <sub>16</sub> H <sub>32</sub> O                               | ✓                      | Srinivasan, et al., 2022  |
| 3                                            | 13.33    | n-Hexadecanoic acid                                                      | Fatty Acids        | 256 | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>                  | ✓                      | Siswadi and Saragih, 2021 |
| 4                                            | 13.44    | Pentadecanoic acid                                                       | Fatty Acids        | 242 | C <sub>15</sub> H <sub>30</sub> O <sub>2</sub>                  | ✓                      | Jogaiah, 2020             |
| 5                                            | 14.09    | dl-Isopulegol/ -                                                         | Terpene            | 154 | C <sub>10</sub> H <sub>18</sub> O                               | ✓                      | Montenegro, et al., 2020  |
| 6                                            | 14.15    | Tricosanoic acid                                                         | Fatty Acids        | 354 | C <sub>23</sub> H <sub>46</sub> O <sub>2</sub>                  |                        |                           |
| 7                                            | 14.9     | Eicosanoic acid                                                          | Fatty Acids        | 312 | C <sub>20</sub> H <sub>40</sub> O <sub>2</sub>                  |                        |                           |
| 8                                            | 15.19    | Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl                 | Phenolic Compounds | 340 | C <sub>23</sub> H <sub>32</sub> O <sub>2</sub>                  | ✓                      | Salim, 2018               |
| 9                                            | 15.5     | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester                | Fatty Acids        | 330 | C <sub>19</sub> H <sub>38</sub> O <sub>4</sub>                  |                        |                           |
| 10                                           | 16.39    | 9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester | Fatty Acids        | 354 | C <sub>21</sub> H <sub>38</sub> O <sub>4</sub>                  |                        |                           |
| 11                                           | 16.51    | Octadecanoic acid, 2,3-dihydroxypropyl ester                             | Fatty Acids        | 358 | C <sub>21</sub> H <sub>42</sub> O <sub>4</sub>                  | ✓                      | Siswadi and Saragih, 2021 |
| 12                                           | 22.19    | γ -Sitosterol                                                            | Sterols            | 414 | C <sub>29</sub> H <sub>50</sub> O                               |                        |                           |
| 13                                           | 22.39    | Epinephrine, (β)-, 3TMS derivative                                       | Catecholamines     | 399 | C <sub>18</sub> H <sub>37</sub> NO <sub>3</sub> Si <sub>3</sub> |                        |                           |
| 14                                           | 22.45    | Ergosta-5,24(28)-dien-3-ol, (3- β)                                       | Sterols            | 398 | C <sub>28</sub> H <sub>46</sub> O                               |                        |                           |
| 15                                           | 26.33    | Friedelan-3-one                                                          | Triterpenoid       | 426 | C <sub>30</sub> H <sub>50</sub> O                               | ✓                      | Odeh, et al., 2016        |

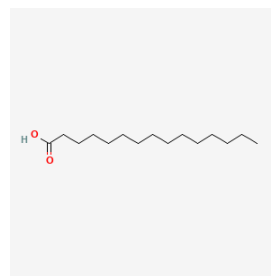
RT: Retention Time, MWT: Molecular Weight.



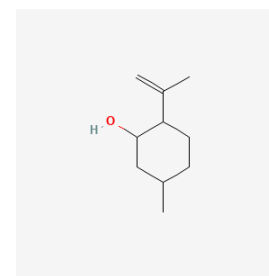
Friedelan-3-one



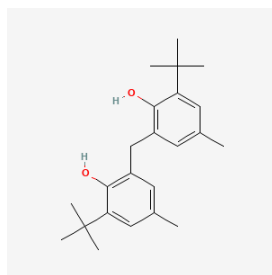
Oxirane, tetradecyl



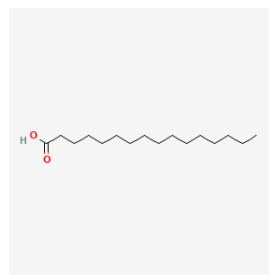
Pentadecanoic acid



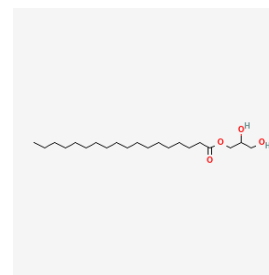
dl-Isopulegol/ -



Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl



n-Hexadecanoic acid



Octadecanoic acid, 2,3-dihydroxypropyl ester

**Figure 24 Chemical Structures of the Compounds with Antimicrobial Activity Available in *P. granatum* (Structures are Generated by PubChem Database)**

The GC-MS analysis conducted on the DE extract of *A. etbaica* revealed a diverse array of phytochemical constituents, as illustrated in Figure 25. The identified chemical compounds have been meticulously cataloged in Table 13. Predominantly, the compounds were characterized by the presence of fatty acids and sterols.

Among the bioactive compounds, fatty acids played a prominent role. Dodecanoic acid was identified at RT of 11.47 min, followed by pentadecanoic acid and n-hexadecanoic acid, which were identified at RT of 12.45 and 13.37 min, respectively. Sterols, including campesterol, stigmasterol,  $\gamma$ -sitosterol, and cholestanol, were also detected at RT of 21, 21.33, 22.3, and 22.46 min, respectively.

In addition to these, a variety of other natural compounds were identified, encompassing phenols, pyrethroids, epoxides, H<sub>2</sub>-receptor antagonists, terpene alcohols, indole derivatives, organic acid esters, fatty acid esters, fatty alcohols, triterpenoids, and monoterpenes. Figure 26 provides a visual representation of the chemical structure of the antimicrobial activity compounds present in the DE extract of *A. etbaica*.

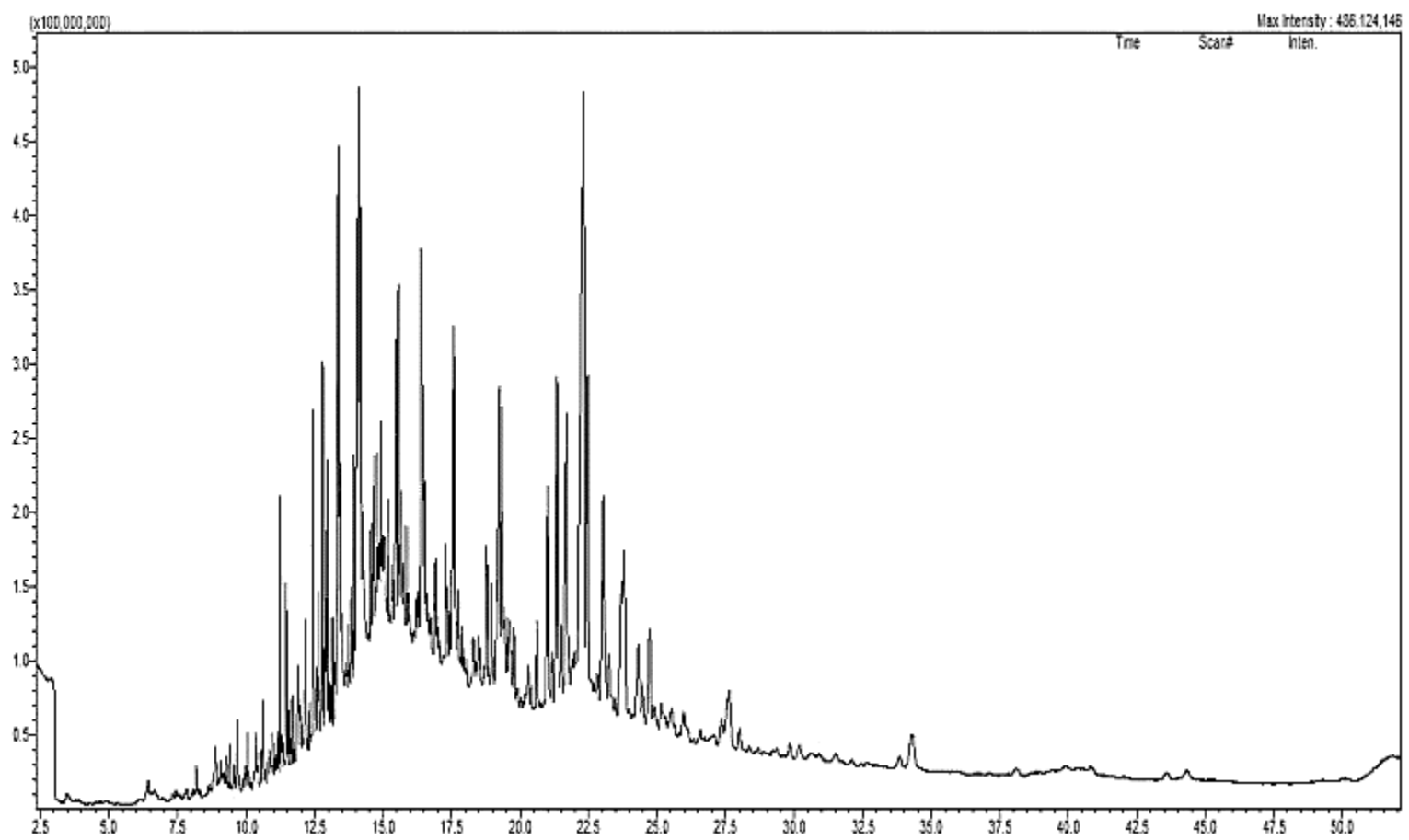
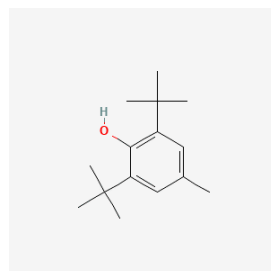


Figure 25 GC-MS Chromatograms of DE fraction of *A. etbaica*

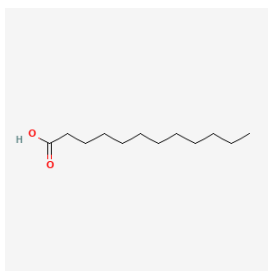
**Table 12 Phytochemical Compounds identified in GC-MS of DE Fraction of *A. etbaica* and their Antimicrobial Activity**

| Chemical Composition of <i>A. etbaica</i> |          |                                                           |                                     |     |                                                               |                        |                           |
|-------------------------------------------|----------|-----------------------------------------------------------|-------------------------------------|-----|---------------------------------------------------------------|------------------------|---------------------------|
| No.                                       | RT (min) | Compound Name                                             | Compound Class                      | MWT | Formula                                                       | Antimicrobial Activity | Ref.                      |
| 1                                         | 11.227   | Butylated Hydroxytoluene                                  | Phenols                             | 220 | C <sub>15</sub> H <sub>24</sub> O                             | ✓                      | Ayaz, et al., 1980        |
| 2                                         | 11.47    | Dodecanoic acid /Lauric Acid                              | Fatty Acid                          | 200 | C <sub>12</sub> H <sub>24</sub> O <sub>2</sub>                | ✓                      | Matsue, et al., 2019      |
| 3                                         | 12.17    | Bioallethrin                                              | Pyrethroids                         | 302 | C <sub>19</sub> H <sub>26</sub> O <sub>3</sub>                |                        |                           |
| 4                                         | 12.45    | Pentadecanoic acid                                        | Fatty Acid                          | 242 | C <sub>15</sub> H <sub>30</sub> O <sub>2</sub>                | ✓                      | Jogaiah, 2020             |
| 5                                         | 12.79    | Oxirane, tetradecyl                                       | Epoxide                             | 240 | C <sub>16</sub> H <sub>32</sub> O                             | ✓                      | Srinivasan, et al., 2022  |
| 6                                         | 12.98    | Oxirane, hexadecyl                                        | Epoxide                             | 268 | C <sub>18</sub> H <sub>36</sub> O                             | ✓                      | Srinivasan, et al., 2022  |
| 7                                         | 13.37    | Cimetidine                                                | H <sub>2</sub> -Receptor Antagonist | 252 | C <sub>10</sub> H <sub>16</sub> N <sub>6</sub> S              | ✓                      | Masoud et al., 2014       |
| 8                                         | 13.37    | n-Hexadecanoic acid                                       | Fatty Acid                          | 256 | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>                | ✓                      | Siswadi and Saragih, 2021 |
| 9                                         | 14.07    | Isopulegol                                                | Terpene Alcohol                     | 154 | C <sub>10</sub> H <sub>18</sub> O                             | ✓                      | Garcia, et al., 2008      |
| 10                                        | 14.19    | 2-Methyl-7-phenylindole                                   | Indole Derivative                   | 207 | C <sub>15</sub> H <sub>13</sub> N                             | ✓                      | Husein, et al., 2019      |
| 11                                        | 15.46    | Heptafluorobutyric acid, undecyl ester                    | Organic Acid Ester                  | 368 | C <sub>15</sub> H <sub>23</sub> F <sub>7</sub> O <sub>2</sub> |                        |                           |
| 12                                        | 15.55    | Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester | Fatty Acid Ester                    | 330 | C <sub>19</sub> H <sub>38</sub> O <sub>4</sub>                |                        |                           |
| 13                                        | 16.41    | 9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester       | Fatty Acid Ester                    | 356 | C <sub>21</sub> H <sub>40</sub> O <sub>4</sub>                |                        |                           |
| 14                                        | 17.55    | n-Pentadecanol                                            | Fatty Alcohol                       | 228 | C <sub>15</sub> H <sub>32</sub> O                             | ✓                      | Jamal, et al., 2022       |
| 15                                        | 21       | Campesterol                                               | Sterol                              | 400 | C <sub>28</sub> H <sub>48</sub> O                             | ✓                      | Uttu, et al., 2023        |
| 16                                        | 21.33    | Stigmasterol                                              | Sterol                              | 412 | C <sub>29</sub> H <sub>48</sub> O                             | ✓                      | Arora and Meena, 2018     |
| 17                                        | 22.3     | γ -Sitosterol                                             | Sterol                              | 414 | C <sub>29</sub> H <sub>50</sub> O                             |                        |                           |
| 18                                        | 22.46    | Cholestanol                                               | Sterol                              | 388 | C <sub>27</sub> H <sub>48</sub> O                             |                        |                           |
| 19                                        | 23.02    | Lup-20(29)-en-3-ol, acetate, (3- β)                       | Triterpenoid                        | 468 | C <sub>32</sub> H <sub>52</sub> O <sub>2</sub>                |                        |                           |
| 20                                        | 23.75    | β -Amyrone                                                | Monoterpene                         | 424 | C <sub>30</sub> H <sub>48</sub> O                             |                        |                           |

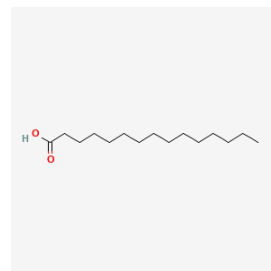
RT: Retention Time, MWT: Molecular Weight.



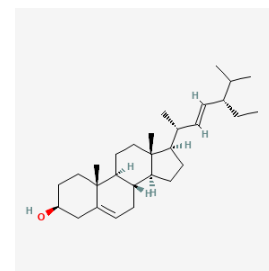
Butylated Hydroxytoluene



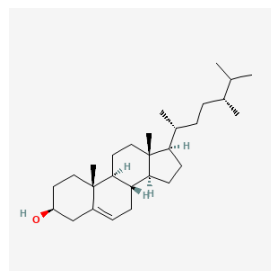
Dodecanoic acid



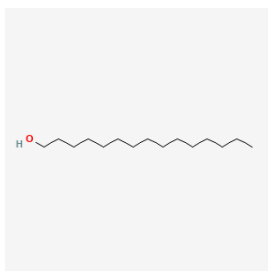
Pentadecanoic acid



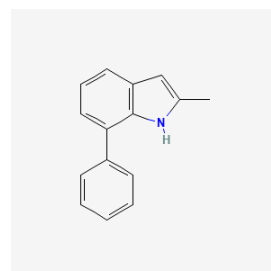
Stigmasterol



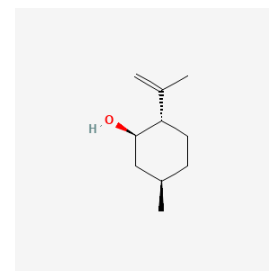
Campesterol



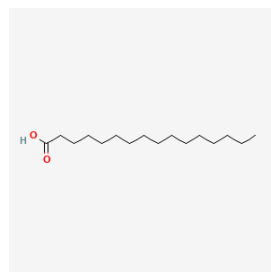
n-Pentadecanol



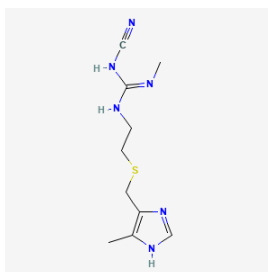
2-Methyl-7-phenylindole



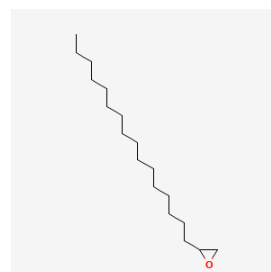
Isopulegol



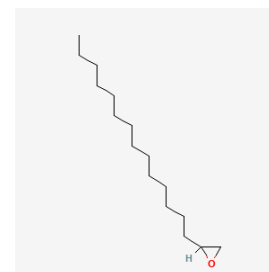
n-Hexadecanoic acid



Cimetidine



Oxirane, hexadecyl



Oxirane, tetradecyl

Figure 26 Chemical Structures of the Compounds with Antimicrobial Activity Available in *A. etbaica* (Structures are Generated by PubChem Database)

The GC-MS analysis conducted on the DE extract of *Rhamnus sp.* revealed a diverse array of phytochemical constituents, as depicted in Figure 27. The identified chemical compounds have been meticulously cataloged in Table 14. Notably, a significant proportion of these compounds are characterized as fatty acids. Among the bioactive compounds, several fatty acids were particularly prominent. These include n-hexadecanoic acid, 9-octadecynoic acid methyl ester, octadecanoic acid, octadecanoic acid ethyl ester, and 1-hexacosanol, identified at RT of 13.33, 14.05, 14.16, 14.25, and 19.27 min, respectively. In addition to fatty acids, the analysis revealed the presence of other active natural compounds, such as epoxides, carboxylic acids, aldehydes, tocopherols, miscellaneous compounds, and sterols. A comprehensive summary of these components, including their RT, molecular formulas, compound classes, and MWT, can be found in Table 14. Figure 28 provides a visual representation of the chemical structure of the antimicrobial activity compounds present in the DE extract of *Rhamnus sp.*

In conclusion, the study employed rigorous extraction and analytical methods to characterize the phytochemical composition of plant extracts. The results unveiled a rich diversity of bioactive compounds, prominently featuring fatty acids, sterols, triterpenes, and phenolic compounds. These findings underscore the significant role of these compounds in the traditional uses and therapeutic benefits associated with these plants.

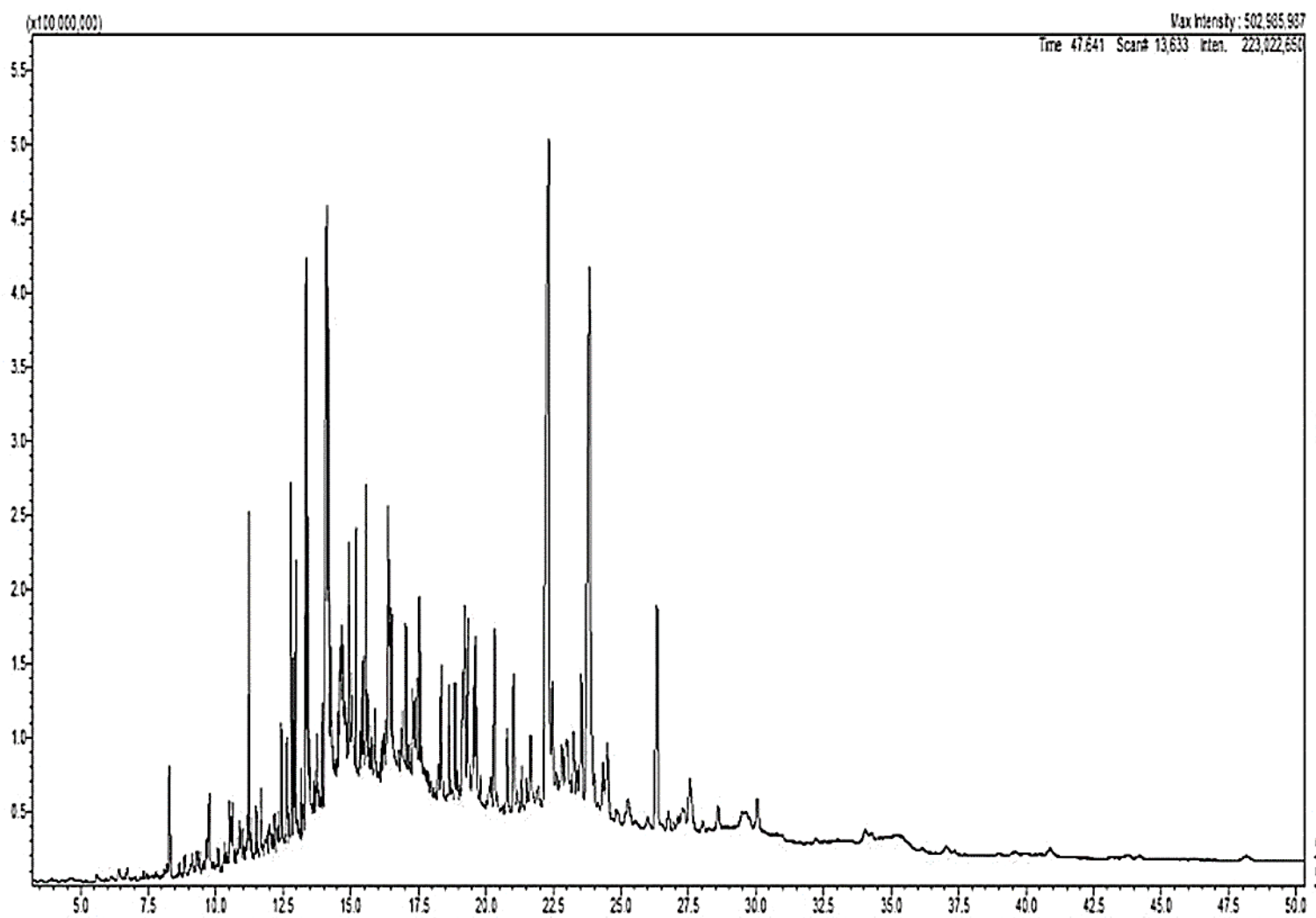
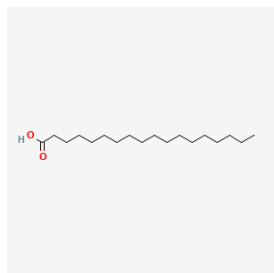


Figure 27 GC-MS Chromatograms of DE fraction of *Rhamnus sp.*

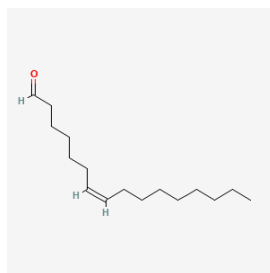
**Table 13 Phytochemical Compounds identified in GC-MS of DE Fraction of *Rhamnus sp.* and their Antimicrobial Activity**

| <b>Chemical Composition of <i>Rhamnus sp.</i></b> |                 |                                                                          |                         |            |                                                |                               |                             |
|---------------------------------------------------|-----------------|--------------------------------------------------------------------------|-------------------------|------------|------------------------------------------------|-------------------------------|-----------------------------|
| <b>No.</b>                                        | <b>RT (min)</b> | <b>Compound Name</b>                                                     | <b>Compound Class</b>   | <b>MWT</b> | <b>Formula</b>                                 | <b>Antimicrobial Activity</b> | <b>Ref.</b>                 |
| 1                                                 | 12.8            | Oxirane, tetradecyl                                                      | Epoxide                 | 240        | C <sub>16</sub> H <sub>32</sub> O              | ✓                             | Srinivasan, et al., 2022    |
| 2                                                 | 13.33           | n-Hexadecanoic acid                                                      | Fatty Acids             | 256        | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | ✓                             | Siswadi and Saragih, 2021   |
| 3                                                 | 14.05           | 9-Octadecynoic acid, methyl ester                                        | Fatty Acids             | 294        | C <sub>19</sub> H <sub>34</sub> O <sub>2</sub> |                               |                             |
| 4                                                 | 14.09           | Cyclododecanecarboxylic acid                                             | Carboxylic Acids        | 212        | C <sub>13</sub> H <sub>24</sub> O <sub>2</sub> |                               |                             |
| 5                                                 | 14.16           | Octadecanoic acid                                                        | Fatty Acids             | 284        | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub> | ✓                             | Srinivasan, et al., 2022    |
| 6                                                 | 14.25           | Octadecanoic acid, ethyl ester                                           | Fatty Acids             | 312        | C <sub>20</sub> H <sub>40</sub> O <sub>2</sub> |                               |                             |
| 7                                                 | 16.38           | 7-Hexadecenal, (Z)                                                       | Aldehydes               | 238        | C <sub>16</sub> H <sub>30</sub> O              | ✓                             | Devakumar, et al., 2017     |
| 8                                                 | 17.05           | 4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl) | Miscellaneous           | 306        | C <sub>20</sub> H <sub>34</sub> O <sub>2</sub> | ✓                             | Chenniappan et al., 2013    |
| 9                                                 | 17.56           | Oxirane, hexadecyl                                                       | Epoxide                 | 268        | C <sub>18</sub> H <sub>36</sub> O              | ✓                             | Srinivasan, et al., 2022    |
| 10                                                | 18.66           | Tridecanal                                                               | Aldehydes               | 198        | C <sub>13</sub> H <sub>26</sub> O              |                               |                             |
| 11                                                | 19.27           | 1-Hexacosanol                                                            | Fatty Acids             | 382        | C <sub>26</sub> H <sub>54</sub> O              | ✓                             | Oscar, et al., 2018         |
| 12                                                | 19.61           | dl- $\alpha$ -Tocopherol                                                 | Tocopherols (Vitamin E) | 430        | C <sub>29</sub> H <sub>50</sub> O <sub>2</sub> | ✓                             | Divyadharsini, et al., 2023 |
| 13                                                | 21.34           | Stigmasterol                                                             | Sterols                 | 412        | C <sub>29</sub> H <sub>48</sub> O              | ✓                             | Arora and Meena, 2018       |
| 14                                                | 22.17           | $\gamma$ -Sitosterol                                                     | Sterols                 | 414        | C <sub>29</sub> H <sub>50</sub> O              |                               |                             |

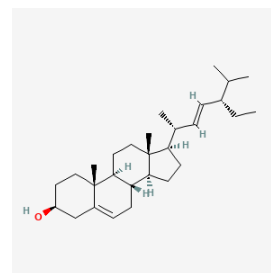
RT: Retention Time, MWT: Molecular Weight.



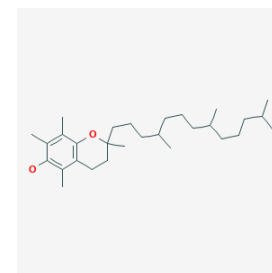
Octadecanoic acid



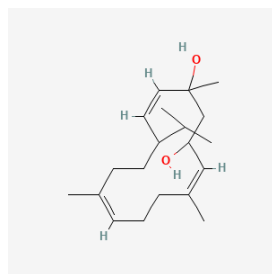
7-Hexadecenal, (Z)



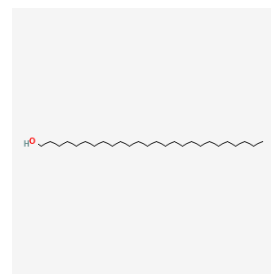
Stigmasterol



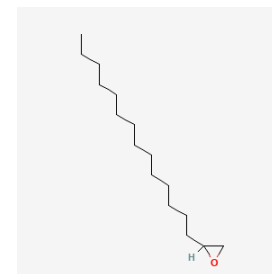
dl- α -Tocopherol



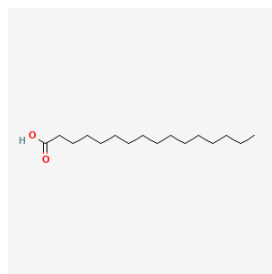
4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)



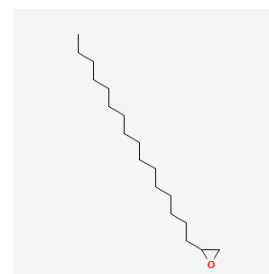
1-Hexacosanol



Oxirane, tetradecyl



n-Hexadecanoic acid



Oxirane, hexadecyl

**Figure 28 Chemical Structures of the Compounds with Antimicrobial Activity Available in *Rhamnus* sp. (Structures are Generated by PubChem Database)**

## CHAPTER 5

### DISCUSSION

The escalating prevalence of antimicrobial resistance to existing agents underscores the imperative for the development of novel drugs. Exploring the antimicrobial potential of plant extracts has emerged as a promising avenue in this endeavor. Various studies have furnished evidence that certain medicinal plants harbor the potential to yield new antibacterial agents, even effective against antibiotic-resistant strains (Kone et al., 2004). The plants scrutinized in this study are reputed for their medicinal properties. This research assessed their efficacy in combating selected model bacterial strains to ascertain their effectiveness. Notably, antimicrobial activity was deemed significant when the zone of inhibition exceeded 5 mm. Additionally, a qualitative analysis was conducted to ascertain the presence of phytochemicals, as they are purportedly responsible for some of the antibacterial properties exhibited by extracts from medicinally valuable plants.

The initial step in investigating medicinal plants involves preparing plant samples, sourced from either fresh or dried plant material. Dried samples are favored due to the fragility of fresh ones. This study utilized pulverized dried parts from *C. procera*, *P. granatum*, *A. etbaica*, and *Rhamnus sp.* for evaluation.

Extraction, the second crucial phase, significantly impacts the quantity and quality of crude extracts. The choice of solvent is vital, influencing extraction efficiency (Altıok et al., 2008). Solvents should exhibit low toxicity, facilitate easy evaporation, promote swift

absorption, act as a preservative, and not induce complexation or dissociation. The solvent selection depends on the intended application of the extract (Eloff, 1998).

Various solvents, including water, methanol, ethanol, acetone, chloroform, hexane, and petroleum ether, are available for extracting plant materials. Water and methanol are commonly used, with water being a universal solvent adept at extracting compounds from medicinal plants. However, organic solvent extracts generally offer a more consistent assessment of antimicrobial activity than water extracts. Active antimicrobial components are often identified in these organic solvent extracts (which are not water-soluble) (Ncube et al., 2008), which are not water-soluble. Water-soluble compounds like polysaccharides and polypeptides primarily function as pathogen adsorption inhibitors rather than antimicrobial agents. Water-soluble flavonoids, such as anthocyanins, lack significant antimicrobial relevance, while water-soluble phenolics are valued primarily as antioxidant compounds (Handa, 2008). In this study, ethanol and chloroform solvents were used in ascending order of polarity for compound extraction from plant samples, revealing varying percentage yields for the same plant with different solvents in increasing order of polarity.

In the present investigation, the highest yield was obtained from the EthOH fraction of *P. granatum* (38.2%), followed by the EthOH fraction of *Rhamnus sp.* (16.7%). Conversely, the lowest yield was from the CHCl<sub>3</sub> fraction of *P. granatum* (1.2%), succeeded by the CHCl<sub>3</sub> fraction of *A. etbaica* (2%). The range of % yield from CHCl<sub>3</sub> solvent is 1.2-3.8, whereas for EthOH, the yield ranges from 4.6-38.2.

A notable distinction in the % yield of extracts from different solvents was observed, even for the same sample. This variance in yield may be attributed to plants containing a higher

proportion of polar compounds compared to non-polar ones. Moreover, several factors influence the % yield from plants, including extraction method, plant material part used, fineness of the powder, degree of dryness, and the nature of the solvent used for extraction. Older and dried plant material tends to yield less than fresh ones. Likewise, incomplete extraction leads to lower yields.

Each fraction underwent testing for antibacterial activity against the selected four bacterial species using the agar WDA. The plant crude extracts were dissolved in 100% DMSO and assessed. The results indicated that extracts from most plants demonstrated antibacterial activity. Furthermore, the extracts exhibited both increased and decreased antibacterial activity when different solvents were utilized.

In our study, polar solvent exhibited stronger antibacterial activities than the less polar solvents, for both *P. granatum* and *Rhamnus sp.* However, the opposite trend was observed for *C. procera* and *A. etbaica*. Several published studies align with the findings of this study. For instance, Arumugam et al. (2011) and Romika et al. (2015) investigated a range of solvents (including acetone, methanol, EthOH, and CHCl<sub>3</sub>) for their ability to solubilize antibacterial compounds from *Centella asiatica L.* These studies revealed that methanol and EthOH extracts exhibited activity against both Gram-positive and Gram-negative bacteria. While chloroform extract displayed very weak activity against all tested bacteria.

### **5.1 *C. procera***

Several activities have been attributed to *C. procera*, including antibacterial (Geraldo, Y. et al., 2021), antifungal (Han, H. L. et al., 2022), and antitumoral (Vahidi, R. et al., 2021), indicating the pronounced biological potential of this genus. The results of this study

revealed that the hydroethanolic extract of *C. procera* exhibited notable antibacterial activity against most bacterial strains, including *S. aureus*, *E. faecalis*, and *P. aeruginosa*, with inhibition zones ranging between 11 and 14 mm. However, no antibacterial activity was observed in ethanol extracts against *E. coli*. Previous studies on the antipathogenic activity align with this finding. For instance, a study on the methanolic extract of *C. procera* leaves demonstrated its potential against *S. aureus* and *Salmonella Typhimurium* (Kumar, A. et al., 2013). Similarly, the findings of Saddiq, A. et al. (2022) were consistent with this study. The plant extract exhibited efficiency in destroying or inhibiting the growth of Gram-positive bacteria like *S. aureus* and Gram-negative bacteria like *E. coli*, although our study did not inhibit the growth of *E. coli*.

From another perspective, data indicates that  $\text{CHCl}_3$  extracts of *C. procera* displayed considerable antibacterial activity against all the tested bacteria, surpassing the activity of ethanolic extracts. However, they differed significantly in their action according to the microorganism tested, with inhibition zones ranging between 11 and 19 mm against the tested bacteria. These results corroborate with studies by Kareem et al. 2008; Ramaprabha, M. and Vasantha, K., 2012; Narayani, M. et al. 2012; and Saadabi. A. et al. 2012, which demonstrated that  $\text{CHCl}_3$  extracts possess antibacterial and antifungal activities. Saadabi, A., et al. (2012) noted that the  $\text{CHCl}_3$  extract of *C. procera* leaf exhibited higher antibacterial activity compared to the aqueous counterpart. They further reported that the  $\text{CHCl}_3$  extract of *C. procera* leaf displayed particularly potent antifungal activity against *Aspergillus flavus* and *Candida albicans*. Both the latex and  $\text{CHCl}_3$  extract of *C. procera* leaf exhibited antibacterial activity against some clinical bacterial isolates using both paper disc and open well diffusion methods (Kareem, S. O., et al., 2008). It is worth noting that

the current findings contrast with the earlier findings of Saddiq, A. et al. (2022), where the CHCl<sub>3</sub> fraction exhibited the least antibacterial activity against *S. aureus*, *E. coli*, and *P. aeruginosa*.

The MIC of the plant extracts against the tested bacterial strains was determined. *C. procera* extracts obtained from all the solvents studied demonstrated antibacterial activity against the test strains at concentrations up to 50 mg/mL. The MIC of ethanol extracts of *C. procera* against *S. aureus* and *E. coli* was 50 mg/mL, while the CHCl<sub>3</sub> extracts showed stronger inhibition of the growth of *S. aureus* and *E. coli* at the lower concentration tested (25 mg/mL). The MIC of the EthOH extracts of *C. procera* against *P. aeruginosa* and *E. faecalis* was 25 mg/mL. The MIC of CHCl<sub>3</sub> extracts of *C. procera* against *P. aeruginosa*, *E. faecalis*, and *S. aureus* appeared to be 25 mg/mL or less.

The MICs of solvent extracts against the test strains in this study contrast with other reports (Kawo, A., et al., 2009; Arekemase, M. O., 2011). Lower MIC values (0.5-1.0 mg/mL) were reported by Kawo, A., et al. (2009) for EthOH extracts of *C. procera* leaf against *E. coli* and *S. aureus*. This slight discrepancy may be attributed to possible differences in the characteristics of bacterial strains used, varying concentrations of plant phytochemicals, or differences in the environment of the plants. These same factors may explain the lower MIC values reported by Nenaah, G. (2013) for plant extracts against all tested bacteria using CHCl<sub>3</sub>. However, the current results do not align with the study conducted by Adamu, L. et al. (2013), where the CHCl<sub>3</sub> extract showed lower activity against *E. coli* and *S. aureus* at 50 and 100 mg/mL, respectively, whereas our study showed similar inhibition at much lower concentrations of 3.125 and 25 mg/mL, respectively. However, Adamu observed higher EthOH activity against the same strains compared to our current study. In

a similar vein, the MBC in this study demonstrated the highest activity of CHCl<sub>3</sub>, especially against *E. coli* and *E. faecalis* at 50 mg/mL, while ethanolic fractions showed higher concentrations between 100 and 200 mg/mL against all strains. Overall, these results revealed that Gram-positive bacteria were more susceptible to the natural products tested than Gram-negative ones. According to Yao, J. D., & Moellering Jr, R. C. (2011), these differences may be attributed to the fact that the cell wall in Gram-positive bacteria consists of a single layer, whereas the Gram-negative cell wall is a multi-layered and complex structure.

## **5.2 *P. granatum***

Results of the current study suggest a class effect antibacterial activity for different bacterial strains and indicate the superiority of the antibacterial activity of *P. granatum* extracts compared to standard antibiotics. The antibacterial activity of ethanolic extracts of *P. granatum* was tested on four bacterial model strains. The antibacterial potency was initially determined by the agar WDA followed by quantitative evaluation of antibacterial activity by the MIC method. Ethanolic extracts showed a higher antibacterial effect than CHCl<sub>3</sub> extracts. CHCl<sub>3</sub> extracts had no activity against most tested bacteria even at high concentrations using the WDA. Conversely, ethanolic extract demonstrated significant inhibition against all tested bacteria even at lower concentrations.

The ethanolic antibacterial activity was recorded, which inhibited all four strains in the following order: *E. faecalis* > *P. aeruginosa* > *E. coli* > *S. aureus* with inhibition zones of 33, 28.5, 26.5, and 22.5 mm, respectively. These results align with Dahham, S., et al. (2010) who reported that the ethanolic extracts of pomegranate demonstrated the highest

antibacterial activity against different bacterial isolates in the order of *S. aureus* > *E. coli* > *P. aeruginosa* > *Klebsiella pneumoniae*. Similar findings were reported by Unnisa, N., et al. (2012) in their study on the activity of ethanolic extracts against different pathogenic bacterial isolates. The antibacterial effects of pomegranate have been previously studied, with reports of the bark, leaves, flowers, and fruits of pomegranate being widely used as phototherapeutic agents in Brazil (Mathabe, M. C., et al., 2006). Ahmad, I., and Beg, A. Z. (2001) also support the current study by reporting that alcohol extracts of pomegranate fruits showed antibacterial activity when tested against *S. aureus*, *E. coli*, and *Shigella dysenteriae*. Furthermore, Prashanth, D., et al. (2001) reported that methanolic extracts of *P. granatum* fruit were active against all bacteria tested in their study. These results aligned with the results obtained in the present study, where antibacterial activity was observed for all four bacterial cultures tested. Duman, A., et al. (2009) also reported the *in vitro* antibacterial activity of extracts obtained from six pomegranate cultivars against bacteria including *P. aeruginosa*, *S. aureus*, *E. coli*, and *E. faecalis*, with inhibition zones ranging from 13-26 mm.

On another hand, the smallest inhibition zone at high concentration was seen for *S. aureus* with a diameter of 14 mm related to the CHCl<sub>3</sub> fraction, and its activity was absent against all other bacteria. The results showed that *S. aureus* (Gram-positive bacteria) could be more sensitive based on the inhibition zones given by the CHCl<sub>3</sub> extract. In the same line, Hegde Chaitra, R., et. al., (2012) conducted a study where the CHCl<sub>3</sub> extracts exhibited maximum inhibition against *S. aureus* (10 mm), but its activity against *E. coli* was absent, and they also presented that the CHCl<sub>3</sub> extract was more active than polar solvents like methanol. This result agreed with this study from the perspective that the CHCl<sub>3</sub> showed activity

against *S. aureus* (14 mm), but it is in contrast with the polar fraction, ethanol, which showed much higher activity against all tested strains. However, the current study agrees with Mahboubi, A., et al. (2015) in their investigation where they showed that the activity of the CHCl<sub>3</sub> fraction was lower than EthOH, but the activity of CHCl<sub>3</sub> was higher than the current result, indicating that the CHCl<sub>3</sub> extracts were active against six different microorganisms including *E. coli* (12 mm) and *S. aureus* (19 mm).

The MIC concentration was significantly lower (indicating higher activity) in ethanolic extracts that inhibited *P. aeruginosa* and *E. faecalis* (3.125 and 6.25 mg/mL, respectively). According to bacterial susceptibility to all tested ethanolic extract, *P. aeruginosa* was the most sensitive species with an MIC of 3.125 mg/mL, and MBC of 25 mg/mL. These results are results obtained by Voravuthikunchai, S., and Kitpipit, L. (2005), where MBCs of ethanolic extract of pomegranate peels for *P. aeruginosa* were at very low concentrations. Regarding another Gram-negative bacteria, *E. coli* was less sensitive to the tested ethanolic extract, with MIC and MBC ranging from 12.5 and 25 mg/mL. In several studies, pomegranate peel extracts were prepared by using a variety of different solvents, leading to sometimes contradictory results that are very difficult to compare. In addition, methanolic peel extracts showed strong broad-spectrum activity against gram-positive and gram-negative bacteria, with the MIC ranging from 0.2 to 0.78 mg/mL (Fawole, O. A., et al., 2012), which agrees with the current study in which the polar solvent showed the highest activity. Also, the 80% methanolic peel extract was a potent inhibitor of *Listeria monocytogenes*, *S. aureus*, *E. coli*, and *Yersinia enterocolitica* (Fawole, O. A., et al., 2012). The result of the MIC test also indicated different levels of concentration depending on the

bacterial strain being tested. The polar fractions generally showed better antibacterial activity, which can be related to the Phyto-compounds content of the fractions.

MIC by broth dilution showed good results compared to the WDA, as there may be a problem with the diffusion of the biological component into the agar. The hydrocarbon components either remain on the surface of the medium or evaporate (Griffin, S. G., et al., 2000). That could be the reason for the better results obtained by the micro-dilution method. The difference in the antibacterial activity with the same source when extracted with different solvents has proven that not all phytochemicals responsible for antibacterial activity are soluble in a single solvent. Hence, solvents of different polarities should be employed as done in this study (polar: EthOH and nonpolar: CHCl<sub>3</sub>). Also, antimicrobial activity is based on pomegranate species and extraction efficiency as well as their active compound's location. This difference may be attributed to location or seasonal variations (Gil, M. I., et al., 2000).

### **5.3    *A. etbaica***

This study also screened the antibacterial activities of *A. etbaica* against different bacterial microorganisms. The results demonstrated potential antibacterial activities obtained from the whole plant against most tested bacterial strains. Variations in inhibition activity were observed based on the type of solvent and the tested microbes. The zones of inhibition against the tested bacterial strains ranged from 12 to 22 mm. Specifically, the ethanolic extract of *A. etbaica* showed no antibacterial activity against all strains except *E. faecalis* (12 mm). On the other hand, certain bacteria exhibited resistance to one type of extract, while other types of extract from the same plant showed more significant effects against

the same bacteria. For instance, the antibacterial activity obtained from CHCl<sub>3</sub> extracts demonstrated the highest activity against *E. coli* (22 mm), whereas the EthOH extract showed no activity. Similarly, the antibacterial activity obtained from CHCl<sub>3</sub> extracts showed significant activity against *S. aureus*, while the ethanolic extract showed no activity against the same pathogen. In contrast, the antibacterial activity obtained from ethanolic, and CHCl<sub>3</sub> extracts showed similar activity against *E. faecalis* and *P. aeruginosa*.

The observed variations in susceptibility between gram-positive and gram-negative bacteria to various extracts could be attributed to differences in bacterial cell components (Nikaido, H., & Vaara, M. 1985). Furthermore, the choice of solvent significantly impacts the extraction capacity of active constituents with strong antibacterial activity from *A. etbaica* against various pathogens. In this study, the polar (EthOH) and non-polar (CHCl<sub>3</sub>) solvents played a crucial role in isolating biologically active compounds with antibacterial effects, aligning with Mahmoud et al. (2016), who reported higher solubility for the dry CHCl<sub>3</sub> extract against *S. aureus* and *Klebsiella pneumoniae* compared to the polar methanol extract.

The obtained results indicate that every solvent has its antibacterial activities. For example, the extract obtained from CHCl<sub>3</sub> showed significant antibacterial effects against all tested bacteria except *P. aeruginosa*. This extract exhibited a potent antibacterial effect against *E. coli*, while the ethanolic extract, in general, was found to be ineffective. These differences in antibacterial activities contradict previous studies. For instance, Sulayli, A. I., et al. (2019) reported that the activity of fresh leaves of *A. etbaica* alcohol extract against *S. aureus* was higher than CHCl<sub>3</sub> extract, 13 mm versus 9.3 mm. Additionally, the leaf alcohol extract against *P. aeruginosa* recorded an inhibition zone with a diameter of 16.3

mm, while the CHCl<sub>3</sub> extract, recorded 17 mm. However, the same study agreed with the current study in that the *A. etbaica* CHCl<sub>3</sub> extract exhibited higher activity than ethanolic extract against *S. aureus* with a nearly identical inhibition zone as the current study. In another study, it was found that EthOH extracts from *A. tortilis* aerial parts had a potent antibacterial effect against *S. aureus*, *P. aeruginosa*, and *Candida albicans* (Alajmi, M. F., et al., 2017).

On another hand, the MIC and MBC concentrations recorded from ethanolic extracts are lower than those from CHCl<sub>3</sub> extracts, which is in the opposite direction of the WDA. Surprisingly, the most susceptible organism to both ethanolic and chloroformed extracts is *P. aeruginosa* at a concentration of 25 mg/mL, followed by *E. faecalis* at 25 mg/mL for CHCl<sub>3</sub> extract and 50 mg/mL for ethanolic extracts. This difference can be attributed to several factors. Firstly, the poor diffusion of the secondary products in the agar, especially those with high molecular weight and complex structure. Secondly, evaporation of the SM in the liquid media before diffusion, or the metabolites requiring a higher amount of solvent or a higher dose to exhibit their activity.

#### **5.4 *Rhamnus sp.***

In the WDA, all microbial species tested exhibited susceptibility to the ethanolic extracts of *Rhamnus sp.*, with zones of inhibition ranging from 13 to 21 mm. Similarly, the CHCl<sub>3</sub> extracts demonstrated susceptibility against all tested microbes except *P. aeruginosa*, with zones of inhibition ranging from 14 to 20 mm. Both extracts displayed commendable antibacterial activity against most microbial species examined. Generally, there were no significant differences in the antibacterial activity between the EthOH and CHCl<sub>3</sub> extracts

of *Rhamnus sp.* Given certain limitations of the diffusion assay, a broth dilution assay was also performed. All *Rhamnus sp.* extracts exhibited antibacterial activity against the tested microbial species, with MIC values equal to or lower than 50 mg/mL. Remarkably, the ethanolic extract was most effective against *E. faecalis* with MIC values of 3.125 mg/mL, while the other tested bacterial species were less sensitive. Intriguingly, the susceptibility to CHCl<sub>3</sub> extract was noted against *P. aeruginosa*, which exhibited resistance in the WDA.

In a Pakistani study (Ahmad, J. 2021), *Rhamnus sp.* (*R. virgata* bark) extracts, including CHCl<sub>3</sub>, were tested for antibacterial activity against *E. coli*, *S. aureus*, and *P. aeruginosa*. The CHCl<sub>3</sub> fraction showed optimal results against *E. coli* (2.9 mm), aligning with the present study. However, our CHCl<sub>3</sub> extract displayed no activity against *P. aeruginosa*, contrasting with Ahmad's findings of significant inhibition (2.1 mm). Additionally, our study revealed activity against *S. aureus*, which was not observed in the Pakistani study.

However, this study contradicts a study conducted by Carranza, M. et al. (2015), where the polar solvent, methanol, used to extract leaves of *Rhamnus sp.*, namely *R. californica*, showed no activity against *P. aeruginosa* even at high doses. The present study, in contrast, demonstrates significant results against it. On another hand, the activity of leaves extract of *R. californica* showed aligned results in activity against *E. coli* and *S. aureus*. Both studies showed no activity at 25 mg, but in the current study, when the concentration was elevated to 30 mg, significant results were observed against these microbes, measuring 14 mm and 13 mm, respectively.

Furthermore, in a study conducted by Zeouk, I., et al. (2021), the ethanolic extract of another species of *Rhamnus*, namely *R. alaternus*, demonstrated activity against the four

target strains of *S. aureus*, including a reference strain of *S. aureus* ATCC25923, but with lower concentrations compared to our study, especially in terms of MIC. Additionally, in the study done by Khuda, et al (2022), the crude extracts of *Rhamnus* sp., namely *R. purpurea* CHCl<sub>3</sub> fraction, revealed significant activity against different bacterial strains, including *P. aeruginosa*, *S. aureus*, and *E. coli*. This study concurs with the present work in that *S. aureus* showed higher inhibition in the CHCl<sub>3</sub> extract, but its activity against *P. aeruginosa* contrasts with this study, where no activity against it was observed. On the other hand, *E. coli* agreed with the finding that CHCl<sub>3</sub> extract showed higher inhibitory activity.

## 5.5 Phytochemical and Secondary Metabolites

The phytochemical analysis indicated that the plants under study harbor a diverse spectrum of bioactive compounds. Notably, some plants exhibited a significant number of metabolites, while others displayed a more limited range. For instance, *C. procera* demonstrated the highest number of phytochemicals, while *Rhamnus* sp. exhibited the lowest count. The presence of these SM in the plant kingdom is pivotal in determining their potential medicinal effects. Therefore, exploring traditional plant-based medicine serves as an initial step towards drug development. Ethnobotanical investigations represent a common method for selecting plants for further study.

All plant extracts were subjected to GC-MS analysis, revealing the presence of specific bioactive SM that are considered to be potential antibacterial agents. It has been noted in the literature that the antimicrobial activity can be attributed to the chemical constituents within the plant mixture. The effectiveness of a medicinal plant may not solely arise from

a single active constituent, but rather from the combined action of various components present in the plant extract (Essawi and Srouf, 2000). This can be attributed to synergistic or additive effects between these components. Moreover, an individual bioactive component can alter its properties in the presence of other compounds within an extract or mixture (Barnabas and Nagarajan, 1988).

The observed antibacterial activity in both microdilution and diffusion assays is likely attributable to the bioactive SM identified in the GC-MS analysis of these four plants. While different distinct SM were observed in each plant, a strong correlation exists between these compounds across the different analyzed plants. Notable compounds include oxiranes (specifically, oxirane, tetradecyl), consistently present in all plants, and oxirane, hexadecyl, found in all plants except those from *P. granatum*. Oxirane activity has been previously documented as an antimicrobial agent by Srinivasan, G. et al. (2022).

Fatty acids, notably n-hexadecanoic acid, primarily contribute to antimicrobial and antioxidant properties (Siswadi and Saragih, 2021; Tamokou et al., 2012). It also exhibits anti-inflammatory attributes (Sindhu and Manorama, 2013). Additionally, 9,12-octadecadienoic acid (Z, Z) and its derivative compounds, specifically 9,12-octadecadienoic acid (Z, Z)-,2-hydroxy-1-(hydroxymethyl) ethyl ester, were identified in *C. procera* and *P. granatum* plants, demonstrating antioxidant and antimicrobial activities (Tamokou et al., 2012).

Octadecanoic acid and its derivatives, octadecanoic acid, ethyl ester, and octadecanoic acid, 2,3-dihydroxypropyl ester, were respectively detected in *Rhamnus sp.* and *P.*

*granatum* extracts. Additionally, pentadecanoic acid was present in all plant extracts except those from *Rhamnus* sp., exhibiting antimicrobial characteristics (Jogaiah, 2020).

Sterols, such as campesterol, were identified in *C. procera* and *A. etbaica* extracts, aligning with their known antimicrobial attributes (Uttu et al., 2023). Furthermore, terpenes, including isopulegol and its derivative dl-isopulegol/-, were respectively identified in *A. etbaica* and *P. granatum* extracts, consistent with their documented antimicrobial properties (Garcia et al., 2008; Montenegro et al., 2020).

The GC-MS analysis of *C. procera* extract revealed triterpenoids, notably lupeol and betulin, which offer a range of health benefits, including antimicrobial, anti-protozoal, anticancer, hypoglycemic, cardioprotective, anti-inflammatory, skin protective, hepatoprotective, and nephroprotective effects (Saddiq et al., 2022). Triterpenes have demonstrated significant antimicrobial properties in prior studies (Hernández-Vázquez et al., 2012). Lupeol acetate, isolated from *Heliotropium marifolium*, exhibited potent activity against *Penicillium chrysogenum*, *E. coli*, and *K. pneumoniae* (Singh and Dubey, 2001). Lupeol acetate also demonstrated moderate antimicrobial activity against *S. aureus* (Awolola et al., 2014).

Phenolic compounds, including phenol,2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl] and friedelan-3-one, were exclusively identified in *P. granatum* extract. These compounds possess documented antimicrobial properties (Salim, 2018; Odeh et al., 2016). The presence of phenolic compounds in the extract suggests their possible involvement in the observed antibacterial activity.

Furthermore, various SM with antimicrobial properties were identified in these plants. For instance, H<sub>2</sub>-antagonists like cimetidine were detected in both *C. procera* and *A. etbaica* extracts (Masoud et al., 2014). Another notable compound, the indole derivative 2-methyl-7-phenylindole, was exclusively found in the *A. etbaica* extract (Husein et al., 2019). Additionally, fatty alcohol, specifically n-pentadecanol, was uniquely present in the *A. etbaica* extract (Jamal et al., 2022). Furthermore, a miscellaneous compound, 4,8,13-cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl), was identified in both *C. procera* and *Rhamnus sp.* extracts (Chenniappan et al., 2013). These diverse SM contribute to the plants' antimicrobial activity and provide a scientific basis for their traditional medicinal use.

Divergent environmental conditions and genetic variations among plant species may contribute to disparities observed in comparison to the existing literature for each solvent. Variations in the quality or composition of the same plants can arise due to these differing conditions, impacting cell wall permeability to SM and the overall chemical composition, resulting in distinct mechanisms of action. The observed bactericidal effect, attributed to bioactive molecules, likely involves multiple mechanisms such as pore formation, inhibition of DNA gyrase and nucleic acid synthesis, and alteration in the physicochemical properties of bacterial membranes (Gradišar et al., 2007). Additionally, these bioactive substances may bind to active sites of enzymes and receptors, influencing cellular flux activity.

In conclusion, *C. procera*, *P. granatum*, *A. etbaica*, and *Rhamnus sp.* have demonstrated the ability to produce SM with antibacterial properties. The diverse array of bioactive compounds underscores the rationale for their use in traditional medicine. This study

emphasizes the need for further research to explore various secondary metabolites with distinct biological activities.

## CHAPTER 6

### CONCLUSION AND RECOMMENDATION

The study delved into the antibacterial potential of four plant species: *Calotropis procera*, *Punica granatum*, *Acacia etbaica*, and *Rhamnus sp.* Each plant displayed distinct bioactive compounds, showcasing promising antibacterial capabilities. Notably, *Calotropis procera* and *Punica granatum* exhibited significant inhibitory effects against various bacterial strains, while *Acacia etbaica* and *Rhamnus sp.* demonstrated efficacy, particularly against specific bacteria. These findings emphasize the potential of these plants as valuable sources for developing antibacterial agents.

In summary, the research highlights the diverse antibacterial properties of the studied plant species, offering promising avenues for the development of effective antibacterial compounds. The unique bioactive compounds found in these plants underscore their potential contributions to addressing bacterial infections and advancing therapeutic options in medicine.

Accordingly, future works need to be conducted to investigate these plants more. Here are some proposed works:

- Further phytochemical investigations are necessary to isolate and identify specific bioactive compounds responsible for the observed antibacterial activity in the selected plant species. This step is crucial for a deeper understanding of their therapeutic potential.

- Other pharmacological studies explore the potential applications of these identified bioactive compounds in cancer, inflammatory conditions, and wound healing. This will contribute to expanding the knowledge about the broader pharmacological benefits of the studied plant species.
- Delving into the mechanisms of action of the identified bioactive compounds is essential. Gaining insights into how these compounds interact at the molecular level with bacterial cells could offer valuable information for drug development.
- Exploration of Synergistic Effects: Investigating potential synergies between compounds within the extracts or in combination with existing antibiotics could enhance their efficacy. Synergistic interactions may lead to the development of more potent antibacterial agents.
- Bioavailability and Toxicity Studies: Assessing the bioavailability and potential toxicological effects of these plant extracts is crucial for their safe and effective use in clinical settings. This would provide valuable information for dosage recommendations.
- Transitioning from *in vitro* assays to *in vivo* studies with animal models is imperative for assessing the efficacy and safety of these extracts. If preclinical studies demonstrate promise, the subsequent phase involves conducting formal clinical trials to rigorously evaluate their efficacy in human subjects, thereby validating their potential integration into mainstream healthcare.
- Formulation Development: Exploring various formulations (e.g., creams, ointments, oral solutions) containing these plant extracts could enhance their applicability in different clinical scenarios, such as topical treatments for skin

infections or oral formulations for systemic infections. Additionally, investigating their potential incorporation into sanitizers and toothpaste formulations for their antibacterial and oral health benefits is recommended.

To sum up, this study has illuminated the substantial antibacterial potential of *Calotropis procera*, *Punica granatum*, *Acacia etbaica*, and *Rhamnus sp.* Future research and development in the highlighted areas offer the potential to harness these natural resources for creating effective antibacterial agents, thereby contributing to the crucial challenge of combating antibacterial resistance.

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