



**Institute of Biodiversity and Environmental Conservation**

**Evaluation of Antimicrobial Compound Effect from *Senna alata* Linn.  
Against Multiple Antibiotic Resistance (MAR) Bacteria from  
Environment**

**Scholastica Ramih Anak Bunya**

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Evaluation of Antimicrobial Compound Effect from *Senna alata* Linn.  
Against Multiple Antibiotic Resistance (MAR) Bacteria from Environment

Scholastica Ramih Anak Bunya

A thesis submitted

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## DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



.....

Signature

Name: Scholastica Ramih Anak Bunya

Matric No.: 19020095

Institute of Biodiversity and Environmental Conservation

Universiti Malaysia Sarawak

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## ABSTRACT

The increasing prevalence of multiple antibiotic-resistant (MAR) bacteria has increased the interest in exploring the potential of medicinal plants as a source of antimicrobial agents. *Senna alata* Linn. commonly known as “gelenggang” and “daun kurap” in Malaysia, is a medicinal plant with various ethnomedical uses and biological features. This study evaluated the antibacterial activity of leaves, stems, and roots against MAR bacteria from the environment, *Enterococcus* sp., and *Mammaliicoccus* sp. The bacteria used in this study were obtained from the environmental bacterial stock provided by the Molecular Laboratory, Institute of Biodiversity and Environmental Conservation, UNIMAS and Department of Animal Science and Fisheries, Universiti Putra Malaysia, Bintulu Campus. From the antibiotic susceptibility test, the MAR value *Enterococcus* sp. was 0.83, which showed resistance against erythromycin, tetracycline, norfloxacin, chloramphenicol and vancomycin. Meanwhile, in *Mammaliicoccus* sp., the MAR value was 0.40, and resistant towards piperacillin, aztreonam, ceftazidime and penicillin. The extraction methods used were soxhlet extraction (SE) and ultrasonic-assisted extraction (UAE), accompanied by ethanol, chloroform and hexane as the choice of solvent. Among all plant parts, the soxhlet and ethanol extracts produced higher yields. However, in the next assay, hexane was removed as it gave a very low yield and was not suitable as most assays needed a high extract concentration. On the other hand, leaves extract showed a greater yield than stems and roots. *S. alata* extracts were also tested for their total phenolic content (TPC) and total flavonoid content (TFC) using Folin-Ciocalteu and aluminium chloride methods, respectively. Both TPC and TFC were exhibited the highest by the leaves of ultrasonic and chloroform extract, UAE-Chlo-L (TPC=117.44±2.78 mg GAE/g DW, TFC=568.78±24.44 mg QE/g DW), followed by the leaves of soxhlet and chloroform extract, SE-Chlo-L. (TPC=80.39±6.60 mg

GAE/g DW, TFC=406.44±7.41 mg QE/g DW). In antibacterial activity using agar well diffusion assay, most of the extracts, especially from soxhlet extraction, were more sensitive and inhibited higher in the *Mammaliicoccus* sp. compared to *Enterococcus* sp. The largest inhibition towards *Mammaliicoccus* sp. was exhibited by SE-Chlo-S (15.00 ± 0.00 mm) with minimum inhibitory concentration and minimum bactericidal concentration at 0.39062 mg/mL and 3.125 mg/mL, respectively. Based on the minimum inhibitory concentration/minimum bactericidal concentration ratio conducted, all the extracts in this study showed potential as bactericidal agents. Besides, the extracts' bioactive compounds associated with antibacterial activity were identified using thin-layer chromatography-direct bioautography (TLC-DB) assay and gas chromatography-mass spectrometry (GC-MS) analysis. Among many spots identified on the TLC plates, only L2 ( $R_f = 0.47$ ), S2 ( $R_f = 0.40$ ), and R1 ( $R_f = 0.42$ ) showed inhibition towards tested bacteria. Various bioactive compounds such as Phenol, 3,5-bis(1,1-dimethylethyl)-, Cholest-5-en-3-ol (3.beta.)-, carbonochloridate, Neophytadiene, Decane, 3,7-dimethyl-, Dodecane, 2,6,11-trimethyl-, 2-Pentadecanone, 6,10,14-trimethyl-, and many other compounds were identified. The findings in this study indicated that *S. alata* possessed antibacterial activities besides having unique bioactive compounds that can be used as a potential antibacterial agent for the production of new drugs in combatting the multiple antibiotic resistance issues in bacteria, particularly bacteria from the environment.

**Keywords:** *Senna alata* Linn., phenolic content, flavonoid content, antibacterial activity, bioactive compounds

**Penilaian Kesan Kompaun Antimikrob dari Senna alata Linn. terhadap Bakteria Rintangan Antibiotik Berbilang (MAR) daripada Persekitaran**

**ABSTRAK**

Peningkatan bakteria dengan perintang pelbagai antibiotik telah menaikkan minat untuk meneroka potensi tumbuhan ubatan sebagai sumber agen antimikrob. Senna alata Linn. atau lebih dikenali sebagai “gelenggang” dan “daun kurap” di Malaysia, merupakan tumbuhan ubatan dengan pelbagai kegunaan etnoperubatan dan ciri biologi. Kajian ini dijalankan untuk menilai aktiviti antibakteria daripada daun, batang dan akar terhadap bakteria dengan perintang pelbagai antibiotik, iaitu Enterococcus sp. dan Mammaliicoccus sp. Bakteria ujian yang digunakan dalam kajian ini diperolehi daripada stok bakteria persekitaran yang disediakan oleh Makmal Molekul, Institut Biodiversiti dan Pemuliharaan Alam Sekitar, UNIMAS dan Jabatan Sains Haiwan dan Perikanan, Universiti Putra Malaysia, Kampus Bintulu. Daripada ujian kerentanan antibiotik, nilai MAR Enterococcus sp. adalah 0.83, yang menunjukkan ketahanan terhadap eritromisin, tetrasiklin, norfloxacin, kloramfenikol dan vankomisin. Manakala, dalam Mammaliicoccus sp., nilai MAR ialah 0.40, dan tahan terhadap piperacillin, aztreonam, ceftazidime dan penisilin. Kaedah pengekstrakan yang digunakan ialah pengekstrakan soxhlet (SE) dan pengekstrakan berbantu ultrasonik (UAE), disertai etanol, kloroform dan heksana sebagai pelarut pilihan. Di antara semua bahagian tumbuhan, ekstrak soxhlet dan etanol menunjukkan pengeluaran hasil yang lebih tinggi. Walau bagaimanapun, dalam ujian seterusnya, heksana telah dikeluarkan kerana ia memberikan hasil yang sangat rendah, dan tidak sesuai kerana kebanyakan ujian memerlukan kepekatan ekstrak yang tinggi. Selain itu, ekstrak daun menunjukkan hasil yang lebih besar daripada batang dan akar.

Ekstrak *S. alata* juga diuji untuk mengenalpasti jumlah kandungan fenolik (TPC) dan jumlah kandungan flavonoid (TFC) di mana masing-masing menggunakan kaedah Folin-Ciocalteu dan aluminium klorida. Kedua-dua nilai TPC and TFC telah dipamerkan tertinggi oleh ekstrak daripada daun yang menggunakan kaedah ultrasonik gabungan kloroform, UAE-Chlo-L (TPC=117.44±2.78 mg GAE/g DW, TFC=568.78±24.44 mg QE/g DW), diikuti oleh ekstrak daun daripada kaedah sokhlet gabungan kloroform, SE-Chlo-L. (TPC=80.39±6.60 mg GAE/g DW, TFC=406.44±7.41 mg QE/g DW). Dalam aktiviti antibakteria menggunakan ujian resapan telaga-agar, kebanyakan ekstrak, terutamanya daripada pengekstrakan sokhlet adalah lebih sensitif dan menghalang lebih tinggi dalam *Mammaliococcus* sp., jika dibandingkan dengan *Enterococcus* sp. Zon perencatan terbesar terhadap *Mammaliococcus* sp. telah dipamerkan oleh SE-Chlo-S (15.00 ± 0.00 mm) dengan nilai kepekatan perencatan minimum dan kepekatan bakteria minimum pada 0.39062 mg/mL dan 3.125 mg/mL, masing-masing. Berdasarkan nisbah nilai kepekatan perencatan minimum/kepekatan bakteria minimum yang dijalankan, kesemua ekstrak dalam kajian ini menunjukkan potensi sebagai agen bakteria. Selain itu, sebatian bioaktif yang dikaitkan dengan aktiviti antibakteria daripada ekstrak telah dikenal pasti menggunakan analisis kromatografi lapisan nipis -bioautografi langsung (TLC-DB) dan kromatografi gas -spektrometri jisim (GC-MS). Di antara banyak tompok yang dikenal pasti pada plat TLC, hanya L2 (Rf = 0.47), S2 (Rf = 0.40) dan R1 (Rf = 0.42) menunjukkan perencatan terhadap bakteria yang diuji. Pelbagai sebatian bioaktif seperti Phenol, 3,5-bis(1,1-dimethylethyl)-, Cholest-5-en-3-ol (3.beta.)-, carbonochloridate, Neophytadiene, Decane, 3,7-dimethyl-, Dodecane, 2,6,11-trimethyl-, dan 2-Pentadecanone, 6,10,14-trimethyl-, dan banyak sebatian lain telah dikenal pasti. Penemuan dalam kajian ini menunjukkan bahawa *S. alata* mempunyai aktiviti antibakteria selain mempunyai sebatian bioaktif unik yang boleh

*digunakan sebagai agen antibakteria yang berpotensi untuk pengeluaran ubat-ubatan baru dalam mengatasi pelbagai isu rintangan antibiotik dalam bakteria, terutamanya bakteria dari persekitaran.*

**Kata kunci:** *Senna alata Linn., kandungan fenolik, kandungan flavonoid, aktiviti antibakteria, sebatian bioaktif*

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## LIST OF ABBREVIATIONS

|       |                                                                  |
|-------|------------------------------------------------------------------|
| ANOVA | Analysis of Variance                                             |
| ATTC  | American Type Culture Collection                                 |
| BLAST | Basic Local Alignment Search Tool                                |
| CFU   | Colony Forming Unit                                              |
| Chlo  | Chloroform                                                       |
| CLSI  | Clinical and Laboratory Standard Institute                       |
| DMSO  | Dimethyl Sulfoxide                                               |
| DNA   | Deoxyribonucleic Acid                                            |
| DW    | Dry Weight                                                       |
| EtOAc | Ethyl acetate                                                    |
| EtOH  | Ethanol                                                          |
| GAE   | Gallic Acid Equivalent                                           |
| GC-MS | Gas Chromatography-Mass Spectrometry                             |
| Hex   | Hexane                                                           |
| kb    | Kilobase                                                         |
| kHz   | Kilohertz                                                        |
| mA    | milliampere                                                      |
| MAR   | Multiple Antibiotic Resistant                                    |
| MBC   | Minimum Bactericidal Concentration                               |
| MHA   | Mueller Hinton Agar                                              |
| MHB   | Mueller Hinton Broth                                             |
| MIC   | Minimum Inhibitory Concentration                                 |
| MTT   | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide |

|        |                                                  |
|--------|--------------------------------------------------|
| NASM   | Non-aureus staphylococci and mammaliicocci       |
| NB     | Nutrient Broth                                   |
| NCBI   | National Center for Biotechnology Information    |
| NIST   | National Institute of Standards and Technology   |
| nm     | Nanometre                                        |
| PCR    | Polymerase Chain Reaction                        |
| Rf     | Retention Factor                                 |
| ROS    | Reactive Oxygen Species                          |
| rpm    | Revolutions per minute                           |
| rRNA   | Ribosomal Ribonucleic Acid                       |
| SE     | Soxhlet Extraction                               |
| TFC    | Total Flavonoid Content                          |
| TLC    | Thin Layer Chromatography                        |
| TLC-DB | Thin Layer Chromatography – Direct Bioautography |
| TPC    | Total Phenolic Content                           |
| UAE    | Ultrasonic Assisted Extraction                   |
| UV     | Ultra-violet                                     |
| UV-Vis | Ultraviolet-Visible spectrophotometry            |
| V      | Volt                                             |

## CHAPTER 1

### INTRODUCTION

#### 1.1 Study Background

Ethnopharmacological aspects of herbal medicine and the discovery of plant-based drugs play a significant role in the evolution of modern therapeutic systems (Süntar, 2020). In traditional practices, medicinal substances were made into herbal remedies with various compositions, including powders, tinctures, pastes, and teas, particularly to address specific health issues (Sreekumar & Nisha, 2022). As in research and development, isolating and identifying physiologically active chemicals and molecules from nature has resulted in the discovery of new treatments, thereby enhancing the health and pharmaceutical industries. These phytochemicals have the potential to serve as a reservoir of novel compounds, which can be utilised in the creation of new pharmaceutical medicines (Lautié et al., 2020).

The World Health Organisation (WHO) has compiled a comprehensive list of 21,000 plants widely utilised for their therapeutic properties worldwide (Anand et al., 2019). In Borneo, the tropical rainforests are an important source of medicinal plants. Its rich biodiversity leads to numerous ethnomedical practices (Baling et al., 2017). Many of the world's population gets their health care from traditional medicines. For example, the ethnic communities in Sarawak, including the Kedayan, Melanau, Bidayuh, Orang Ulu, and Iban, have utilised medicinal plants for various treatments for generations. The utilisation of herbal medicines, rooted in a knowledge of traditional medicine, has been passed across many generations (Bakar et al., 2023).

*Senna alata* Linn. (previously known as *Cassia alata*) is a native plant from the Fabaceae family with various therapeutic uses. This species is found in various tropical

countries, including Africa, America, India, Fiji, Indonesia, and Malaysia (Oladeji et al., 2020). It is known as "*gelenggang*" and "*daun kurap*" in Malaysia, whereas in Indonesia it is referred as "*ketepang badak*" (Fatmawati et al., 2020). Different parts from *S. alata* have been known to have therapeutic properties. Traditionally, *S. alata* has been utilised for centuries to treat a wide range of skin disorders in several Asian and African nations. Furthermore, it has registered as an herbal medication in Thailand and developed as a topical fungal skin infection treatment agent (Silalahi, 2022).

Fresh leaves of the plant are utilised to treat skin infections, mycosis, and dermatitis in Malaysia (Oladeji et al., 2020). The Muruts, Kedayan, and Kadazandusun of Borneo often used the leaves to treat skin disorders such as ringworm by pressing the pounded leaves into the infected area. Some use it as a tonic to treat yellowish skin and asthma (Kulip, 2005; Ahmad & Holdsworth, 1994 cited in Bakar et al., 2023). Besides, the leaf extract that initially demonstrated clinically significant antifungal activity against the yeast *Malassezia furfur* originated in the Philippines. The leaf decoction also appeared to have therapeutic potential for tinea imbricata, a fungal infection caused by *Trichophyton concentricum* (Eusebio-Alpapara et al., 2020). Aside from that, the leaf decoction was utilised to treat constipation in Thailand. Meanwhile, In China, the seeds are brewed into a beverage beneficial for asthma and eyesight (Yon et al., 2022).

The various ethnomedicinal benefits of *S. alata* have prompted researchers to analyse plant sources for bioactive compounds. This approach is considered a novel strategy for developing new pharmaceutical and biological resources in healthcare systems (Vaou et al., 2021; Rahman et al., 2022). Bioactive compounds are plants' most abundant secondary metabolites broadly distributed in plant sources. Plant secondary metabolites are not considered necessary for fundamental plant functions (Samtiya et al., 2021). Nevertheless,

both traditional and contemporary medicine rely extensively on these bioactive compounds. The chemical composition of these substances includes glycosides, flavonoids, tannins, terpenoids, lignans, alkaloids, peptides, and many other compounds. They possess significant natural antioxidant properties and are crucial sources of remedies and pharmaceuticals (Gürbüz et al., 2018).

Various studies have suggested that the bioactive compounds in *S. alata* possess a wide spectrum of biological properties such as anti-inflammatory, antioxidant, antidiabetic, antimicrobial, anticancer and antidepressant (Fatmawati et al., 2020). Phenolic compounds, alkaloids, anthraquinones, tannins, steroids and flavonoids were the common bioactive compounds in them. These compounds' presence correlates to the medicinal and biological properties reported in the plants (Adelowo & Oladeji, 2017).

## **1.2 Problem Statement**

Bacteria have been discovered in the water system, aquaculture sectors, pharmaceutical industries, and food production throughout the last several years (González-Plaza et al., 2019; Peters et al., 2019). Multiple antibiotic resistant (MAR) bacteria were discovered in the environment even before the use of antibiotics in clinical, agricultural, and aquaculture settings (Serwecinska, 2020). The widespread use of antibiotics in many sectors has widened the pathways for the emergence and spread of resistant bacteria (Li & Webster, 2018; Serwecinska, 2020).

Low- and middle-income countries are more susceptible to antibiotic resistance. Hospital overcrowding, poor hygiene, a surge in meat and fish output, a lack of affordable second or third-line antibiotics, and a lack of surveillance and diagnostics all contribute to this problem (Laxminarayan et al., 2020). As antibiotic resistance grows, it restricts the efficacy of treatment for various bacterial, fungal, and viral infections. It also jeopardises the

ability to manage common diseases such as typhoid and influenza, resulting in protracted illness and treatment, lifelong impairment, or even death. Besides, patients with antibiotic-resistant infections require longer treatment and more expensive medications, which raises their healthcare expenses (Abushaheen et al., 2020).

Gram-negative bacterial pathogens are accorded a higher priority than Gram-positive bacteria because of their greater evolution and resistance mechanism levels. This gap can be seen in the surveillance and research on antibiotic resistance in Gram-positive bacteria (Osei Sekyere & Mensah, 2020). *Enterococcus* sp. is a type of Gram-positive bacteria present in various settings, including soil, water, and sewage, as well as the digestive tracts of humans, animals, and insects. While some *Enterococcus* sp. are commensal, others have the potential to be pathogens (Krawczyk et al., 2021).

Besides, *Mammaliicoccus* sp. is also recognised as Gram-positive bacteria. *Mammaliicoccus sciuri* (*M. sciuri*) is the type species of the novel genus *Mammaliicoccus*, which was originally *Staphylococcus sciuri* (Madhaiyan et al., 2020). While most *Mammaliicoccus* isolates are considered commensals, they can occasionally infect both people and animals. They may serve as a repository for resistance genes and were detected in both wild and farmed animals and the environment (Schwendener & Perreten, 2022).

Because of the increasing prevalence of MAR bacteria, researchers are particularly interested in the study of antimicrobial compounds derived from medicinal plants (Vaou et al., 2021). The emergence of bacteria resistant to various antibiotics and the high treatment costs associated with the resistance necessitates the development of novel, safe, efficient, and cost-effective natural medications (Hashempour-Baltork et al., 2019). Medicinal plants have become a prospect for discovering novel bioactive compounds, hence capable of

serving as possible antimicrobial agents and reducing the use of antibiotics in need to overcome the growth of MAR bacteria (Talib, 2011; Vaou et al., 2021).

Although various pharmacological have been studied from *S. alata*, there needs to be more experimental data available on the antibacterial activity of this plant against MAR bacteria, *Enterococcus* sp. and *Mammaliicoccus* sp. The study of phenolic and flavonoid contents in the leaves, stems, and roots of *S. alata* would also provide a better understanding of the plant's significance as a potential source for antioxidant agents. Besides, the compound separation would help to increase the purity of the isolated bioactive compounds upon characterisation. Thus, this study is intended to provide scientific evidence of *S. alata*'s antibacterial efficacy on MAR bacteria and its corresponding bioactive compounds, which can be exploited to manufacture health products from the plant.

### 1.3 Objectives

This research is conducted to achieve the following objectives:

- i. To extract and characterise the extraction efficiency, total phenolic content and total flavonoid content in leaves, stems and roots of *S. alata* Linn. by using soxhlet extraction and ultrasonic extraction methods;
- ii. To evaluate the antibacterial activities in leaves, stems and roots of *S. alata* Linn. against multiple antibiotic resistant bacteria, *Enterococcus* sp. and *Mammaliicoccus* sp.; and
- iii. To separate and analyse the extracted bioactive compounds in the leaves, stems and roots of *S. alata* Linn. by using thin-layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS) analysis.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 *Senna alata* Linn.

*Senna alata* Linn. (*S. alata*), also known as *Cassia alata* Linn. Roxb is a medicinal plant from the family of Fabaceae (Legaspi & Maramba-Lazarte, 2020). It is commonly referred to as “*daun kurap*” and “*gelenggang*” by the Malay (Saidin et al., 2019). As for the taxonomy classification, *S. alata* is classified as kingdom: Plantae; order: Fabeles; family: Fabaceae; subfamily: Caesalpiniondeae; tribe: Cassieae; subtribe: Cassiinae; genus: *Senna*; species: *S. alata* (Oladeji et al., 2020).

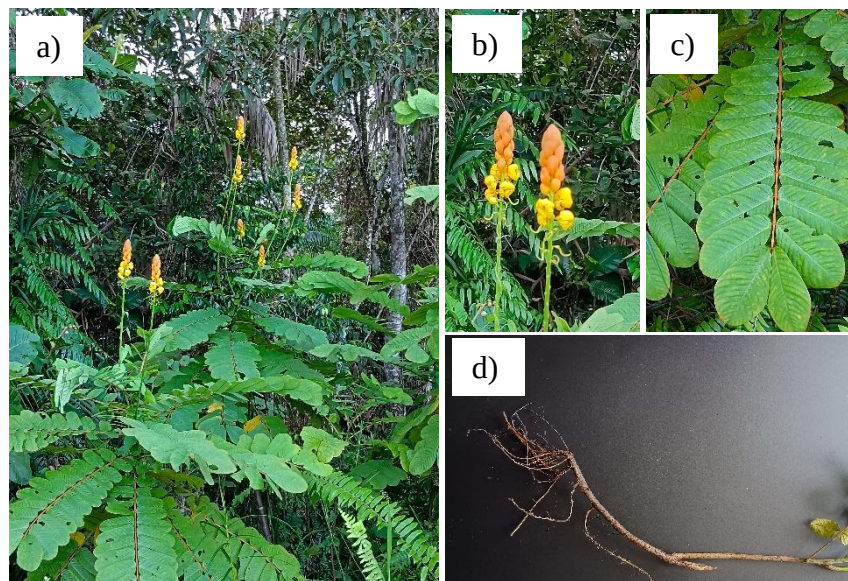


Figure 2.1: The Overview of *S. alata* Linn. a) The Whole Plants. b) Flowers. c) Leaves. d) Roots and Stems (Photo Taken on 8<sup>th</sup> December 2023)

*S. alata* is a shrub with horizontally spread branches that grows to a height of 1 to 5 metres. Its leaves are oblong-rounded ends with par pinnate and range in length from 30 to 60 cm, with 8 to 20 pairs of leaflets (Adelowo & Oladeji, 2017). Its flowers resemble a

golden candle and are tightly arranged in auxiliary racemes that are 20 to 50 cm long and 3 to 4 cm wide. The plant's fruits are thick, flattened, and glabrous, with wings. This species can be spread by seed and found up to 1,500 metres above sea level (Oladeji et al., 2020). It is typically found in tropical locations with warm and humid climates, such as Southeast Asia, Northern Australia, Africa, and Latin America (Fazwa et al., 2020).

Various ethnomedicinal uses have been reported over the years. Among the local ethnic communities of Borneo, leaves of *S. alata* were most often used to treat constipation, skin diseases and ringworm, where decoction and pounding were the most common techniques for medicine preparation (Bakar et al., 2023). In Sabah, the Kadazandusun and Murut communities used leaves for treating skin disease, jaundice, and as a tonic (Kulip, 2003). Meanwhile, in India, the Philippines and China, the stem, bark, and leaf decoction of *S. alata* were found to be effective in treating haemorrhoids, inguinal hernia, syphilis, intestinal parasitosis, and diabetes (Igoli et al., 2005 cited in Oladeji et al., 2020).

#### **2.1.1 Pharmacological Activity of *S. alata* Linn.**

According to Fatmawati et al. (2020) and Oladeji et al. (2020), *S. alata* was claimed to have various ethnobiological and pharmacological activities. The stems, leaves, and roots of *S. alata* are utilised in a variety of traditional remedies, including those for intestinal parasitosis, skin disorders, and diarrhoea. Pharmacological activities have been observed in extracts derived from this plant, including antibacterial, antidiabetic, anti-lipogenic, antifungal, antioxidant, dermatophytic, anti-hyperlipidemic, and anthelmintic properties.

Based on a study by Saidin et al. (2019), ethanolic extracts of the leaves have been proposed as potential active constituents for skin care products due to their antioxidant and anti-inflammatory properties. A facial serum with *S. alata* extracts was formulated as a prototype product and has passed the skin irritation test using an *in vitro* reconstructed human

epidermal model (EpiDerm™). This test was conducted by The Institute Biotechnology Research Centre, SIRIM Berhad.

In addition, using a molecular docking technique, it was observed that acetone and methanolic extracts of the leaves demonstrated antidiabetic properties through the inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase. The methanolic extract exhibited a comparable level of inhibition to the standard acarbose, in which the percentage inhibition of  $\alpha$ -glucosidase at 40  $\mu$ g/mL was 62.586% and 65.274%, respectively (Thilak et al., 2023). An effective suppression of alpha glucosidase significantly decreases postprandial blood glucose levels (Thodi et al., 2022).

Other therapeutic behaviours, antidepressant and antianxiety, were reported in the leaves of *S. alata*, where the aqueous leaves extracts (200 mg/kg) showed a significant decrease in antidepressant and antianxiety activities in all of the test animals (Pamulaparathi et al., 2016). Antiplasmodial was also reported in a study conducted by Onyegeme-Okerenta et al. (2022). The aqueous extracts of *S. alata*, *Dennettia tripetalla*, and the combination of the two exhibited dose-dependent chemo-suppressive and curative effects on *Plasmodium berghei*-infected rodents.

Besides, ethanolic leaf extracts of *S. alata*, *S. alexandrina*, and *S. occidentalis* were reported to have crucial antitrepatodal action against the ruminant parasite *Paramphistomum gracile*. This action suggested the presence of anthelmintic properties in *S. alata*, a drug that kill or remove the helminths or other parasitic worm in the body (Roy & Lyndem, 2019).

### 2.1.2 Antibacterial Activity of *S. alata* Linn.

The antibacterial activity of *S. alata* were observed and documented in numerous plant parts (Fatmawati et al., 2020). A study by Pham et al. (2021) reported that the ethyl acetate-soluble extract from leaves and its metabolite rhein exhibited a good inhibition towards plant pathogenic bacteria, *Acidovorax avenae* subsp. *Cattlyae*. The rhein's bacteriostatic action interfered with the action of  $\alpha$ -hemolysin and catalase, which destroyed the biofilm structure, exposing bacteria to antibiotics and immune determinants (Dell'Annunziata et al., 2022). In another study by Hussain et al. (2021), the inhibition activity of *E. coli* and *S. aureus* by the plant protein from *S. alata* indicated the presence of antimicrobial compounds in it. Similar to Anibijuwon et al. (2018), antibacterial activity was found against the MAR clinical isolates (i.e., *S. aureus*, *S. typhi* and *P. aeruginosa*) by the ethanolic and methanolic extract of *S. alata* leaves.

In addition to the leaves, *S. alata* extracts from the bark and roots demonstrated inhibition zones against *Salmonella enterica* serovar Typhimurium, *S. aureus*, and *E. coli*. Consistently, the inhibition zones were greatest in the extracts derived from the roots (Halim-Lim et al., 2020). A study conducted by Toh et al. (2023) also found that the roots of *S. alata* exhibited a greater antibacterial activity against *S. aureus*, with the average of inhibition zone at 15.30 mm (roots) and 14.70 mm (leaves). Meanwhile, the extracted dye from the flowers of *S. alata* also showed antibacterial activity against *Staphylococcus* sp., *Pseudomonas* sp., *Vibrio* sp., *Klebsiella* sp., *Micrococcus* sp. (Muruganandham et al., 2023). The findings on the pharmacological activity are significant as they support its traditional usage for ages (Goh et al., 2017).

## **2.2 Plant Secondary Metabolites**

Pharmacologically active substances called phytochemicals have always been thought to be the reason why plants are used as medicine (Süntar, 2020). The observed antimicrobial efficacy of the plant extracts against the targeted bacterial strains suggests the existence of diverse bioactive compounds with the potential for therapeutic application in treating bacterial infections (Anibijuwon et al., 2018). Plant secondary metabolites have biological activity as they play a vital role in growth, development, innate immunity, defence against pathogenic threats and environmental challenges response (Yang et al., 2018).

They consist of a broad range of structurally varied chemicals derived from either primary metabolites or intermediates in these primary metabolites' metabolic pathways (Piasecka et al., 2015). These include phenolic acids, flavonoids, terpenoids, steroids and alkaloids (Ashraf et al., 2018). Due to their biological activity, they are used as medicinal ingredients and food additives for therapeutic and aromatic purposes (Yang et al., 2018). Nonetheless, the extraction and purification of the secondary metabolites from plants are complicated, time-consuming, and the yield of the isolated compounds is usually relatively low (Ruan et al., 2016).

Herbal medications have a complex chemical composition, with secondary metabolites and biological effects changing with the environment, whereas high-quality medicinal plants need controlled settings to synthesise specific secondary metabolites (Yang et al., 2018). The accumulation of secondary metabolites is significantly dependent on a wide range of environmental conditions, including light, temperature, soil water, soil fertility, and salinity (Jan et al., 2021). For the majority of plants, a change in a single component can alter the content of secondary metabolites even if the other elements remain the same (Ashraf et al., 2018).

### **2.2.1 Phenolics Compounds**

Phenolic compounds are one of the plants' most abundant classes of secondary metabolites (Rahman et al., 2022). In terms of their chemical structures, they are made up of one or more aromatic rings that are linked to one or more hydroxyl groups. This gives them different chemical structures. These compounds are categorised into phenolic acids, flavonoids, tannins, stilbenes, and lignans (Albuquerque et al., 2021). Furthermore, phenolic compounds are mostly synthesised by phenylpropanoid metabolism, which requires the presence of precursors synthesised via two major pathways: the shikimic acid pathway and the malonic acid pathway (de la Rosa et al., 2018).

These phenolic compounds have a wide range of biological activity that could be used in therapy, including antioxidant, antibacterial, anticancer, and cardioprotective properties (Cosme et al., 2020). However, the amount or presence of different phenolic chemicals varies amongst plant kinds. These factors also include plant growing circumstances, location, and plant maturity (Vu et al., 2018). In terms of pre-treatment methods, drying processes, extraction conditions, and the type of solvent used can all have an impact on phenolic recovery in plants (Alara et al., 2021).

### **2.2.2 Flavonoids Compounds**

Flavonoids are the most abundant type of phenolic compound (Albuquerque et al., 2021). They are heterocyclic compounds made up of two aromatic rings connected by an oxygen atom. Flavonoids are classified into six sub-groups based on hydrogenation status and heterocyclic substitution identities: flavones, flavonols, anthocyanins, and isoflavones (Rahman et al., 2022). According to Karak (2019), flavonoid molecules exhibit a variety of biological features, including antioxidative activity, free radical scavenging capability, cardioprotective, antidiabetic, anti-inflammatory, and anti-allergic.

In addition, flavonoids have been found to have a broad range of anticancer properties. Modulation of reactive oxygen species (ROS)-scavenging enzyme activities, participation in cell cycle arrest, induction of apoptosis and autophagy, and suppression of cancer cell proliferation and invasiveness are among those (Kopustinskiene et al., 2020). The quantity of flavonoids in fruits and vegetables varies depending on species variety, edaphoclimatic conditions, plant part, cultivation, and degree of ripeness (Vuolo et al., 2019).

### **2.2.3 Bioactive compounds of *S. alata* Linn.**

*S. alata* is capable of producing a large number of bioactive compounds that can have a variety of different structural configurations and beneficial biological applications (Fatmawati et al., 2020). The investigation of the origins of bioactive compounds associated with biological activity has attracted the attention of researchers. This is because extracting and collecting the compounds is advantageous, particularly for use in healthcare (Goh et al., 2017). Besides, drug resistance gained by microbes poses a significant concern to the public, so more emphasis should be placed on employing medicinal plants with antibacterial characteristics, which require further research into their plant's components (Pachorkar & Patil, 2021).

A study conducted by Mohanasundaram et al. (2021) revealed that the leaves of hydroethanolic extract *S. alata* contained kaempferol 3-e-O-gentiobioside, phytol, stigmasterol, sitosterol, dimethyl derivatives, methyl esters, and octanoic acid derivatives. This extract was found to have an antidiabetic action in experimental rodents and was suggested as a potential anti-diabetes agent. Chimi Fotso et al. (2021) also found chrysophanol, emodin and  $\beta$ -Sitosterol-3-O- Glucoside which isolated from the leaves and trunks of *S. alata* showed antibacterial activity against various strains (i.e., *S. aureus*

ATCC43300, *P. aeruginosa* (HM801), *S. pneumoniae* ATCC491619, *E. coli* ATCC25322 and clinical isolates of *S. typhi* and *Enterobacter cloacae*). The highest was showed by emodin (7.8 µg/mL) against *P. aeruginosa*. In a study by Duong et al. (2017), methyl 2,4,6-trihydroxybenzoate, kaempferol, (-)epiafzelechin, kaempferol-3-O-glucoside and kaempferol-3-O-gentiobioside was identified from the antibacterial activity of ethyl acetate leaves extract against *S. aureus* and *B. cereus*.

Meanwhile, Yang et al. (2022) discovered anti-rotavirus activities in the stems of *S. alata*, with therapeutic index values of 9.7515.3. Three novel indole alkaloids, alataindoleins, one new chromone, alatachromone A, and a new dimeric chromone-indole alkaloid, alataindolein D, were recovered from this extract. Similar to Zhou et al. (2020), two structurally unique polyphenols, alatains A and alatains B were identified from the bark of *S. alata*. Both alatains A and B showed significant anti-tobacco mosaic virus inhibition. Apart from that, 2 new flavones, 2-hydroxy-7-(3-hydroxypropyl)-8-methoxyflavone and 4-hydroxy-7-(3-hydroxypropyl)-6-methoxyflavone, together with 2 known flavones, 6-prenylapigenin and cycloartocarpesin were identified from bark extract of *S. alata*. This extract was found with anti-methicillin-resistant *S. aureus* (MRSA) activity and antioxidant activity (Liu et al., 2018).

*S. alata* seed extract, on the other hand, has been discovered to have an anti-hyperglycemic effect (Umaru et al., 2022). This extract contained major components such as kaempferol, naringenin, 3-Oxabicyclo[3.3.0]octan-2-one, 7-Isopropylid e, pyrrolo[1,2-a] pyrazine-1,4-dione,hexahydro-3-, gallic acid, tocopherol, quercetin, and octadecanoic acid. In contrast to the seed extract, the floral extract contained cyclotrisiloxan, b-Sitosterol, tocopherol, eicosane, beta carotene, omega 3 derivatives, omega 6 derivatives, and cyclotrisiloxan derivative chemicals (Salamatullah et al., 2021). This flower extract inhibited

the development of HT-115 human colon cancer cells via mitochondrial dependent apoptotic stimulation. Besides, in a study conducted by Toh et al. (2023), the roots found with antimicrobial activity against *S. aureus* discovered with the presence of tetradecanoic acid, heptadecanoic acid, methyl ester, 9-Octadecenoic acid (Z)-, methyl ester, cyclohexasiloxane, dodecamethyl, phytol, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, n-heptadecanol-1 and 2-pentadecanone, 6,10,14-trimethyl-.

### **2.3 Extraction Methods of Bioactive Compounds from Medicinal Plants**

The choice of extraction process is extremely important when it comes to the analysis of phytochemicals derived from solid materials (Koparde, 2017). Using certain solvents, the bioactive fractions of plant materials are isolated and separated from the inactive or inert components of the plant during the normal extraction technique. The initial crude extracts that are obtained by utilising these procedures contain a complex mixture of many different plant metabolites, such as flavonoids, alkaloids, glycosides, phenolics, and terpenoids. The extraction of natural products involves four steps: the solvent penetrates the solid matrix, the solute dissolves in the solvents, the solute diffuses out of the matrix, and the extracted solutes are collected (Zhang et al., 2018).

The extraction method can be divided into two separate groups, namely the conventional extraction method and the modern extraction method. Maceration, infusion, decoction, percolation, reflux extraction, and soxhlet extraction are all examples of conventional extraction techniques (Bitwell et al., 2023). These techniques usually require high usage of organic solvent and a long extraction period (Garcia-Vaquero et al., 2020). The extraction of natural products has also led to the use of some more modern and sustainable extraction techniques, such as supercritical fluid extraction (SFC), pressurised liquid extraction (PLE), ultrasonic assisted extraction (UAE) and microwave assisted extraction

(MAE) (Bitwell et al., 2023). These techniques offer a variety of advantages, including a decrease in the amount of organic solvent consumed, a reduction in the amount of time required for extraction, and an increase in selectivity (Picot-Allain et al., 2021).

### **2.3.1 Soxhlet Extraction**

Soxhlet extraction is a continuous extraction method with high extraction efficiency that uses less solvent and time than maceration or percolation (Bitwell et al., 2023). In a soxhlet apparatus thimble chamber, pulverised plant material is inserted in a filter paper or cellulose thimble. The bottom flask heats extraction solvents, which vaporise into the sample thimble, condense in the condenser, and drip back. When the liquid reaches the syphon arm, it goes back into the bottom flask again, and the process continues (Azwanida, 2015). Soxhlet extraction requires more solvent and time than ultrasonic assisted extraction. The use of a high temperature and a prolonged extraction time in the soxhlet method may, however, increase the risk that the substance will degrade due to the heat, which is not ideal for thermolabile compounds (Zhang et al., 2018).

### **2.3.2 Ultrasonic Assisted Extraction**

The ultrasonic assisted extraction method employs ultrasonic wave energy within a frequency range from 20 kHz to 2000 kHz. The application of ultrasound in the solvent induces cavitation, leading to enhanced dissolution and diffusion of the solute, as well as improved heat transfer. Consequently, this results in increased extraction efficiency (Bitwell et al., 2023). The phenomenon of acoustic cavitation induced by ultrasound enhances the area of contact between solvents and plant samples, as well as the permeability of cellular membranes (Azwanida, 2015). The phenomenon of bubble formation, its growth, and eventual collapse is commonly referred to as cavitation. However, cavitation can be

influenced by various physical characteristics of the solvent, including viscosity, surface tension, and saturation vapour pressure (Vernès et al., 2019).

When compared to conventional methods for extracting phytochemicals, this process is more environmentally friendly, green, and cost-effective (Bitwell et al., 2023). Low energy and solvent utilisation, as well as a reduction in extraction temperature and duration, are another advantage of ultrasonic assisted extraction. Hence, the extraction of unstable and thermolabile compounds is applicable to this method (Zhang et al., 2018). Both an ultrasonic bath and a probe can be used to provide high-intensity ultrasound. Most ultrasonic baths have a frequency of 40 kHz and some even have adjustable heat. They are simple to operate, accessible, and cheap (Altemimi et al., 2017). However, when compared to probe systems, the low power and poor reproducibility of the ultrasound that is applied to the sample are significant limitations. Indeed, the water in the bath and the glassware used in the experiment significantly affected the intensity (Vernès et al., 2019).

### **2.3.3 Solvent Extraction**

For bioactive compound extraction, the choice of solvent is crucial. In selecting solvents, selectivity, solubility, cost, and safety should be considered (Truong et al., 2019). The selection of solvents for the extraction of bioactive compounds from plants depends on the type of plant, the part of the plant to be extracted, the nature of the bioactive compounds, and the availability of solvent. According to the law of similarity and impermissibility, the solute will be effectively dissolved when a solvent with similar polarity is used (Altemimi et al., 2017).

Polar solvents, intermediate polar solvents, and non-polar solvents are the three types of solvents used in the extraction of medicinal plants. In general, polar solvents like water, methanol, and ethanol are used to extract polar compounds, while nonpolar solvents

like hexane, chloroform, and ether are used to extract nonpolar compounds. Similarly, acetone and dichloromethane are intermediate polar solvents (Abubakar & Haque, 2020). The use of different solvents resulted in various extraction yields. This is due to differences in the polarity of the extraction solvents; the extraction yields of the various solvents varied. This means that it results in a broad range of bioactive compound concentrations in the extract (Truong et al., 2019).

## **2.4 Antibiotic Resistance**

Antibiotic resistant bacteria are a major global public health issue. They cause infections that are more difficult to treat, demanding the use of more toxic and expensive medications (Larsson & Flach, 2022). Antibiotic resistance genes have been present in the environment since ages, and natural environments contain a broad range of resistance genes (Koch et al., 2021). The overuse and misuse of antibiotics significantly increase the concentrations and rates of antibiotics in the environment. This excessive use of antibiotics for purposes other than medical treatment has increased the rates of antibiotic excretion and environmental release, resulting in the emergence of drug resistance in bacterial strains, posing a serious threat to human health (Serwecinska, 2020). Hospitals, sewage treatment plants, agriculture, aquaculture, and manufacturing are potential sources of exposure (Zainab et al., 2020). Additionally, there is evidence of a potential transfer of genes for antibiotic resistance from environmental bacteria to human pathogens (McInnes et al., 2020).

### **2.4.1 Emergence of Antibiotic Resistance in the Environment**

Antibiotic resistance arises when a medication loses its ability to effectively hinder or regulate the activity of a microorganism, such as bacteria, fungus, virus, or parasite. Numerous pathways exist for the transmission of antibiotic-resistant microorganisms into the environment. (Lepper et al., 2022). The environment serves as a repository for mobile

genetic elements that propagate to other organisms or to animal and human hosts through interactions. For example, the ecological network connects different parts of animals to soil, water, sand, and sewage, which in turn allows the transmission to spread among humans, animals, and the environment (Ahmad et al., 2021).

Furthermore, it has been found that techniques used to clean wastewater and drinking water are unable to eradicate antibiotic resistance genes (ARGs). Antibiotic-resistant bacteria and ARGs containing effluents from wastewater treatment plants can end up in aquatic habitats such as rivers and lakes (Amarasiri et al., 2020). In the absence of antibiotic-induced selective pressure, antibiotic resistance can develop naturally (intrinsic) as a result of a spontaneous gene mutation. ARGs are thought to have evolved from environmental bacteria that generate and release antibiotics to influence microbial populations with whom they compete for resources (Hamdani et al., 2020).

On the other hand, this natural resistance can also contribute to the emergence of acquired resistance, and both forms of resistance can be passed either horizontally or vertically (Lepper et al., 2022). Horizontal gene transfer (HGT) is the process by which organism or species acquires foreign genes or mobile genetic components. This process is critical to bacterial evolution and greatly accelerates the spread of resistance genes. The genes shared through this pathway make a significant contribution to the global spread of antibiotic resistance genes (Tao et al., 2022). HGT can take place in several environments, especially where there is a high concentration of bacteria. This includes environments such as soil, wastewater treatment plants (Nguyen et al., 2021), manure (Lima et al., 2020), and the gut microbiome of both people and animals (McInnes et al., 2020).

#### 2.4.2 Multiple Antibiotic Resistant Bacteria from Environment

The environment such as aquatic ecosystems, aquaculture areas, recreational rivers, lakes, soils, humans, and animals are examples of antibiotic resistance reservoirs (Kim and Cha, 2021; Xu et al., 2020). Until now, antibiotic resistance has been monitored in several sectors to assess the burden of antibiotic resistance and ensure the availability of data. This aspect is significant as it encompasses initiatives for prevention and control, provision of vital antibiotics, and investigation and advancement of novel antibiotics and vaccines (Murray et al., 2022).

According to a study conducted by Nadella et al. (2021), 11.9% of the bacteria (n=2304) isolated from shrimp (*Penaeus vannamei*) aquaculture ponds were multiple antibiotic resistant. These bacterial isolates were mostly identified as gram-negative bacteria. These bacterial isolates showed resistance the highest in oxytetracycline (23.4%), followed by erythromycin (12.7%), co-trimoxazole (10%) ciprofloxacin (9.6%), and chloramphenicol (6%). According to their findings, this was due to the recycling of untreated water from the previous crop (Su et al., 2017), the placement of seed (post-larvae) from different hatching facilities (Nadella et al., 2022), diverse culture practices, the release of water from the plant waters during harvest into the environment, and the use of a contaminated water source (Lai et al., 2018).

Besides, Rajendiran et al. (2022) showed that 62.5% of Enterococci isolates from poultry settings (soils and effluent water) in Selangor, Malaysia, had multiple antibiotic resistance out of a total of 80 isolates. Every linezolid-resistant enterococci (LRE) is resistant to various antibiotics, making up 40 % of all resistance among the isolates. *E. faecium* isolates had a higher multidrug resistance rate of 69 % compared to *E. faecalis* isolates at 49 %. One *E. faecium* strain showed extended drug resistance by being resistant to seven types

of antibiotics. The significant prevalence of linezolid-resistant enterococci and multidrug resistance causes serious attention, given that linezolid is a viable substitute for vancomycin in treating enterococcal infections (Echeverria-Esnal et al., 2019).

In another study conducted by Boonchuay et al. (2023), methicillin-resistant *M. sciuri* isolates (n=11) from animals in Southern Thailand were resistant to 11 drugs. All the isolates were particularly from horses, cows and chickens. The resistance rate was higher than 50 %, where all isolates exhibited sensitivity to cefalexin, gentamicin, neomycin, enrofloxacin, marbofloxacin, and nitrofurantoin. In all isolates, the staphylococcal chromosomal cassette (SCC) *mec* was detected in addition to the *mecA* and *blaZ* genes. Methicillin-resistant staphylococci are pathogenic bacteria that cause nosocomial infections in humans and animals (Turner et al., 2019). Large mobile genetic elements, SCC*mec*, that carry resistance genes allow intraspecies and interspecies transmission between staphylococci and mammaliicocci. This element facilitates bacterial resistance gene transfer, causing antibiotic treatment problems (Amirsoleimani et al., 2021).

## **2.5 *Enterococcus* sp.**

*Enterococcus* sp. is a Gram-positive bacteria found in the intestinal tracts of humans, mammals, invertebrates, reptiles, and birds (Torres et al., 2018). In addition, they are also found in environments such as water, soil, and vegetation (Cho et al., 2020). They can live in a wide range of temperatures (5-60°C) and pH (4.6-9.9), as well as in the presence of high salt concentrations (6.5% NaCl) and bile salts (40%), as well as extensive desiccation (Shin et al., 2019). *E. faecalis* and *E. faecium* are the most prevalent enterococcal species in the intestinal microbiota of humans, which are responsible for the majority of human enterococcal infections (Dadashi et al., 2021). Enterococci are also used as an indicator of faecal contamination in freshwater. In marine water, enterococci were thought to be a better

indicator of faecal pollution than *E. coli* because their concentrations in marine water are related to the number of swimming-associated gastrointestinal illness rates (Devane et al., 2020).

Other than that, *Enterococcus* sp. can also cause endocarditis, surgical wound infection, neonatal sepsis, bacteremia, and, in rare cases, meningitis, which makes it a major pathogen responsible for dangerous nosocomial and community acquired infections (Raza et al., 2018). Due to their resistance to multiple antimicrobials, especially  $\beta$ -lactams and aminoglycosides, they have emerged as a prominent nosocomial pathogen (Raza et al., 2018). However, enterococci have only recently gained attention as multiple antibiotic resistant bacteria due to the development of vancomycin resistance (CDC, 2019). Infections caused by vancomycin-resistant enterococci are often treated with linezolid, quinupristin/dalfopristin, daptomycin, and tigecycline. However, the resistance to these antibiotics is increasingly observed, making vancomycin-resistant enterococci infections difficult to cure. The limited number of antimicrobials that can treat vancomycin-resistant enterococci infections has caused major concern since the development of these bacteria (Pöntinen et al., 2021). Furthermore, there is also the chance that a gene that confers resistance could be passed horizontally, resulting in higher virulence factors (Chajęcka-Wierzchowska et al., 2019).

## **2.6 *Mammaliicoccus* sp.**

Gram-positive bacteria, including *Mammaliicoccus* sp., are non-motile, spore-free cocci that can be found single, in pairs, or in a typical cluster. Previously known as non-aureus *Staphylococcus* species (NASM), *Mammaliicoccus* sp. was recently transferred to the novel genus *Mammaliicoccus*, with *Mammaliicoccus sciuri* (*M. sciuri*) serving as the type species (Madhaiyan et al., 2020). Opportunistic bacteria like staphylococci and

mammaliicocci can still cause a variety of life-threatening illnesses (Belhout et al., 2022). Occasionally, they are present in environment, such as river and soil, due to the untreated manure from the livestock. For most of the time, *Mammaliicoccus* sp. inhabits the skin and mucous membranes of various part of body in humans and animals (Martins-Silva et al., 2023). Bovine mastitis, dairy sheep and goats, piglets, as well as canine dermatitis have all been shown to be carrying *M. sciuri* (Rosa et al., 2022).

In events of severe clinical mastitis, milk abnormalities are readily identified, and the milk must be thrown to ensure it does not enter the food chain (Hussein et al., 2022). But when milk from animals with subclinical mastitis, such as that caused by NASM but with no obvious changes, is accidentally mixed with bulk milk, it gets into the food chain and could be bad for human health (Hussein et al., 2022). Due to the high level of contamination from animals, pastures, milking machinery, and containers, raw milk consumption is not advised due to the presence of NASM. Heikkilä et al. (2018) state that heat treatment is required to assure food safety and extend its shelf life to protect human health. It has been reported that the pathogenicity factors of *Mammaliicoccus* can form biofilms and transmit virulence factors such as enterotoxins and hemolysins (Dhaouadi et al., 2020).

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Collection and Preparation of Plant Sample

Fresh plant of *S. alata* including the roots, stems, and leaves were obtained from a sampling site located in the Kota Samarahan region (1°27'40.968" N, 110°24'50.5" E). The plant materials were directly sorted according to their parts. Then, each part of the plant was washed thoroughly using running tap water. At a temperature of 40 °C, the samples were dried in an oven for 5 days or until they reached a constant weight. The dried samples were ground into a fine powder using an electronic blender and grinder. The powdered samples were stored inside an airtight container at -4 °C before extraction was performed (Mugao et al., 2020).



Figure 3.1: The *Senna alata* Plant (Photo Taken on 11<sup>th</sup> November 2020)

## **3.2 Extraction of Plant Sample**

### **3.2.1 Extraction using Soxhlet Extraction**

The extraction process from *S. alata* through soxhlet extraction was performed according to Mahyuddin et al. (2020) with modifications. Fifteen grams of the powdered *S. alata* was weighed and placed in an extraction thimble inside the soxhlet apparatus. A volume of 200 mL of extraction solvent was added. The extraction solvent used in this study was hexane (C<sub>6</sub>H<sub>14</sub>) (Merck, Germany), chloroform (CHCl<sub>3</sub>) (Merck, Germany) and ethanol (C<sub>2</sub>H<sub>5</sub>OH) (HmbG, Malaysia). The sample was allowed to extract for 6 h with boiling points 69 °C, 60 °C and 78 °C, respectively.

### **3.2.2 Extraction using Ultrasonic Assisted Extraction**

The ultrasonic assisted extraction was conducted using an ultrasonic bath (Branson 2510MT) as described by Saifullah et al. (2020) with modifications. One gram of plant samples was mixed with 40 mL of extraction solvent inside a Schott bottle. The ultrasonic bath was allowed to be degassed for 20 min, and then the Schott bottles were put in an ultrasonic bath. The temperature was set at 30 °C and extracted for 30 min with a fixed frequency of 40kHz. A similar extraction solvent was used: hexane (C<sub>6</sub>H<sub>14</sub>) (Merck, Germany), chloroform (CHCl<sub>3</sub>) (Merck, Germany) and ethanol (C<sub>2</sub>H<sub>5</sub>OH) (HmbG, Malaysia).

## **3.3 Determination of Yield of Extraction**

The extracted extracts were then filtered through filter paper (Whatman No. 1, UK) and left evaporated under vacuum conditions by rotary evaporation at 40 °C until a small amount was left inside the flask. The remaining extracts were transferred into a pre-weighed

beaker and allowed to dry in the fume hood. The yield of extraction (%) was calculated as below:

$$\text{Yield of extraction (\%)} = W_0 / W_1 \times 100 \quad \text{Equation 3.1}$$

Where  $W_0$  and  $W_1$  are the weighed of *S. alata* extracted from the sample (g) and the weighed of the sample (g), respectively (Singh et al., 2021).

### 3.4 Determination of Total Phenolic Content (TPC)

Folin-Ciocalteu reagent (Sigma Aldrich, USA) was used to determine total phenolic content (TPC) in *S. alata* extracts Ling et al. (2019). Gallic acid (HiMedia, India) was used as a standard to construct the calibration curve. An aliquot of 0.125 mL of standard gallic acid solution (10, 20, 40, 60, 80, 100 µg/mL) or extract was added into a test tube containing 0.5 mL of ultrapure water and 0.125 mL of Folin-Ciocalteu phenol reagent. The mixture was allowed to stand for 3 min upon addition of 1.25 mL of 7% Na<sub>2</sub>CO<sub>3</sub> (Merck, Germany). The mixture was made up to a total volume of 3 mL using distilled water. The reaction mixture was mixed well and allowed to stand for 1 h in the dark at room temperature. The absorbance was observed using a UV-Vis spectrophotometer (Shimadzu UV-1900i) at a wavelength of 760 nm. The extract was done in triplicates, and ultrapure water was used as blank. The total phenolic content of extracts was expressed as mg of gallic acid equivalent (GAE) per g of dry weight (mg GAE/ g DW) using a calibration curve, as shown in Appendix B1.

### 3.5 Determination of Total Flavonoid Content (TFC)

Aluminium chloride colourimetric assay was used to determine total flavonoid content (TFC) in *S. alata* extracts, according to Ling et al. (2019). Quercetin (Sigma Aldrich, USA) solution with concentrations of 100, 200, 400, 600, 800 and 1000 µg/mL was used to construct the calibration curve. An aliquot of 0.2 mL of standard quercetin solution or extract

was transferred into a test tube, containing 4.8 mL of ultrapure water. Briefly, 0.3 mL of 5% NaNO<sub>3</sub> (Merck, Germany) solution was added and allowed to stand for 5 min. After that, 0.3 mL of 10% AlCl<sub>3</sub> (Merck, Germany) was added, followed by the addition of 2 mL of 1M NaOH (Merck, Germany), and the final volume was made up to 10 mL with ultrapure water. The absorbance was determined using a UV-Vis spectrophotometer (Shimadzu UV-1900i) at a wavelength of 414 nm. The total flavonoid content of the extracts was calculated in mean  $\pm$  SD (n=3) and expressed as mg quercetin equivalent per gram of dry weight (mg QE/ g DW) by comparing to the calibration curve as shown in Appendix C1. All samples were performed in triplicate and ultrapure water was used as blank.

### **3.6 DNA Extraction**

The test bacteria used in this study were obtained from the environmental bacterial collection stocks in which provided by the Molecular Laboratory, Institute of Biodiversity and Environmental Conservation, UNIMAS and Department of Animal Science and Fisheries, Universiti Putra Malaysia, Bintulu Campus. The DNA of the bacterial isolates was extracted by boiling-centrifugation method, according to Kathleen et al. (2011 cited by Mohammad Hamdi et al., 2022). Two mL of the overnight culture in Nutrient Broth (NB) (HiMedia, India) was centrifuged for 5 min at 10,000 rpm. The supernatant obtained was removed while 500  $\mu$ L of sterile ultrapure water was added to the pellet. The suspension was then heated at 95°C for 10 min and quickly chilled in ice at 4°C for 5 min before being centrifuged at 10,000 rpm for 10 min. The supernatant obtained was used as the DNA template in 16S rRNA polymerase chain reaction (PCR).

### 3.7 16S rRNA Amplification

The DNA of bacterial isolates that were extracted were subjected to 16S rRNA gene amplification, in which 27F: (5'-AGAGTTTGATCCTGGCTCAG-3') and 519R: (5'-GWATTACCGCGGCKGCTG-3') primer sets were used (Kathleen et al., 2011 cited by Mohammad Hamdi et al., 2022). The PCR was carried out in a total volume of 40 µL with a reaction mixture of a master mix (Promega, USA), as shown in Table 3.1 below.

Table 3.1: Master Mix of 16S rRNA PCR

| <b>Solution</b>         | <b>Volume (µL)</b> |
|-------------------------|--------------------|
| 5× Taq Green Buffer     | 4                  |
| 25 mM MgCl <sub>2</sub> | 4                  |
| 25 mM dNTPs             | 0.3                |
| 10 µM 27 F Primer       | 2                  |
| 10 µM 519 R Primer      | 2                  |
| Taq DNA Polymerase      | 0.3                |
| DNA template            | 2                  |
| Sterile ultrapure water | 25.4               |
| Total                   | 40                 |

All the reaction mixtures were obtained from Promega Corporation, USA. The samples were then amplified in Mastercycler® nexus X2 with thermal parameters as below:

Table 3.2: Thermal Parameters in 16S rRNA PCR Amplification

| <b>Process</b>       | <b>Temperature (°C)</b> | <b>Time</b> | <b>Number of cycles</b> |
|----------------------|-------------------------|-------------|-------------------------|
| Initial denaturation | 95                      | 2 min       | -                       |
| Denaturation         | 95                      | 30 sec      | 30                      |
| Annealing            | 58                      | 30 sec      | 30                      |
| Extension            | 72                      | 30 sec      | 30                      |
| Final elongation     | 72                      | 3 min       | -                       |

### **3.8 Agarose Gel Electrophoresis**

The amplified PCR products were then run on gel electrophoresis at 90 V, 200 mA for 45 minutes. Briefly, 3  $\mu$ L of the DNA product and 3  $\mu$ L of 1000 bp of Promega DNA ladder were electrophoresed on 1.5% (w/v) agarose gel. After electrophoresis, the gel was stained in ethidium bromide solution for 10 min and then rinsed with distilled water. Finally, the DNA bands were visualised and observed by an Ultraviolet (UV) Transilluminator (Mir et al., 2022).

### **3.9 Sequencing Analysis**

The PCR products were submitted to Apical Scientific Sdn. Bhd., Malaysia, for the sequence analysis. The full gene sequences of isolates were compared automatically using the Basic Local Alignment Search Tool (BLAST) with the sequences of bacteria available in databanks at the National Centre for Biotechnology Information (NCBI).

### **3.10 Preparation for Standardised Concentration of Bacterial Culture**

A few colonies from the bacterial stocks that were obtained from the Molecular Laboratory, Institute of Biodiversity and Environmental Conservation, UNIMAS and Department of Animal Science and Fisheries, UPM Bintulu Campus were cultured into 5 mL of Mueller Hinton Broth (MHB) (Oxoid, UK) and incubated at 37 °C in an incubator shaker for 18 h. The turbidity of the bacterial suspension was standardised to 0.5 McFarland solution at 600 nm using a spectrophotometer. This standardised bacterial broth culture was now prepared for use in the *in-vitro* antibacterial bioassay evaluation (Toh et al., 2023).

### **3.11 Antibiotic Susceptibility Profile of Test Bacteria**

The antibiotic susceptibility profile of both test bacteria was retested using the disc diffusion method as described by Lihan et al. (2020) and Clinical and Laboratory Standard

Institute guidelines (CLSI, 2021). The freshly prepared standardised bacterial culture was swabbed onto the surface of Mueller Hinton Agar (MHA) (Oxoid, UK) using a sterile cotton bud and allowed to dry for 15 min. After that, the antibiotic discs from different classes of antibiotics were placed on inoculated MHA and incubated at 37 °C for 18 h. The results from the inhibition zone were interpreted as resistant (R), intermediate (I) or susceptible (S) by referring to (CLSI, 2021). Each isolate's multiple antibiotic resistance (MAR) index was calculated by dividing the number of antibiotics it was resistant to by the total number of antibiotics tested (Mohammad Hamdi et al., 2023). The antibiotic discs used in this study are listed in Table 3.3.

Table 3.3: Antibiotic Disc Used for Antibiotic Susceptibility Test

| <b>Abbreviation</b> | <b>Antibiotic</b>       | <b>Concentration (µg)</b> |
|---------------------|-------------------------|---------------------------|
| AMP                 | Ampicillin              | 10                        |
| ATM                 | Aztreonam               | 30                        |
| C                   | Chloramphenicol         | 30                        |
| CAZ                 | Ceftazidime             | 30                        |
| CN                  | Gentamicin              | 10                        |
| E                   | Erythromycin            | 15                        |
| NOR                 | Norfloxacin             | 10                        |
| P                   | Penicillin              | 10 units                  |
| PRL                 | Piperacillin            | 100                       |
| S                   | Streptomycin            | 10                        |
| TE                  | Tetracycline            | 30                        |
| TIM                 | Ticarcillin-Clavulanate | 5                         |
| VA                  | Vancomycin              | 30                        |

### **3.12 In-Vitro Antibacterial Assay**

#### **3.12.1 Agar Well Diffusion Assay**

The antibacterial activity of crude extracts was determined using the agar well diffusion method, with several modifications, as described by Toh et al. (2023). Using a sterile cotton bud, the standardised bacteria were applied to the surface of MHA and allowed to dry for 15 min. For SE, the concentration of the extract was prepared at 10, 50, and 100 mg/mL. Meanwhile, UAE extracts concentration was prepared at 5, 10 and 25 mg/mL. Then, the bores were made using a cork borer (6 mm) and 50 µL of extracts with prepared concentration were added to the wells. In this assay, Vancomycin (30 µg) and Gentamicin (10 µg) served as a positive control for *Enterococcus* sp. and *Mammaliicoccus* sp., respectively, while DMSO (100%) (Nacalai Tesque, Japan) was used as a negative control. All plates were incubated at 37 °C for 18 h. The experiments were conducted in triplicate, and the diameter of the inhibition zone (mm) was recorded as the extracts' antibacterial activity.

#### **3.12.2 Minimum Inhibitory Concentration (MIC)**

A colourimetric broth microdilution assay was used to determine the MIC according to Nurunnabi et al. (2020) and CLSI (2021) standards with slight modifications. This method was performed by a two-fold serial dilution method. The bacterial suspension was standardised to 0.5 McFarland and further diluted to the ratio of 1:100 using MHB to obtain  $2.8 \times 10^6$  CFU/mL. In a 96 wells plate, 50 µL of MHB was dispensed into all wells. Then, 100 µL of 100 mg/mL of plant extracts was dispensed into the first column of wells from rows A to C for assays and rows D to F as the extract control (negative control). Meanwhile, Row H, columns 1 to 12, was used as growth control (positive control). As for antibiotic

control, 100  $\mu$ L of 128 mg/mL antibiotic was added into row G column 1. Penicillin (128 mg/mL) was the antibiotic control for *Enterococcus* sp., while Tetracycline (128 mg/mL) was used for *Mammaliicoccus* sp. A volume of 50  $\mu$ L from row A to G, column 1 was serially diluted until column 12 and discarded 50  $\mu$ L of the solution from column 12. Briefly, 30  $\mu$ L of MHB and 10  $\mu$ L of resazurin (0.02%) were added into all wells. After that, 10  $\mu$ L of bacterial suspensions was dispensed into all wells, excluding the extract control. For the extract control at rows D to F, columns 1 to 12, another 10  $\mu$ L of MHB was added. The plates were done in triplicate and incubated at 37 °C for 24 h. The wells that showed colour changes from purple to pink were recorded as positive. The lowest concentration for the colour change was recorded as the MIC endpoint value.

### **3.12.3 Minimum Bactericidal Concentration (MBC)**

After overnight incubation from 96 wells plates, the MBC was determined by transferring 10  $\mu$ L of each solution in the well into MHA (Asan et al., 2022). The solution in rows D to F was used as an extract control (negative control), while the solution in row H was used as a growth control (positive control). The plate was incubated for 24 h at 37 °C. The MBC value was determined by the lowest concentration that showed no bacterial growth on MHA plates. The test was done in triplicates.

### **3.13 Thin-Layer Chromatography**

The crude extracts that obtained the highest antibacterial activity from each plant part were subjected to thin layer chromatography (TLC) analysis according to Muhammad et al. (2021) with modifications. TLC was performed on silica gel G60 F<sub>254</sub> aluminium plates (Merck, Germany) using 1 mg of crude extract diluted in 1 mL of dichloromethane (Merck, Germany). A glass capillary tube was used to repeatedly apply samples to TLC plates (8 x 4

cm) with a 0.3 mm diameter area. The TLC plate was then positioned in a developing chamber with its lower marked edge (1 cm from the bottom) dipped into a developing solvent below the samples were spotted. The plates were allowed to develop to the upper mark (6 cm from the bottom) before being removed and allowed to dry. The separated compounds on TLC plates were then viewed directly under UV light (365 nm), and their  $R_f$  values were calculated. The developing solvent used was Hex: EtOAc: Chlo (8:2:1), according to Ogunlaja et al. (2022), with modifications.

### **3.14 Thin Layer Chromatography-Direct Bioautography Assay**

For bioautography, the bacteria were grown in MHB at 37 °C for 18 h and standardised to 0.5 McFarland standard solution using a spectrophotometer at 600 nm. This assay was done according to Jesionek et al. (2015) modifications. The developed plates were dipped for 10 seconds in standardised bacterial suspension before drying at room temperature for 2 min. The inoculated plates were then placed into a Petri Dish (150 × 15 mm) with wetted tissue paper and incubated at 37 °C for 17 h. After incubation, the plates were treated by spraying with an aqueous solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 2.5 mg/ml) (Sigma Aldrich, USA) and re-incubated for another 2 h. Dehydrogenase activity was detected using tetrazolium salt-based reagents to visualise the inhibition zones of the isolated compounds. A drop of Triton X-100 (Merck, Germany) was added per 10 mL of aqueous MTT solution to increase colour intensity. Metabolically active bacteria on the TLC plate transform the MTT into formazan dye, resulting in the inhibition zones appearing as pale spots against a dark blue background. The developing solvent Hex: EtOAc: Chlo (8:2:1) was used as the negative control. All tests were done in triplicate.

### **3.15 Semi-preparative Thin Layer Chromatography**

In this study, semi-preparative TLC was used for the isolation of antibacterial compounds identified in TLC-DB, according to Dar et al. (2015), with modifications. Using a tip with cotton wool at the end, 1mg/mL of crude extracts in dichloromethane was applied as 20 cm bands on TLC Silica gel 60G F<sub>254</sub> 25 Glass plates (Merck, Germany). The plates were then developed to a distance of 6 cm using the same developing solution as before. After drying, the separated compounds on TLC plates were viewed under UV light (365 nm) to confirm the presence of targeted compounds. The active compound on silica gel was scrapped and eluted with 20 mL of methanol. The eluted solvent was filtered through filter paper (Whatman No. 1, UK), allowed to dry in the fume hood and subjected to GC/MS for compound identification.

### **3.16 Gas Chromatography-Mass Spectrophotometry Analysis**

Gas chromatography-mass spectrometry (GC-MS) was used to determine the chemical constituents of the active compound isolated in the previous assay and conducted as described by Samling et al. (2021) with several modifications. Shimadzu GCMS QP2010 Plus, Japan, was used for the analysis, which included a quadrupole mass analyser and a non-polar GC BPX-5 cross-linked column (5% Phenyl Polysilphenylene-siloxane) of 30 m length, 0.25 mm internal diameter, and 0.25 m film thickness. Briefly, 1 µL of the sample (1mg/mL) was injected into GC-MS. The ionisation energy of the mass spectrophotometer was set at 70 eV. The temperature was programmed to rise from 50 °C to 260 °C at a rate of 10 °C/min and hold for 10 min. The injector and detector temperatures were both set at 280 °C. The chemical constituents found were identified and compared to the known compounds in the National Institute of Standards and Technology's (NIST) mass spectral library.

### 3.17 Statistical Analysis

In the data collection for extraction yield, TPC and TFC, univariate analysis of variance (ANOVA) was used to determine the relationship between a single numerical dependent variable with different control factors. The control factors were extraction techniques (SE and UAE), plant parts (leaves, stems and roots) and extraction solvents (Hex, EtOH and Chlo). The adjustment for multiple comparisons between each level was made by the Bonferroni method.

As for antibacterial activity using the agar well diffusion method, univariate ANOVA was also used. The control factors were plant parts (leaves, stems and roots), extraction solvents (EtOH and Chlo) and extracts concentrations (SE: 100, 50 and 10 mg/mL, while UAE: 25, 10 and 2 mg/mL). Meanwhile, for the determination of significant differences between SE and UAE, one-way ANOVA was performed on the data of antibacterial activity at the extract concentration of 10 mg/mL.

The Pearson correlation coefficient was also performed on the TPC and TFC to determine the strength and direction of the relationship between those two variables. In all statistical analyses, the significance of the difference between the mean of the test was established, and  $p$  value  $< 0.05$  was considered significant. All the analyses were performed using SPSS Statistics Software version 27.

## CHAPTER 4

### RESULTS AND DISCUSSION

#### 4.1 Yield of Extraction for the Crude Extract of *S. alata* Linn.

The extraction yield refers to the percentage of a group of compounds that are extracted from plants relative to the total amount of those compounds present in plants. This provides an idea of the plant's extractability under various conditions (Adam et al., 2019; Anigboro et al., 2022). The accumulation of bioactive compounds in crude extract is essential, particularly for determining their biological properties, such as antimicrobial activity (Dhanani et al., 2017). In addition, significant extraction efficacy can help to reduce solvent volume, dry sample amount, sampling time, energy costs, and extraction time per extraction (Zhang et al., 2018).

Therefore, it is important to choose the suitable extraction method and solvent based on the properties of the sample matrix, the chemical properties of the analytes, the way the matrix and analytes interact, how effectively they work, and the target properties (Dhanani et al., 2017). The present study used ethanol, chloroform, and hexane as extraction solvents, and employed soxhlet (SE) and ultrasonic assisted extraction (UAE) as the extraction techniques. Figure 4.1 shows the extraction yield of *S. alata*, utilising three different solvents and two extraction techniques for the extraction of leaves, stems, and roots.

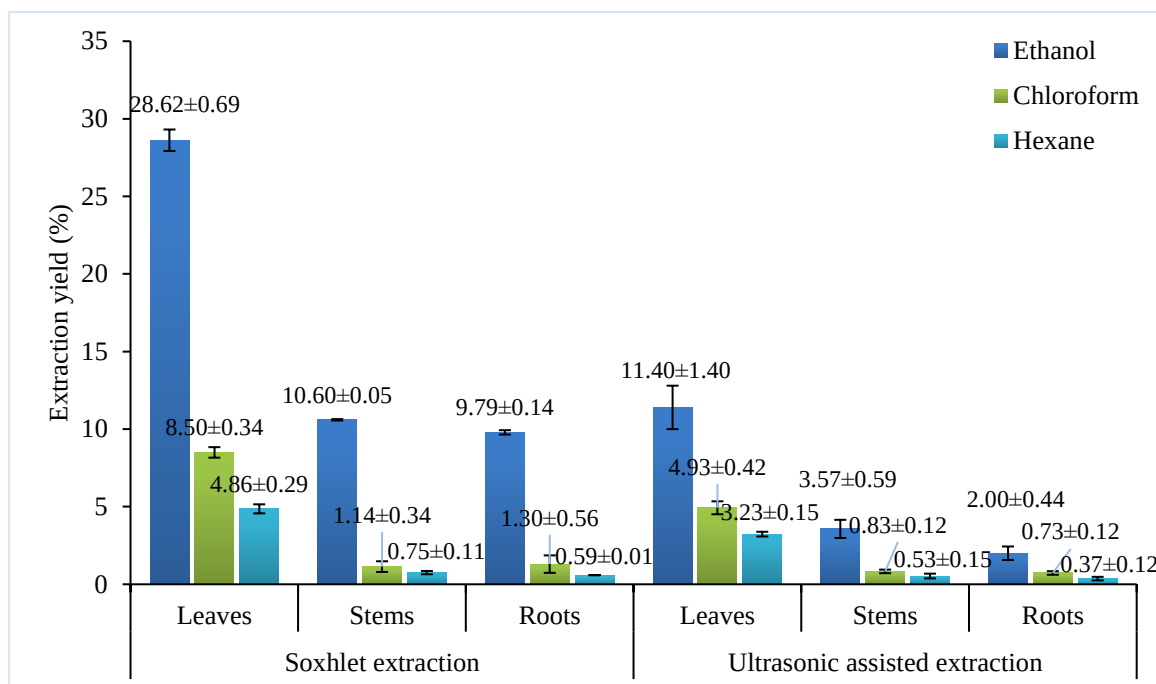


Figure 4.1: Extraction Yield (%) of Leaves, Stems and Roots of *S. alata* by Soxhlet Extraction and Ultrasonic Assisted Extraction Using Ethanol, Chloroform, and Hexane Solvents

In all plant parts, the extraction yield increased from solvents with low polarity to those with high polarity ( $p$  value < 0.001). For example, in leaves extracted using SE, the extraction yield was as follows: ethanol (28.62±0.69%) > chloroform (8.50±0.34%) > hexane (4.86±0.29%). A similar trend in results was reported by Kusumaningrum et al. (2023), who found that within 6 h of SE conducted, the Binahong leaves extract yielded the highest in ethanol (3.12%), chloroform (0.26%), and hexane (0.24%). Aside from that, in this study, the stems and roots showed a similar pattern to the leaves. An increased extraction yield of stems and roots were extracted using ethanol as compared to chloroform and hexane.

A greater yield in ethanol showed that the presence of polar compounds was higher in *S. alata*. This was due to the solvent's ability to dissolve the polar compounds of secondary metabolites. A compound with a close polarity with the solvent will readily dissolve, and a

maximum yield of the metabolites is highly obtained (Dirar et al., 2019). Therefore, the use of hexane in this study is not highly recommended because of the low yield production. Besides, hexane can be deemed harmful at high levels of exposure since it may have a neurotoxic effect (de Jesus et al., 2019). This is similar to methanol; it is highly flammable and toxic to humans if ingested, inhaled or in contact with the skin (Gonfa et al., 2020). Thus, ethanol and chloroform were used as the extraction solvent.

SE, on the other hand, produced greater yields than UAE extracts, regardless of solvent extraction and plant material used ( $p$  value < 0.001). A similar trend in extraction yield percentage was reported by Sharma and Cannoo (2017). For methanol and hexane extracts, the order of extraction yield was SE (12.16 and 1.10%) > UAE (9.69 and 1.03%) > maceration (M) (7.99 and 0.98%), whereas for chloroform extracts, the order was SE (3.08%) > M (2.47%) > UAE (2.35%). Another similar result was reported by Das et al. (2019), in which the leaves of *Piper betle* yielded the highest in SE (11.00%), followed by M (10.33%) and UAE (8.00%).

Compared to the UAE, the greatest percentage of extraction yield may be caused by the complete extraction of plant material by recurrent washings with a warm solvent in the case of SE. In addition, the use of high temperatures in SE reduces the surface tension and viscosity of the solvent, allowing the solvent to permeate deeper into the plant matrix and thereby increasing the extraction rate (Sharma & Cannoo, 2017). On the other hand, high temperatures aided in loosening the particles of the leaves, allowing the extract to dissolve readily in solvent (Muhamad et al., 2019).

Additionally, it was also observed that the quantity of dry samples extracted influenced the extraction yield. In this study, 12 g of dry sample was used in SE, while only 1 g of dry sample was used in UAE. In previous research by Ramandi et al. (2017), it was

determined that as the amount of *Borage officinalis* L flowers extracted increased, so did the extraction yield, regardless of the temperature used. For instance, at 50 °C, the yield obtained was 0.63%, 0.99%, and 1.03% for 20 mL/g, 30 mL/g, and 40 mL/g (ratio between extracting solvent and plant weight), respectively. A higher solvent-to-raw material ratio increases the concentration gradient during solid-to-solution diffusion and extraction yield. The solvent-to-solid ratio increases plant material swelling, increasing solvent contact surface area (Kaderides et al., 2019).

Besides that, the time of harvesting also affects the extraction yield. The plant starts from seed and will flower in about 6 to 7 months. According to Kamble et al. (2020), the best time to harvest the plant parts, especially leaves, is 6 months after planting. This is because active constituents are mostly abundant in leaves before flowering. After harvesting, the plant parts such as leaves, stems and roots are air-dried, grounded and finally stored in an airtight container at room temperature for further use (Ugbogu et al., 2016; Guerra et al., 2020). To conclude, it was proved that extraction techniques and extraction solvents greatly affect the production of yield (El Mannoubi, 2023). According to the univariate ANOVA, there were significant differences in the extraction yield influenced by the extraction techniques ( $F = 10.88.742$ ,  $p$  value  $< 0.001$ ), extraction solvents ( $F = 2105.712$ ,  $p$  value  $< 0.001$ ), and plant's parts ( $F = 1638.547$ ,  $p$  value  $< 0.001$ ).

The findings suggested that ethanol and SE served as good extraction solvent and technique for the production of higher yield, even though in SE, higher extraction temperatures, higher solid-to-solvent ratios, and longer extraction time were used (Garcia-Vaquero et al., 2020). This makes the UAE still considered in this study because of their advantages of using a small quantity of solid-to-solvent ratio and shorter extraction time (Bernardo et al., 2018). Thus, both SE and UAE were used as a choice of extraction

techniques for the study. As for extraction solvent, both ethanol and chloroform extracts were chosen. At the same time, hexane extract was removed because of low yield of extraction. This is because hexane has lower extractive efficiency of non-polar compounds in *S. alata* (Kusumaningrum et al., 2023).

## 4.2 Determination of Phytochemical Contents of *S. alata* Linn. Extracts

### 4.2.1 Total Phenolic Content

This study examined the total phenolic content (TPC) from the leaves, stems, and roots of *S. alata* using the Folic-Ciocalteu reagent. The values of TPC were obtained from the calibration curve  $y = 0.0013x + 0.0005$  with  $R^2 = 0.9963$ , where 'x' is the absorbance at 725 nm and 'y' is the concentration of a gallic acid solution (mg/mL) which can be referred in Appendix B2. The TPC values were expressed as mg GAE/g DW. The TPC values of the leaves, stems and roots of *S. alata* are shown in Figure 4.2.

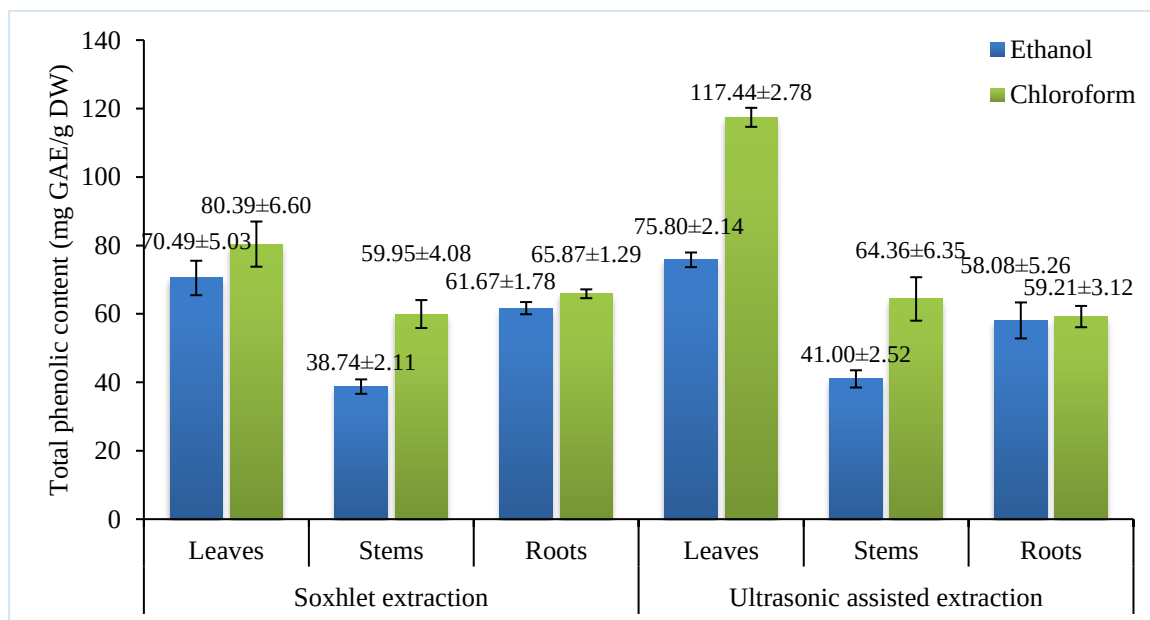


Figure 4.2: Total Phenolic Content (mg GAE/g DW) of Leaves, Stems and Root of *S. alata* Detected for Soxhlet Extraction and Ultrasonic Assisted Extraction Using Ethanol and Chloroform Solvents

Various TPC values were obtained from the 12 crude extracts due to different extraction techniques, solvent extractions, and parts of the plants extracted. According to the univariate ANOVA performed, all three factors were significant to the TPC, in which the plant's parts showed  $F = 244.309$  ( $p$  value  $< 0.001$ ), followed by extraction solvents ( $F = 161.480$ ,  $p$  value  $< 0.001$ ) and extraction techniques ( $F = 23.595$ ,  $p$  value  $< 0.001$ ).

In this study, leaves extracts showed the most significant high TPC, regardless of the extraction techniques and solvent used ( $p$  value  $< 0.001$ ). The highest was obtained from UAE-Chlo-L ( $117.44 \pm 2.78$  mg GAE/g DW), followed by SE-Chlo-L ( $80.39 \pm 6.60$  mg GAE/g DW), UAE-EtOH-L ( $75.80 \pm 2.14$  mg GAE/g DW), and SE-EtOH-L ( $70.49 \pm 5.03$  mg GAE/g DW). This was supported by Nurhaslina et al. (2019), which in *Physalis minima* Linn ethanol extract, TPC was found the highest in leaves, followed by whole plant, stems, roots, and fruits. Halim-Lim et al. (2020) also reported that the leaves of *S. alata* water extract showed higher TPC than barks and roots. The genetic determinants, changing growth conditions, especially nitrogen availability, environmental influences, and their interaction affect the plant parts, which may be explained by the phenolic compounds varied by its parts (Nantongo et al., 2018).

However, the lowest TPC was obtained from stems part, SE-EtOH-S ( $38.74 \pm 2.11$  mg GAE/g DW) and UAE-EtOH-S ( $41.00 \pm 2.52$  mg GAE/g DW), for both of extraction methods. Different plant parts may contain distinct phytochemical compounds, which may contribute to each part's unique pharmacological effect (Sembiring et al., 2018). The production of phenolics compounds is a common plant response to these stresses, as these compounds can absorb UV light, repair damaged tissues and deter pests and pathogens (Clement et al., 2021). The stems and roots are less exposed to these factors. Thus, the evolutionary pressure to produce high levels of phenolics is lower in stems and roots than in

the leaves of *S. alata* (Panchorkar & Patil, 2021). The leaves are more directly exposed to environmental stress factors such as UV radiation, pests and pathogens (Nantongo et al., 2018; Aguirre-Becerra et al., 2021). Therefore, the leaves of *S. alata* have high level of phenolic compounds than its stems and roots.

According to Lefebvre et al. (2021), regardless of the extraction method applied, the chemical characteristics of the extraction solvent were of the utmost significance for enhancing the dispersion of the compound, i.e. the extraction recovery and extraction preference. This included the solvent polarity index and the solubility of the target compounds in the extraction solvent (Nguyen et al., 2022). Compared to ethanol, chloroform was the optimal extraction solvent for phenolic recovery for all crude extracts ( $p < 0.001$ ). This was supported by Gan et al. (2017); in their study, the chloroform fraction of *Lepidium meyenii* had the maximum antioxidant activity and secondary metabolite concentration, indicating that chloroform is a good solvent for extracting antioxidant chemicals from plant materials. Similar to Lee et al. (2014), *Curcuma domestica* chloroform extract reported the highest TPC with 47.8 µg/mL. This halogenated solvent with medium polarity can isolate a wide range of chemicals from the plant material and was often used for non-targeted natural product isolation and analysis (Altemimi et al., 2017).

As for the extraction techniques, interestingly, UAE showed a better phenolic recovery with four out of six extracts in UAE, which had higher TPC than SE extracts ( $p$  value  $< 0.001$ ). This can be supported by Thilakarathna et al. (2023), which the TPC of *Madhuca longifolia* oil yield the highest in UAE (301.81 mg GAE/kg DW), M (121.20 mg GAE/kg DW), and SE (85.59 mg GAE/kg DW). The ultrasound treatment successfully damaged the plant cell wall, reducing particle size and increasing solvent penetration into the plant matrix. This increased the surface area of contact between the trapped bioactive

compounds and the solvent, improving extraction efficiency (Das et al., 2019). Long periods of high temperature exposure in SE may result in the destruction of bioactive chemicals in plants (Mohammadpour et al., 2019). Additionally, UAE is effective as it reduces process durations and temperatures, which is advantageous for the extraction of thermolabile compounds, such as phenolic compounds (Medina-Torres et al., 2017).

The screening of TPC was significant because a phenolic compound is one of the largest groups of secondary metabolites in plants. This compound participated in various biological properties, including anti-ageing (Dhalaria et al., 2020), anti-proliferative activities, and anti-inflammatory and antioxidant properties (Cianciosi et al., 2018; Cardoso et al., 2020). One or more hydroxyl groups bonded to the benzene ring in polyphenols. This structural property makes phenol more acidic than other alcohols. This chemical reaction serves as the reason for the antioxidant properties of polyphenols (Subramanian et al., 2015). Tanase et al. (2019) cited woody vascular plants, particularly bark, as a significant source of phenolic compounds with a possible biological effect. The proper solvent, extraction procedure, and conditions must be considered for phenolic recovery to produce more reliable results.

#### **4.2.2 Total Flavonoid Content**

The total flavonoid content (TFC) of all crude extracts from *S. alata* was also performed using an aluminium chloride colourimetric assay. The values of TFC were obtained from the calibration curve,  $y = 0.0003x - 0.001$  with  $R^2 = 0.9982$ , where 'x' is the absorbance at 414 nm and 'y' is the concentration of quercetin solution (mg/mL), which can be referred in Appendix C2. The TFC values were expressed as mg QE/g DW. The TFC values of the leaves, stems and roots of *S. alata* are shown in Figure 4.3.

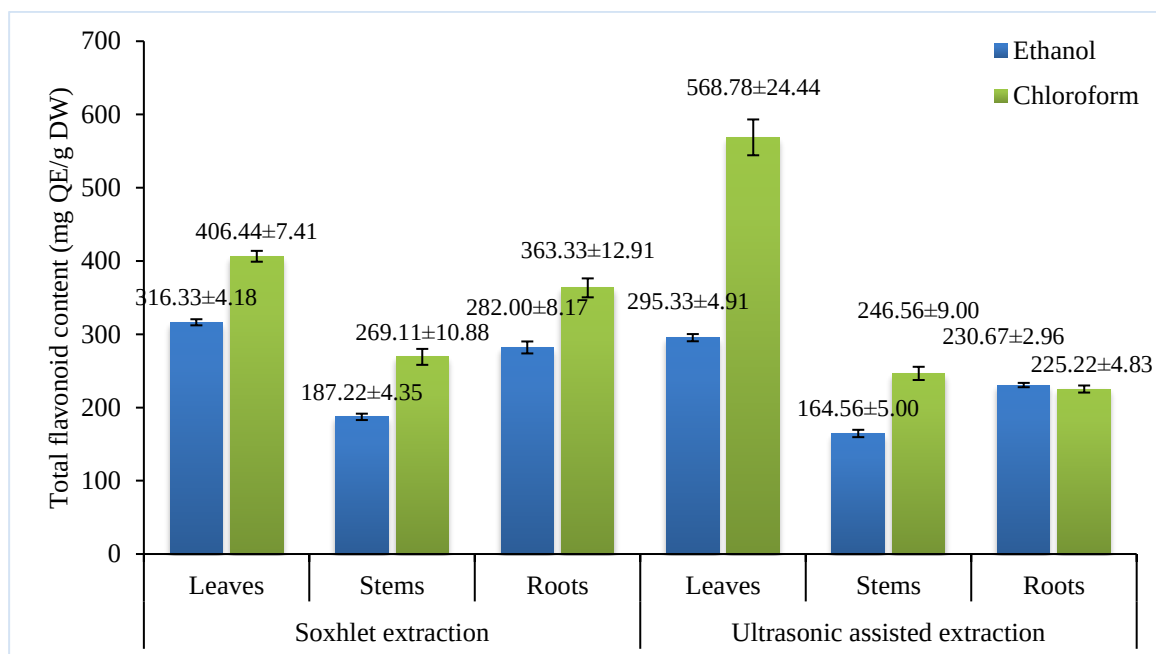


Figure 4.3: Total Flavonoid Content (mg QE/g DW) of Leaves, Stems and Roots of *S. alata* Detected for Soxhlet Extraction and Ultrasonic Assisted Extraction Using Ethanol and Chloroform Solvents

The flavonoid, however, belongs to a class of polyphenolic-structured secondary plant metabolites. Nearly two-thirds of dietary phenolic compounds are flavonoids, the most abundant and bioactive phenolic compounds in fruits and vegetables (de la Rosa et al., 2018). Furthermore, flavonoids have a variety of biological actions, including hepatoprotective, anti-inflammatory (Jiang et al., 2019), antibacterial, antioxidant (Li et al., 2019) and anti-hyperlipidaemic (Bencheikh et al., 2021). Similar to TPC results in Figure 4.2, the TFC values of *S. alata* were the highest in leaves and the lowest in stems for both SE and UAE. According to the Pearson correlation coefficient analysis, a positive correlation between TPC and TFC was observed, in which  $r = 0.949$  and  $p$  value  $< 0.01$ .

Similar in TPC, according to the univariate ANOVA performed, all the three factors showed significant differences to TFC in *S. alata*, in which plant's parts showed  $F = 1004.494$  ( $p$  value  $< 0.001$ ), followed by extraction solvents ( $F = 922.797$ ,  $p$  value  $< 0.001$ )

and extraction techniques ( $F = 24.112$ ,  $p$  value  $< 0.001$ ). As displayed in Figure 4.3, the highest was UAE-Chlo-L ( $568.78 \pm 24.44$  mg QE/g DW) and SE-Chlo-L ( $406.44 \pm 7.41$  mg QE/g DW). Interestingly, the third highest was from roots extract, SE-Chlo-R ( $363.33 \pm 12.91$  mg QE/g DW), followed by SE-EtOH-L ( $316.33 \pm 4.18$  mg QE/g DW) and UAE-EtOH-L ( $295.33 \pm 4.91$  mg QE/g DW). In the pairwise comparisons, all plant parts also showed a significant difference towards TPC, with a  $p$  value  $< 0.001$ . A study by Tanweer et al. (2020) also reported that the leaves part of the ginger extract contained the most flavonoid than rhizome and flower extracts.

As shown above, in comparison with ethanol, chloroform was still preferred as the choice for extraction solvent ( $p$  value  $< 0.001$ ). In other studies, polar and intermediate polarity solvents such as ethanol and acetone were commonly preferred as extraction solvents (Nguyen et al., 2022). However, a recent study found that a non-polar solvent, chloroform is a good solvent for extracting flavonoids. The utilisation of chloroform as the extraction solvent gave a good flavonoid recovery in all extracts except for UAE-Chlo-R ( $225.22$  mg QE/g DW). However, it did not show much difference with ethanol extract, UAE-EtOH-R, which was  $230.67$  mg QE/g DW. This suggests that the majority of flavonoids found in *S. alata* are non-polar; as a result, their extractability was enhanced in a non-polar solvent. This was supported by Zombe et al. (2022), the TFC of crude cashew nutshell liquid chloroform extract was higher than ethanol extract with  $27.2$  mg QE/g and  $26.1$  mg QE/g DW, respectively.

Unlike in TPC results, SE was more effective for flavonoid recovery. From the six crude extracts in SE, five of them showed a higher flavonoid than UAE ( $p$  value  $< 0.001$ ). Although SE was not effective in phenolic recovery, for flavonoid recovery, it was suggested that SE should be taken into consideration for its application in extracting flavonoids from

plants. However, the UAE is still competent because, despite using a minimal amount of sample and solvent, the extraction effectively extracted flavonoids. This was consistent with Das et al. (2019), who utilised a similar solid-to-solvent ratio for sonication, maceration, and soxhlet extraction, demonstrating that sonication recovers more flavonoids than other methods. According to Elboughdiri (2018) and Jovanović et al. (2021), when the solid-to-solvent ratio increased, the concentration gradient increased, which increased the diffusion rate of the compounds from the sample into the solvent.

To conclude, TFC values of *S. alata* were the highest in leaves for both SE and UAE. Also, the utilisation of chloroform as the extraction solvent gave a good flavonoid recovery in all extracts. This suggest that majority of flavonoids found in *S. alata* are non-polar compounds. Besides that, the stems and roots recorded the lowest value of TFC than leaves. This can be attributed to several factors such as metabolic activity and concentration of bioactive compounds in different parts of plants (Nantongo et al., 2018). The leaves are more directly exposed to environmental stress factors such as UV radiation, pests and pathogens. For instance, the leaves of *S. alata* has higher content of flavonoids in response to these stresses. This compound is crucial in plant's metabolism mechanisms such as attracting pollinators and protecting against UV radiation (Aguirre-Becerra et al., 2021). Therefore, leaves are rich sources of flavonoid than stem and roots.

### 4.3 Bacterial Molecular Identification and Antibiotic Susceptibility Profile

A series of molecular identification methods were performed to identify and confirm the bacterial isolates down to the genus and/or species level. In Figure 4.4, the product which a band size of around 550 bp indicated the presence of bacterial genomic DNA. The primer used in the 16S rRNA identification was 27F primer (5'-AGAGTTTGATCMTGGCTCAG-3'), and 519R primer (5'-GWATTACCGCGGCKGCTG-3'). At the domain taxonomic level, the coverage for the bacterial-specific 27F/515R primer set was 90.1% with a maximum of one mismatch, and it reached 79.6% with no mismatches permitted (Hathaway et al., 2021).

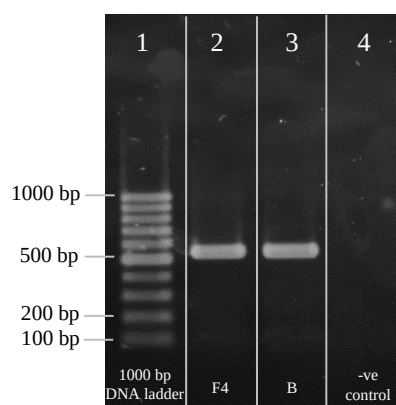


Figure 4.4: Amplified Bacterial DNA on Agarose Gel. Lane 1 - 1000 bp DNA Ladder. Lane 2 to 3 – PCR Product. Lane 4 – Negative Control

Table 4.1: Molecular Identification of Bacteria Using BLAST

| Isolates | Species                   | Family            | Identity (%) | Accession number |
|----------|---------------------------|-------------------|--------------|------------------|
| F4       | <i>Enterococcus</i> sp.   | Enterococcaceae   | 99.61        | MT044902.1       |
| B        | <i>Mammaliicoccus</i> sp. | Staphylococcaceae | 99.80        | MT527534.1       |

The test bacteria used in this study were obtained from the environmental bacterial collection stocks in the Molecular Laboratory, Institute of Biodiversity and Environmental

Conservation, UNIMAS and Department of Animal Science and Fisheries, Universiti Putra Malaysia, Bintulu Campus. Table 4.1 shows the molecular identification of the bacterial isolates identified by BLAST. It was identified that the isolates were *Enterococcus* sp. and *Mammaliicoccus* sp., with the identity matched by 99.61% and 99.80%, respectively. *Enterococcus* sp. is a gram-positive coccus that occurs singly, in pairs, in brief chains, or clusters and is a member of the Enterococcaceae family. *Mammaliicoccus* sp., on the other hand, is a Gram-positive, non-motile, non-sporulating cocci found singly, in pairs, and in irregular clusters (Belhout et al., 2022; Lienen et al., 2022).

Bacterial identification was necessary before determining their antibiotic susceptibility profile because different species within a genus can have vastly different pathogenicity and resistance profiles, causing a spectrum of infections of varying severity and necessitating distinct antibiotic treatment regimens. Antimicrobial susceptibility testing (AST) is the one method that reliably determines whether microorganisms are susceptible or resistant to a particular antimicrobial (Váradi et al., 2017). In this study, antibiotic discs for *Enterococcus* sp. and *Mammaliicoccus* sp. were selected by following the CLSI (2021) standard protocol.

Table 4.2: The Inhibition Zone (mm) of *Enterococcus* sp. and its MAR Index for Antibiotic Susceptibility Profile

| Bacteria                |                      | Antibiotic disc  |                 |                 |                  |                 |                  | MAR Index |
|-------------------------|----------------------|------------------|-----------------|-----------------|------------------|-----------------|------------------|-----------|
|                         |                      | AMP              | E               | TE              | NOR              | C               | VA               |           |
| <i>Enterococcus</i> sp. | Inhibition zone (mm) | 25.0±<br>0.0 (S) | 0.0±<br>0.0 (R) | 8.0±<br>0.0 (R) | 12.0±<br>0.0 (R) | 9.0±<br>0.0 (R) | 12.0±<br>0.0 (R) | 0.83      |

Antibiotics: AMP-Ampicillin (10 µg), E-Erythromycin (15 µg), TE-Tetracycline (30 µg), NOR-Norfloxacin (10 µg), C-Chloramphenicol (30 µg), and V-Vancomycin (30 µg)

Table 4.3: The Inhibition Zone (mm) of *Mammaliicoccus* sp. and its MAR Index for Antibiotic Susceptibility Profile

| Bacteria                  | Antibiotic disc      |                 |         |         |         | MAR Index |      |
|---------------------------|----------------------|-----------------|---------|---------|---------|-----------|------|
|                           | AMP                  | PRL             | TIM     | ATM     | S       |           |      |
| <i>Mammaliicoccus</i> sp. | Inhibition zone (mm) | 17.0±           | 17.0±   | 26.0±   | 0.0±    | 13.0±     | 0.40 |
|                           |                      | 0.0 (S)         | 0.0 (R) | 0.0 (S) | 0.0 (R) | 0.0 (I)   |      |
|                           |                      | Antibiotic disc |         |         |         |           |      |
|                           |                      | TE              | C       | CAZ     | CN      | P         |      |
|                           |                      | 24.0±           | 24.0±   | 14.0±   | 20.0±   | 20.0±     |      |
|                           |                      | 0.0 (S)         | 0.0 (S) | 0.0 (R) | 0.0 (S) | 0.0 (R)   |      |

Antibiotics: AMP-Ampicillin (10 µg), PRL-Piperacillin (100 µg), TIM-Ticarcillin-Clavulanate (5 µg), ATM-Aztreonam (30µg), S-Streptomycin (10 µg), TE-Tetracycline (30 µg), C-Chloramphenicol (30 µg), CAZ-Ceftazidime (30 µg), CN-Gentamicin (10 µg), and P-Penicillin (10 units)

Table 4.2 and Table 4.3 showed the antibiotic susceptibility profile for both *Enterococcus* sp. and *Mammaliicoccus* sp., respectively. The antibiotic susceptibility profiles were classified as "Resistant," "Intermediate," and "Susceptible". It appears that both of the bacteria tested were resistant to multiple antibiotics; in which the MAR value for *Enterococcus* sp. was 0.83, while for *Mammaliicoccus* sp., the MAR value was 0.40. Only ampicillin was susceptible to *Enterococcus* sp. among the six antibiotics tested, in which it resistant to erythromycin, norfloxacin, chloramphenicol, and vancomycin. In contrast, among the ten antibiotics tested, *Mammaliicoccus* sp. was resistant to piperacillin, aztreonam, ceftazidime, and penicillin. These results indicated that the bacteria examined were multiple antibiotic resistant (MAR), meaning that the isolate was resistant to more than two classes of antibiotics among all antibiotics tested (Mir et al., 2022).

Apart from that, these bacteria have a MAR index value of more than 0.2. Bacteria with a MAR value of 0.2 or higher are likely to have originated from highly antibiotic-dependent environments, such as those found in humans, pigs, dairy cattle, and commercial

poultry farms (Tangwa et al., 2019). Furthermore, it shows that plasmids containing one or more resistance genes, each of which encodes a distinct antibiotic resistance trait, are present, suggesting that these genes may have originated in the environment. Meanwhile, MAR index values below 0.2 are thought to have come from strains in animals that rarely or never take antibiotics (Afunwa et al., 2020). Although these isolates are part of the environmental bacteria, they pose a threat due to their role in the development of antibiotic resistance. Besides, there is a possibility that the antibiotic resistance gene spreads through horizontal gene transfer from the environment to humans (Tao et al., 2022). Therefore, the isolates were used as test organisms in this study.

#### **4.4 Antibacterial Activity of *S. alata* Linn.**

##### **4.4.1 Agar Well Diffusion Assay**

The antibacterial activity of all crude extracts from the leaves, stems, and roots of *S. alata* was performed using the agar well diffusion method. In this assay, various concentration of the extracts was prepared and tested against the MAR bacteria, *Enterococcus* sp. and *Mammaliicoccus* sp. For SE extract, the concentration of the extract tested was 10 mg/mL, 50 mg/mL and 100 mg/mL. Meanwhile, for the UAE extract, since the yield from UAE was lower, the concentration of the extract prepared was 2 mg/mL, 10 mg/mL and 25 mg/mL only. The inhibition zone of *Mammaliicoccus* sp. against SE extracts and UAE extracts was shown in Table 4.4 and Table 4.5, respectively. On the other hand, the inhibition zone of *Enterococcus* sp. against SE extracts and UAE extracts was presented in Table 4.6 and Table 4.7, respectively. The 100% DMSO negative control does not exhibit an inhibitory zone as DMSO does not hinder microbial growth (Wenji et al., 2019). The inhibitory zone created by *S. alata* extract is a result of its phytochemical components.

Table 4.4: Mean Zone of Inhibition (mm) by *S. alata* Crude Extracts from Soxhlet Extraction (mg/mL) Against MAR *Mammaliicoccus* sp.

| Extraction                                 | Parts  | Solvent    | Zone of inhibition (mm) |              |              |
|--------------------------------------------|--------|------------|-------------------------|--------------|--------------|
|                                            |        |            | 10 mg/mL                | 50 mg/mL     | 100 mg/mL    |
| Soxhlet extraction                         | Leaves | Ethanol    | 7.50 ± 0.00             | 10.00 ± 0.00 | 10.67 ± 0.58 |
|                                            |        | Chloroform | 8.00 ± 0.00             | 10.00 ± 0.00 | 11.00 ± 0.00 |
|                                            | Stems  | Ethanol    | 0.00 ± 0.00             | 7.67 ± 0.29  | 9.50 ± 0.00  |
|                                            |        | Chloroform | 9.00 ± 0.00             | 10.00 ± 0.00 | 15.00 ± 0.00 |
|                                            | Roots  | Ethanol    | 7.50 ± 0.00             | 10.00 ± 0.00 | 12.67 ± 0.00 |
|                                            |        | Chloroform | 8.50 ± 0.00             | 9.00 ± 0.00  | 13.00 ± 0.00 |
| Positive control: Gentamicin disc (10 µg)  |        |            |                         | 20.00 ± 0.00 |              |
| Negative control: 100% (v/v) DMSO solution |        |            |                         | 0.00 ± 0.00  |              |

Table 4.5: Mean Zone of Inhibition (mm) by *S. alata* Crude Extracts from Ultrasonic Assisted Extraction (mg/mL) Against MAR *Mammaliicoccus* sp.

| Extraction                                 | Parts  | Solvent    | Zone of inhibition (mm) |              |             |
|--------------------------------------------|--------|------------|-------------------------|--------------|-------------|
|                                            |        |            | 2 mg/mL                 | 10 mg/mL     | 25 mg/mL    |
| Ultrasonic assisted extraction             | Leaves | Ethanol    | 0.00 ± 0.00             | 0.00 ± 0.00  | 9.00 ± 0.00 |
|                                            |        | Chloroform | 0.00 ± 0.00             | 0.00 ± 0.00  | 8.50 ± 0.00 |
|                                            | Stems  | Ethanol    | 0.00 ± 0.00             | 0.00 ± 0.00  | 8.00 ± 0.00 |
|                                            |        | Chloroform | 0.00 ± 0.00             | 0.00 ± 0.00  | 8.17 ± 0.29 |
|                                            | Roots  | Ethanol    | 0.00 ± 0.00             | 0.00 ± 0.00  | 7.50 ± 0.00 |
|                                            |        | Chloroform | 0.00 ± 0.00             | 0.00 ± 0.00  | 8.00 ± 0.00 |
| Positive control: Gentamicin disc (10 µg)  |        |            |                         | 20.00 ± 0.00 |             |
| Negative control: 100% (v/v) DMSO solution |        |            |                         | 0.00 ± 0.00  |             |

For the *Mammaliicoccus* sp., the previous MAR Index value was 0.40 with a resistant activity towards piperacillin, aztreonam, ceftazidime and penicillin. In this assay, it was observed that the SE extracts exhibited good antibacterial activity against the

*Mammaliicoccus* sp. Among SE extracts, as shown in Figure 4.4, SE-Chlo-S exhibited the highest antibacterial activity with  $15.00 \pm 0.00$  mm at 100 mg/mL. It was followed by SE-Chlo-R, SE-EtOH-R and SE-Chlo-L with  $13.00 \pm 0.00$  mm,  $12.67 \pm 0.00$  mm and  $11.00 \pm 0.00$  mm at 100 mg/mL, respectively. In this assay, it was observed that none of the extracts, neither SE nor UAE, were able to exhibit antibacterial activity higher than the positive control, which was gentamicin, with  $20.00 \pm 0.00$  mm inhibition. The example of observations from agar well diffusion for *Mammaliicoccus* sp. is shown in Appendix D2.

In contrast, the UAE extracts showed lower antibacterial activity against the *Mammaliicoccus* sp., as shown in Figure 4.5. Among the UAE extracts, UAE-EtOH-R exhibited the least antibacterial activity, with  $7.50 \pm 0.00$  mm at 25 mg/mL. According to the one-way ANOVA used in this study for *Mammaliicoccus* sp. (10 mg/mL), when it was subjected to the extraction methods, there was a significant effect in the inhibition zone of bacteria, with  $F = 82.437$  and  $p$  value  $< 0.001$ . In SE extracts, at 10 mg/mL, SE-EtOH-R showed a similar inhibition zone,  $7.50 \pm 0.00$  mm, which noted that the extracts from SE had a better antibacterial activity than the extracts from UAE.

It has been shown that the antimicrobial efficacy of the *S. alata* extracts correlates with their TFC from the previous assays. The high TFC was obtained by the SE. Furthermore, many flavonoids have demonstrated anti-infective properties by forming interactions with various proteins within bacterial cell walls or extracellular proteins (Mahboubi et al., 2015). For example, in *Mammaliicoccus* sp., the inhibition by SE-Chlo-R was  $13.00 \pm 0.00$  mm (100 mg/mL), while the TFC was 363.33 mg QE/g DW. The other extract such as UAE-EtOH-R, inhibited *Mammaliicoccus* sp. the lowest by  $7.50 \pm 0.00$  mm (100mg/mL), while the TFC obtained was 230.67 mg QE/g DW. Such a relation between antibacterial effects with flavonoid content suggested that the high antimicrobial activity in

SE, and low antimicrobial activity in UAE were due to the presence of flavonoids in *S. alata* extracts.

Table 4.6: Mean Zone of Inhibition (mm) by *S. alata* Crude Extracts from Soxhlet Extraction (mg/mL) Against MAR *Enterococcus* sp.

| Extraction                                 | Parts  | Solvent    | Zone of inhibition (mm) |              |             |
|--------------------------------------------|--------|------------|-------------------------|--------------|-------------|
|                                            |        |            | 10 mg/mL                | 50 mg/mL     | 100 mg/mL   |
| Soxhlet extraction                         | Leaves | Ethanol    | 0.00 ± 0.00             | 0.00 ± 0.00  | 0.00 ± 0.00 |
|                                            |        | Chloroform | 0.00 ± 0.00             | 0.00 ± 0.00  | 0.00 ± 0.00 |
|                                            | Stems  | Ethanol    | 0.00 ± 0.00             | 0.00 ± 0.00  | 7.50 ± 0.00 |
|                                            |        | Chloroform | 0.00 ± 0.00             | 0.00 ± 0.00  | 0.00 ± 0.00 |
|                                            | Roots  | Ethanol    | 0.00 ± 0.00             | 0.00 ± 0.00  | 7.50 ± 0.00 |
|                                            |        | Chloroform | 0.00 ± 0.00             | 0.00 ± 0.00  | 0.00 ± 0.00 |
| Positive control: Vancomycin disc (30 µg)  |        |            |                         | 12.00 ± 0.00 |             |
| Negative control: 100% (v/v) DMSO solution |        |            |                         | 0.00 ± 0.00  |             |

Table 4.7: Mean Zone of Inhibition (mm) by *S. alata* Crude Extracts from Ultrasonic Assisted Extraction (mg/mL) Against MAR *Enterococcus* sp.

| Extraction                                 | Parts  | Solvent    | Inhibition zone (mm) |              |             |
|--------------------------------------------|--------|------------|----------------------|--------------|-------------|
|                                            |        |            | 2 mg/mL              | 10 mg/mL     | 25 mg/mL    |
| Ultrasonic assisted extraction             | Leaves | Ethanol    | 0.00 ± 0.00          | 0.00 ± 0.00  | 0.00 ± 0.00 |
|                                            |        | Chloroform | 0.00 ± 0.00          | 0.00 ± 0.00  | 0.00 ± 0.00 |
|                                            | Stems  | Ethanol    | 0.00 ± 0.00          | 0.00 ± 0.00  | 0.00 ± 0.00 |
|                                            |        | Chloroform | 0.00 ± 0.00          | 0.00 ± 0.00  | 0.00 ± 0.00 |
|                                            | Roots  | Ethanol    | 0.00 ± 0.00          | 0.00 ± 0.00  | 0.00 ± 0.00 |
|                                            |        | Chloroform | 0.00 ± 0.00          | 0.00 ± 0.00  | 0.00 ± 0.00 |
| Positive control: Vancomycin disc (30 µg)  |        |            |                      | 12.00 ± 0.00 |             |
| Negative control: 100% (v/v) DMSO solution |        |            |                      | 0.00 ± 0.00  |             |

Meanwhile, among the SE extracts as shown in Figure 4.6, it was observed that only SE-EtOH-R and SE-EtOH-S were showing antibacterial activity against *Enterococcus* sp., with inhibition of  $7.50 \pm 0.00$  mm for both at 100 mg/mL. In contrast, as shown in Figure 4.7, none of the extracts from the UAE showed antibacterial activity. It was also noted that none of SE-EtOH-R and SE-EtOH-S exhibited antibacterial activity higher than the positive control, vancomycin, with  $12.00 \pm 0.00$  mm inhibition. This indicated that the extracts in this study were not strong enough to inhibit *Enterococcus* sp., which had the highest MAR value of 0.83, and showed a resistant activity towards erythromycin, tetracycline, norfloxacin, chloramphenicol and vancomycin. The example of observations on agar well diffusion for *Enterococcus* sp. is shown in Appendix D1.

Apart from that, univariate ANOVA was conducted on *Mammaliicoccus* sp. to determine if other factors, such as parts of the plants, extraction solvents and extract concentrations, affect its inhibition zone. As for *Enterococcus* sp., no statistical analysis was performed because only a few and no inhibition was reported. In this study, all three factors were found to significantly affect the inhibition of *Mammaliicoccus* sp., with  $p$  value  $< 0.001$ . Among these factors, the concentration of the extract was found to influence the inhibition zone the most ( $F = 2946.333$ ), followed by the choice of extraction solvents ( $F = 1296.000$ ) and parts of the plants ( $F = 277.000$ ). Similar trends were observed in UAE, except no significant differences in inhibition zone were observed from the extraction solvents ( $F = 1.000$ ,  $p$  value = 0.324).

In the pairwise comparisons, all parts of the plants showed a mean significant difference at  $p$  value  $< 0.001$ . It can be deduced that the crude extracts from leaves, stems, and roots of *S. alata* had various degrees of antibacterial performance, which could be due to their availability and the number of bioactive compounds. The extracts with high

antibacterial activity likely have a significant number of bioactive compounds. On top of that, the extracts with low and no antibacterial activity may contain bioactive compounds as well, but in lesser numbers or not exhibiting antibacterial activity (Tolulope et al., 2015).

Between the SE and UAE, extracts from the SE exhibited antibacterial activity efficiently. In other studies where SE and ethanol were used, *Staphylococcus* sp. was inhibited by *S. alata* leaves extract with 10.85 mm and 14.10 mm at the concentration of 50 mg/mL and 100 mg/mL, respectively (Ekpiken et al., 2021). This was in line with the recent study, in which the SE-EtOH-L inhibited *Mammaliicoccus* sp. with  $10.00 \pm 0.00$  mm and  $10.67 \pm 0.58$  mm at the concentrations of 50 mg/mL and 100 mg/mL.

However, the UAE extracts in this study were less effective in exhibiting antibacterial activity. Based on studies by Mar'ie et al. (2022), the extract of *S. alata* stems by UAE and ethanol exhibited *S. aureus* at 25% concentration with 17.6 mm. In the phytochemical study, the same plant component had different antibacterial properties. The concentration of plant constituents of the same plant part can vary depending on the age of the plant, topographical factors, soil nutrients, extraction method, and antimicrobial study method (Vu et al., 2018; Alara et al., 2021). In addition, the bacteria status in this study was a multiple antibiotic resistant bacterium, suggesting that a stronger extract concentration is needed (Li et al., 2017).

Among the extraction solvents utilized, chloroform and ethanol extracts shown strong antibacterial activity, particularly against *Mammaliicoccus* sp. Pairwise comparisons revealed a substantial difference in the inhibitory zone between ethanol and chloroform extracts in SE ( $p < 0.001$ ). The majority of chloroform extracts exhibited the strongest inhibition. Alam et al. (2009) found that the chloroform extract from both the leaves and the stems of *S. alata* had antibacterial activity against *Bacillus subtilis*, whereas the ethanol

extract had no inhibitory effect. However, when tested with *S. aureus*, both ethanol extracts from leaves and stems showed antibacterial action, although chloroform extracts did not.

This shows that different phytoconstituents are soluble to different degrees in different solvents based on their polarity (Vivekanandan, 2018). Metsämuuronen & Siren (2014) state that antibacterial activity can result from various antibacterial compounds or a synergistic effect of multiple compounds. The presence of a significant quantity of phenolics and flavonoids in the extracts may be one of the reasons for its activity. To form structures with the walls of bacteria cells, flavonoids may have antibacterial activity due to their capability to create structures with extracellular and soluble proteins (Cowan, 2016) and to suppress pathogenic quorum-sensing signal receptors, enzymes, and toxins (Cushnie & Lamb, 2011).

#### **4.4.2 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration**

The previous antimicrobial activities using agar well diffusion assay were performed to obtain the preliminary observation on the crude extract with possible bioactive compounds that inhibited *Enterococcus* sp. and *Mammaliicoccus* sp. growth. The drug diffusion interference in the agar well diffusion experiment makes it unreliable for accurate crude extract assessment and comparison (Panda et al., 2011). Thus, the evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of all crude extracts from leaves, stems and roots of *S. alata* were performed to study their antibacterial activity further. Table 4.8 and Table 4.9 presented the MIC and MBC of *S. alata* crude extracts against *Enterococcus* sp. and *Mammaliicoccus* sp., respectively.

The MIC is referred to as the minimum concentration for the *S. alata* extract to inhibit the growth of bacteria. The MIC of the extracts was performed by colourimetric broth microdilution assay according to Nurunnabi et al. (2020) and CLSI (2021) standards with

slight modifications. The MIC endpoint was determined by observing the colour changes in wells from purple to pink. However, since the viscosity of the extracts was high at higher concentration, the colour from the tested extracts (Row A to C) were compared with the extracts control (Row D to F). The example of the observation from MIC values for *Enterococcus* sp. was shown in Figure 4.5, whereas for *Mammaliicoccus* sp. in Figure 4.6.

Table 4.8: Minimum Inhibitory Concentration, Minimum Bactericidal Concentration and MBC/MIC Ratio of *S. alata* Crude Extracts Against MAR *Enterococcus* sp.

| Extraction                           | Parts  | Solvent    | MIC<br>(mg/mL) | MBC<br>(mg/mL) | MBC/MIC<br>ratio |
|--------------------------------------|--------|------------|----------------|----------------|------------------|
| Soxhlet<br>extraction                | Leaves | Ethanol    | 6.25           | 25.0           | 4                |
|                                      |        | Chloroform | 12.5           | 25.0           | 2                |
|                                      | Stems  | Ethanol    | 3.125          | 12.5           | 4                |
|                                      |        | Chloroform | 3.125          | 3.125          | 1                |
|                                      | Roots  | Ethanol    | 1.5625         | 3.125          | 2                |
|                                      |        | Chloroform | 3.125          | 3.125          | 1                |
| Ultrasonic<br>assisted<br>extraction | Leaves | Ethanol    | 12.5           | 12.5           | 1                |
|                                      |        | Chloroform | 12.5           | 12.5           | 1                |
|                                      | Stems  | Ethanol    | 6.25           | 12.5           | 2                |
|                                      |        | Chloroform | 6.25           | 6.25           | 1                |
|                                      | Roots  | Ethanol    | 6.25           | 12.5           | 2                |
|                                      |        | Chloroform | 6.25           | 12.5           | 2                |
| Antibiotic control                   |        | Penicillin | 0.5            | 1.0            | 2                |



Figure 4.5: Colorimetric Broth Microdilution Using 96-Wells Plate for MIC of Soxhlet Extracts Using Ethanol (SE-EtOH) Against MAR *Enterococcus* sp. (L: Leaves, S: Stems, R: Roots)

Table 4.9: Minimum Inhibitory Concentration, Minimum Bactericidal Concentration and MBC/MIC Ratio of *S. alata* Crude Extracts Against MAR *Mammaliicoccus* sp.

| Extraction                           | Parts        | Solvent    | MIC<br>(mg/mL) | MBC<br>(mg/mL) | MBC/MIC<br>ratio |
|--------------------------------------|--------------|------------|----------------|----------------|------------------|
| Soxhlet<br>extraction                | Leaves       | Ethanol    | 6.25           | 3.125          | 0.5              |
|                                      |              | Chloroform | 6.25           | 6.25           | 1                |
|                                      | Stems        | Ethanol    | 3.125          | 3.125          | 1                |
|                                      |              | Chloroform | 0.78125        | 1.5625         | 2                |
|                                      | Roots        | Ethanol    | 1.5625         | 1.5625         | 1                |
|                                      |              | Chloroform | 0.390625       | 3.125          | 1                |
| Ultrasonic<br>assisted<br>extraction | Leaves       | Ethanol    | 12.5           | 6.25           | 0.5              |
|                                      |              | Chloroform | 6.25           | 6.25           | 1                |
|                                      | Stems        | Ethanol    | 3.125          | 6.25           | 2                |
|                                      |              | Chloroform | 3.125          | 6.25           | 1                |
|                                      | Roots        | Ethanol    | 3.125          | 6.25           | 1                |
|                                      |              | Chloroform | 3.125          | 3.125          | 1                |
| Antibiotic control                   | Tetracycline |            | 0.25           | 0.25           | 1                |



Figure 4.6: Colorimetric Broth Microdilution Using 96-Wells Plate for MIC of Soxhlet Extracts Using Ethanol (SE-Chlo) Against MAR *Mammaliicoccus* sp. (L: Leaves, S: Stems, R: Roots)

From Table 4.8, SE-EtOH-R (1.5625 mg/mL) exhibited the lowest MIC value against the *Enterococcus* sp. Then, it was followed by SE-Chlo-R, SE-Chlo-S and SE-EtOH-S, which all had similar MIC values at 3.125 mg/mL. The MIC range identified in SE was from 1.5625 mg/mL to 12.5 mg/mL. In UAE extracts, the MIC value of all extracts against *Enterococcus* sp. ranged from 6.25 mg/mL to 12.5 mg/mL. In other studies, the MIC value of SE-EtOH-R against *S. aureus* was 1.250 µg/mL, much lower than in this study (Toh et al., 2023). This suggested that a stronger concentration of extract is needed to fight the multiple antibiotic resistant bacteria in this study (Li et al., 2017).

On the other hand, as shown in Table 4.9, the MIC value for SE extracts ranged from 0.390625 mg/mL to 6.25 mg/mL for *Mammaliicoccus* sp. For UAE extracts, the MIC value ranged from 3.125 mg/mL to 12.5 mg/mL. The lowest MIC value was identified in SE-Chlo-R with 0.390625 mg/mL, which in the previous agar well diffusion assay with the highest inhibition. Then, the MIC value was followed by SE-Chlo-S and SE-EtOH-R with 0.78125 mg/mL and 1.5625 mg/mL, respectively. Similar to *Enterococcus* sp., it was identified that

SE accompanied with either ethanol or chloroform was suitable for the extraction of *S. alata* with antibacterial activity.

Next, the MBC value was determined by the lowest concentration that showed no bacterial growth on MHA plates. It was further expressed in the ratio of MBC/MIC to characterise their antibacterial properties. The example of the observation of MBC values for *Enterococcus* sp. was shown in Figure 4.7, whereas *Mammaliicoccus* sp. was shown in Figure 4.8. For *Enterococcus* sp., the MBC value ranged from 3.25 mg/mL to 25.0 mg/mL for SE, and 6.25 to 12.5 mg/mL for UAE. Meanwhile, the MBC value for *Mammaliicoccus* sp. ranged from 1.5625 mg/mL to 6.25 mg/mL for SE, whereas 3.125 mg/mL to 6.25 mg/mL for UAE. It was identified that SE-EtOH-R and SE-Chlo-S exhibited the lowest MBC with 1.5625 mg/mL.

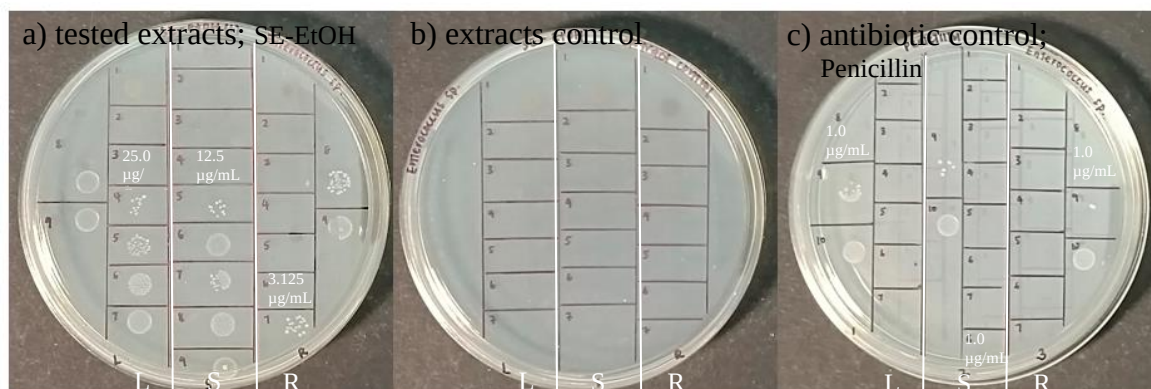


Figure 4.7: Observation of MBC of Soxhlet Extracts Using Ethanol Against MAR *Enterococcus* sp. a) Tested Extracts; SE-EtOH. b) Extracts Control. c) Antibiotic Control; Penicillin (L: Leaves, S: Stems, R: Roots)

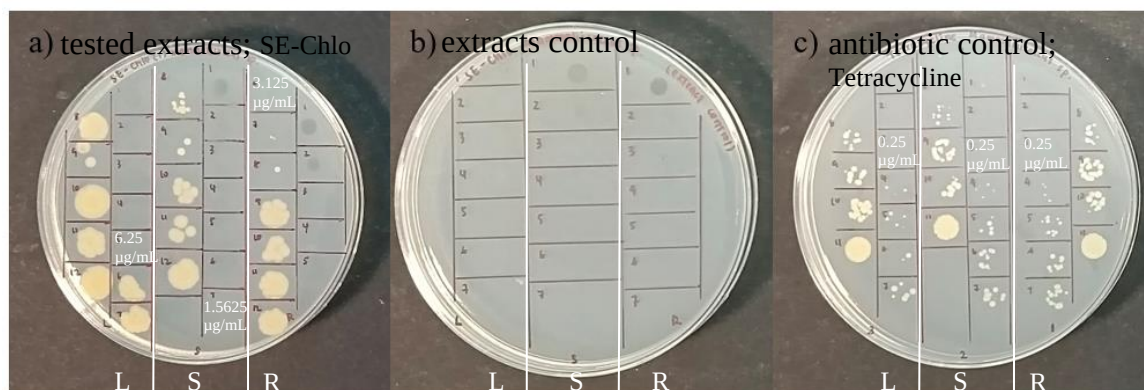


Figure 4.8: Observation of MBC of Soxhlet Extracts Using Ethanol Against MAR *Mammaliicoccus* sp. a) Tested Extracts; SE-Chlo. b) Extracts Control. c) Antibiotic Control; Tetracycline (L: Leaves, S: Stems, R: Roots)

In this assay, the MBC/MIC ratio for *S. alata* was identified between the range of 0.5 to 4. If the MBC/MIC ratio is below or equivalent to 4, the extracts are bactericidal, whereas those with a value above 4 are considered bacteriostatic (Keepers et al., 2014). Thus, all the crude extracts from *S. alata* in this study were identified as bactericidal. This was in line with a previous study by Toh et al. (2023), in which most bactericidal agents (MBC/MIC ratio  $\leq 4$ ) were generated by maceration and SE of *S. alata* stems, leaves, and roots using n-hexane, ethyl acetate, and ethanol solvents.

#### 4.5 Thin Layer Chromatography

The extracts, SE-Chlo-L, SE-Chlo-S and SE-Chlo-R, that showed the highest antibacterial activity in the previous assay were subjected to the separation of the compound using a thin layer chromatography technique. Hex: EtOAc: Chlo (8:2:1) was the solvent system used to separate compounds in all extracts. Figure 4.9 shows the observed TLC plates under UV light at 365 nm, while Table 4.10 shows the  $R_f$  values calculated from each identified spot.

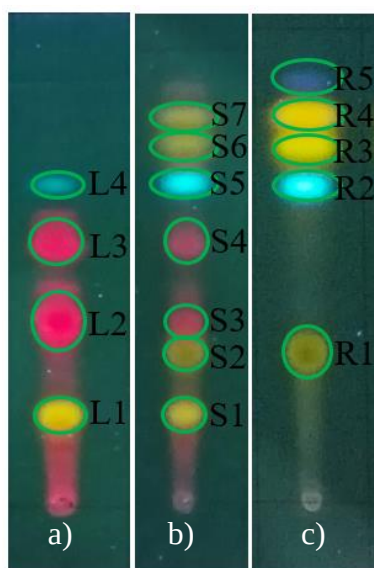


Figure 4.9: TLC Plates Viewed Under Long UV Light at 365 nm. a) SE-Chlo-L. b) SE-Chlo-S. c) SE-Chlo-R

There were 4 spots spotted from the SE-Chlo-L extract, 7 spots from the SE-Chlo-S extract, and 5 spots from the SE-Chlo-R extract. Among these spots, several spots from different extracts were similar in colour and  $R_f$  values, which were likely the same compound, though this was not necessary (Sanjeet et al., 2013). This includes L1 and S1 with  $R_f$  values = 0.23. Other spots, such as S5, S6 and S7, were similar to R2, R3 and R4, respectively. Their  $R_f$  values were 0.80, 0.87 and 0.92. L4 spots, on the other hand, were also similar to S5 and R4, with  $R_f$  values = 0.80.

Table 4.10: R<sub>f</sub> Values of Spots Spotted Under Long UV Light at 365 nm

| Extracts  | Spots | Colour of spots | R <sub>f</sub> values |
|-----------|-------|-----------------|-----------------------|
| SE-Chlo-L | L1    | Yellow          | 0.23                  |
|           | L2    | Red             | 0.47                  |
|           | L3    | Yellow          | 0.67                  |
|           | L4    | Light blue      | 0.80                  |
| SE-Chlo-S | S1    | Yellow          | 0.23                  |
|           | S2    | Yellow          | 0.40                  |
|           | S3    | Red             | 0.50                  |
|           | S4    | Red             | 0.72                  |
|           | S5    | Light blue      | 0.80                  |
|           | S6    | Yellow          | 0.87                  |
|           | S7    | Yellow          | 0.92                  |
| SE-Chlo-R | R1    | Yellow          | 0.42                  |
|           | R2    | Light blue      | 0.80                  |
|           | R3    | Yellow          | 0.87                  |
|           | R4    | Yellow          | 0.92                  |
|           | R5    | Dark blue       | 0.97                  |

The application of TLC is essential in analysing drugs and is frequently used for targeting medicinal plants in search of various physiological properties of botanical materials (Kowalska & Sajewicz, 2022). Observations under 365 nm revealed that TLC plates appeared with dark-coloured chemical compounds as a background emission. The occurrence of conjugated double bonds and aromatic was often found within a wavelength between 200 and 400 nm (Doskočil et al., 2018). These chemical compounds may have biological activities, and the crude extract's mixture may boost the active component's additive or synergistic effects, while others may be inert or inhibitory (Dhankhar et al., 2012).

#### 4.6 Thin Layer Chromatography - Direct Bioautography

The detection of antibacterial compounds by TLC-direct bioautography assay was performed on *Enterococcus* sp. and *Mammaliicoccus* sp. A chromatography, in conjunction with a bioassay consisting of a TLC-bioautography experiment, was used to identify the occurrence of bioactive compounds that developed from chromatograms. The chromatographic separation of microbial extracts improved the purity of bioactive compounds' purity and helped with partial identification using the dereplication technique. Fractionated active metabolites on the chromatogram inhibit the growth of bacteria, creating a clear white zone (Nuthan et al., 2020). A dehydrogenase activity-detecting reagent (tetrazolium salt) was employed to visualise the inhibition zones. The biologically active bacteria change it into the highly coloured formazan that goes with it (Dewanjee et al., 2015).

The detection of antibacterial compounds through the TLC-DB confirmed the presence of bioactive compounds in all three extracts. The  $R_f$  values from the inhibition spots (zones that appeared as clear or pale white) were compared with the  $R_f$  values of identified spots from the previous TLC in Table 4.9. The TLC-DB plates of *S. alata* extracts against *Enterococcus* sp. and *Mammaliicoccus* sp. are shown in Figure 4.10 and Figure 4.11. One inhibition spot was spotted on each TLC-DB plate; L2 from SE-Chlo-L plate ( $R_f = 0.47$ ), S2 from SE-Chlo-S plate ( $R_f = 0.40$ ), and R1 from SE-Chlo-R plate ( $R_f = 0.42$ ).

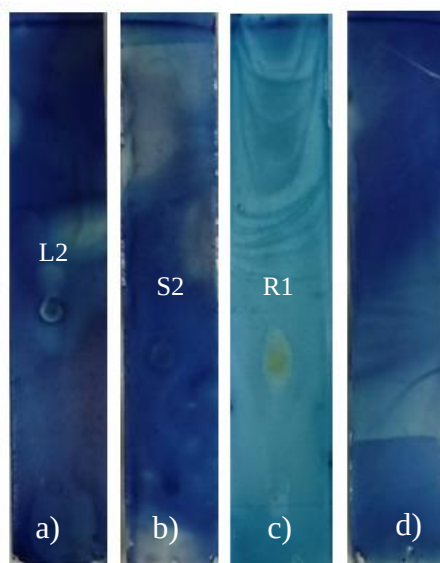


Figure 4.10: TLC-DB of *S. alata* Extracts Against *Enterococcus* sp. After 24 h Incubation and Application of MTT Solution. a) SE-Chlo-L. b) SE-Chlo-S. c) SE-Chlo-R. d) Control - Hex: EtOAc: Chlo (8:2:1)

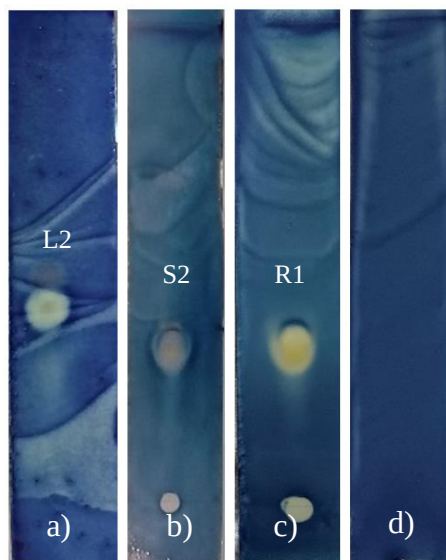


Figure 4.11: TLC-DB of *S. alata* Extracts Against *Mammaliicoccus* sp. After 24 h Incubation and Application of MTT solution. a) SE-Chlo-L. b) SE-Chlo-S. c) SE-Chlo-R. d) Control - Hex: EtOAc: Chlo (8:2:1)

As shown in Figure 4.10 and Figure 4.11, the inhibition spots identified in both *Enterococcus* sp. and *Mammaliicoccus* sp. were at similar locations. This explained that the potential compounds responsible for the previous antibacterial activity likely came from similar compounds (Sanjeet et al., 2013). In this study, polar compounds were exhibited with inhibition among the spot identified. Based on the previous study by Salem et al. (2021), the polar fraction of star anise could be utilised in the biopharmaceutical industry as a potent antibiotic and antioxidant.

However, there was a difference in the intensity of the inhibition spots on TLC-DB plates. In Figure 4.11, the inhibition spots for *Mammaliicoccus* sp. appeared clear, and the antibacterial activity observed was likely stronger. Unlike in Figure 4.10, the inhibition spots showed on TLC-DB plates were very weak, almost not resulting in any activity. This was in line with the previous antibacterial activity, in which a greater inhibition zone was observed on the *Mammaliicoccus* sp. in agar well diffusion assays. In other circumstances, no microbe growth suppression was detected due to evaporation, photo-oxidation, or a low amount of active chemicals (Belaqziz et al., 2017). In extracts that were active when the least inhibitory concentration of the mixture was calculated but lacked antibacterial activity when isolated by bioautography, synergism may be important (Mundy et al., 2016).

Bioautography cannot be relied upon as a reliable method for quantifying antibiotic efficacy. It only indicates the number of identified antibacterial active compounds (Horváth et al., 2010). The fact that *Mammaliicoccus* sp. had the greatest inhibition against the extract does not necessarily indicate that it was the most susceptible bacterial species because different compounds have varying degrees of activity and were selective against bacteria and fungi (Suleiman et al., 2010).

#### **4.7 Identification of Bioactive Compounds Using GC-MS Analysis**

The bioactive compounds in active spots from the TLC-DB assays were identified using the Gas Chromatography-Mass Spectrometry (GC-MS) analysis. This analysis determined the name, molecular formula, molecular weight, retention time, area (%) and biological properties of the bioactive compounds. The mass spectrum of the unknown compound was compared with the spectrum of the known compounds from the National Institute of Standards and Technology 2017 (NIST 2017, USA) database.

The bioactive compounds present in isolate L2, isolate S2 and isolate R1 are shown in Table 4.11, Table 4.12 and Table 4.13, respectively. There was a total of 16 compounds from isolate L2 (SE-Chlo-L), another 16 compounds from isolate S2 (SE-Chlo-S), and 7 compounds from isolate R1 (SE-Chlo-R). Among these compounds, 13 compounds from isolate L2, 11 from isolate S2, and 7 from isolate R1 had been reported with biological properties.

Table 4.11: Major Bioactive Compounds Identified in Isolate L2 from SE-Chlo-L Extract of *S. alata* and its Biological Activities

| Spots | Compound name                          | Molecular formula                 | Molecular weight | RT (min) | Area (%) | Reported biological activities                                                                                               |
|-------|----------------------------------------|-----------------------------------|------------------|----------|----------|------------------------------------------------------------------------------------------------------------------------------|
| L2    | Dodecane, 2,6,10-trimethyl-            | C <sub>15</sub> H <sub>32</sub>   | 212              | 6.848    | 5.56     | Antimicrobial and cytotoxicity (Jaradat et al., 2022)                                                                        |
|       | Dodecane, 2,6,11-trimethyl-            | C <sub>15</sub> H <sub>32</sub>   | 212              | 10.118   | 5.99     | Antioxidant and antibacterial (Khanal et al., 2022)                                                                          |
|       | Dodecane, 2,6,11-trimethyl-            | C <sub>15</sub> H <sub>32</sub>   | 212              | 12.975   | 5.75     | Antioxidant and antibacterial (Khanal et al., 2022)                                                                          |
|       | Phenol, 3,5-bis(1,1-dimethylethyl)-    | C <sub>14</sub> H <sub>22</sub> O | 206              | 13.515   | 32.07    | Antioxidant, antimicrobial (Ndiege et al., 2021), antibacterial, antiseptic, disinfectant and flavouring (Igwe et al., 2015) |
|       | Neophytadiene                          | C <sub>20</sub> H <sub>38</sub>   | 278              | 17.065   | 11.87    | Antioxidant and antimicrobial (Al-Rajhi et al., 2022)                                                                        |
|       | 2-Pentadecanone, 6,10,14-trimethyl-    | C <sub>18</sub> H <sub>36</sub> O | 268              | 17.180   | 9.73     | Antibacterial (Amos-tautua et al., 2020)                                                                                     |
|       | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol | C <sub>20</sub> H <sub>40</sub> O | 296              | 17.525   | 4.05     | Antimicrobial (Zayed & Samling, 2016)                                                                                        |

Table 4.12: Major Bioactive Compounds Identified in Isolate S2 from SE-Chlo-S Extract of *S. alata* and its Biological Activities

| Spots | Compound name                                   | Molecular formula                                | Molecular weight | RT (min) | Area (%) | Reported biological activities                                                                                                                                   |
|-------|-------------------------------------------------|--------------------------------------------------|------------------|----------|----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| S2    | Decane, 3,7-dimethyl-                           | C <sub>12</sub> H <sub>26</sub>                  | 170              | 6.849    | 4.86     | -                                                                                                                                                                |
|       | Dodecane, 2,6,11-trimethyl-                     | C <sub>15</sub> H <sub>32</sub>                  | 212              | 10.119   | 4.16     | Antioxidant and antibacterial (Khanal et al., 2022)                                                                                                              |
|       |                                                 |                                                  |                  | 12.976   | 3.60     |                                                                                                                                                                  |
|       |                                                 |                                                  |                  | 15.496   | 2.56     |                                                                                                                                                                  |
|       | Phenol, 3,5-bis(1,1-dimethylethyl)-             | C <sub>14</sub> H <sub>22</sub> O                | 206              | 13.518   | 35.69    | Antioxidant, antimicrobial (Ndiege et al., 2021), antibacterial, antiseptic, disinfectant and flavouring (Igwe et al., 2015)                                     |
|       | Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)-   | C <sub>31</sub> H <sub>50</sub> O <sub>2</sub>   | 454              | 24.936   | 4.44     | Source of synthetic progesterone and exhibits antihepatotoxic, antiviral, antioxidant, cancer preventive, and hypocholesterolemic activity (Arora & Kumar, 2017) |
|       | Cholesta-4,6-dien-3-ol, (3.beta.)-              | C <sub>27</sub> H <sub>44</sub> O                | 384              | 26.835   | 7.52     | Antibacterial and antifungal (Zhu et al., 2018)                                                                                                                  |
|       | Cholest-5-en-3-ol (3.beta.)-, carbonochloridate | C <sub>28</sub> H <sub>45</sub> ClO <sub>2</sub> | 448              | 27.841   | 17.69    | Antimicrobial activity (Santhi et al., 2016) and nematocidal (Maulidia et al., 2020)                                                                             |

Table 4.13: Major Bioactive Compounds Identified in Isolate R1 from SE-Chlo-R Extract of *S. alata* and its Biological Activities

| Spots | Compound name                       | Molecular formula                 | Molecular weight | RT (min) | Area (%) | Reported biological activities                                                                                               |
|-------|-------------------------------------|-----------------------------------|------------------|----------|----------|------------------------------------------------------------------------------------------------------------------------------|
| R1    | Decane, 3,7-dimethyl-               | C <sub>12</sub> H <sub>26</sub>   | 170              | 6.847    | 11.73    | -                                                                                                                            |
|       | Dodecane, 4,6-dimethyl-             | C <sub>14</sub> H <sub>30</sub>   | 198              | 9.810    | 6.89     | Antioxidant and antibacterial (Khanal et al., 2022)                                                                          |
|       | Dodecane, 2,6,11-trimethyl-         | C <sub>15</sub> H <sub>32</sub>   | 212              | 10.117   | 10.46    | Antioxidant and antibacterial (Khanal et al., 2022)                                                                          |
|       |                                     |                                   |                  | 12.974   | 8.86     |                                                                                                                              |
|       | Phenol, 3,5-bis(1,1-dimethylethyl)- | C <sub>14</sub> H <sub>22</sub> O | 206              | 13.517   | 51.11    | Antioxidant, antimicrobial (Ndiege et al., 2021), antibacterial, antiseptic, disinfectant and flavouring (Igwe et al., 2015) |
|       | 9-Eicosene, (E)-                    | C <sub>20</sub> H <sub>40</sub>   | 280              | 14.342   | 5.39     | Antibacterial (Tiji et al., 2021)                                                                                            |
|       | 1-Hexadecanol                       | C <sub>16</sub> H <sub>34</sub> O | 242              | 16.615   | 5.56     | Antibacterial (Tan et al., 2019) and (Dawwam et al., 2022)                                                                   |

As shown in Table 4.11-Table 4.13, phenol, 3,5-bis(1,1-dimethylethyl)- was found to be the most dominant compound in all isolates; L2 (32.07%), S2 (35.69%) and R1 (51.11%). Based on the previous study, phenol, 3,5-bis(1,1-dimethylethyl)- is a phenolic compound that has various biological properties, such as antioxidant and antimicrobial activity, isolated from *Biden pilosa* leaves (Ndiege et al., 2021). In Igwe et al. (2015), a similar compound was also identified from *Vernonia amygdalina* and reported several biological properties, including antiseptic, disinfectant, flavouring, and antibacterial activity.

Another major compound identified from isolate L2 was followed by neophytadiene (11.87%), 2-pentadecanone, 6,10,14-trimethyl- (9.73%), dodecane, 2,6,10-trimethyl- (5.99%, 5.75%, 5.56%) and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (4.05%). In the study of *Mentha pulegium* extract by Al-Rajhi et al. (2022), neophytadiene was analysed by molecular docking for its binding affinity with *P. aeruginosa* and showed the compound has antibacterial activity. Other compounds such as 2-pentadecanone, 6,10,14-trimethyl- and 3,7,11,15-tetramethyl-2-hexadecen-1-ol were also exerting antibacterial (Amos-tautua et al., 2020) and antimicrobial activity (Zayed & Samling, 2016), respectively. As for dodecane, 2,6,10-trimethyl-, it was found as a major compound in *Alhagi mannifera* extracts, with an antimicrobial and cytotoxic activity reported (Jaradat et al., 2022).

Meanwhile, from isolate S2, cholest-5-en-3-ol (3.beta.)-, carbonochloridate (17.69%) and cholesta-4,6-dien-3-ol, (3.beta.)- (7.52%) were the second and third major compounds identified (Table 4.12). Cholest-5-en-3-ol (3.beta.)-, carbonochloridate found in *B. thuringiensis* AK08 was reported with nematicidal activity. Cholestane is a saturated tetracyclic triterpene with 27 carbons (C-27), present in Rhodopyhtes organisms in low concentrations (Maulidia et al., 2020). In mollusc *P. persica* extract, it exhibited antimicrobial activity (Santhi et al., 2016), as well as an emulsion stabiliser on toothpaste

made from *Citrus reticulata* extract (Pratiwi et al., 2017). Meanwhile, cholesta-4,6-dien-3-ol, (3.beta.) obtained from marine *Urechis unicinctus* exhibited antibacterial and antifungal activities (Zhu et al., 2018).

Other major compounds from isolate S2 were followed by decane, 3,7-dimethyl- (4.86%), dodecane, 2,6,11-trimethyl- (4.16%, 3.60%, 2.56%) and stigmasta-5,22-dien-3-ol, acetate, (3.beta.)- (4.44%). Decane, 3,7-dimethyl- and dodecane, 2,6,11-trimethyl- were also found in isolate R1. In the previous study by Geethalakshmi and Sarada (2013), decane, 3,7-dimethyl- was only identified as a minor compound from the essential oil of *Trianthema decandra* L and has no reported biological properties.

Dodecane, 2,6,11-trimethyl-, on the other hand, was identified as the second major compound in *Ephedra pachyclada* with an area of 16.35%. The extracts were reported with antioxidant and antibacterial activities (Khanal et al., 2022). As for stigmasta-5,22-dien-3-ol, acetate, (3.beta.), it has been reported as a source of synthetic progesterone and exhibits antihepatotoxic, antiviral, antioxidant, cancer preventive, and hypocholesterolemic activity. It was also known as stigmasterol acetate and was identified from the methanolic and ethyl acetate extract of *Cenchrus setigerus* Vahl (Arora & Kumar, 2018).

Meanwhile, in isolate R1, the major compound was followed by decane, 3,7-dimethyl- (11.73%), dodecane, 2,6,11-trimethyl- (10.46%, 8.86%), dodecane, 4,6-dimethyl- (6.89%), 1-hexadecanol (5.56%) and 9-eicosene, (E)- (5.39%). Based on the previous study by Khanal et al. (2022), dodecane, 4,6-dimethyl- was one of the major compounds identified from *E. pachyclada* with an area of 11.59%. In this study, the methanol extract of the plant exhibited antioxidant and significant antibacterial activities against *E. coli* and *S. aureus* (Khanal et al., 2022).

Antibacterial activity was also exhibited by the 1-hexadecanol compound. In Tan et al. (2019), 1-hexadecanol was found as a major compound in *Crinum asiaticum* that exhibited antibacterial activity against *Shigella boydii*, *Candida albicans* and *Rhizopus* sp. A similar compound was also identified as a major compound from probiotic bacteria, *Lactobacillus plantarum* and *Lactobacillus acidophilus* strains. In their study, both probiotics exhibited antibacterial activity as well (Dawwam et al., 2022). As for 9-eicosene, the compound was identified in the fractions SH7 and SH8, from *Nigella sativa* essential oil, which exhibited antibacterial activity on both Gram-positive and Gram-negative bacteria (Tiji et al., 2021).

Therefore, it can be concluded that phenol, 3,5-bis(1,1-dimethylethyl)-, cholest-5-en-3-ol (3.beta.)-, carbonochloridate, neophytadiene and dodecane, 2,6,11-trimethyl- are the most major compounds that have a potential as the alternative antibacterial agent in combatting the MAR bacteria, especially against the *Mammaliicoccus* sp. The GC-MS analysis showed the bioactive compounds identified from one isolate were also present in other isolates. However, it was not varying much, which could be a marker for the differentiation of leaves, stems and roots of *S. alata*. The different bioactive compounds present explained the difference in antibacterial activity from different parts of *S. alata*. This is because the therapeutic efficacy of medicinal plant extracts relies on synergistic interactions between their components (Vaou et al, 2022).

## CHAPTER 5

### CONCLUSION AND RECOMMENDATIONS

#### 5.1 Conclusion

From this research, both soxhlet extraction and ultrasonic assisted extraction were managed to extract a significant amount of phenolics and flavonoids contents from the *S. alata* leaves, stems and roots. The highest phenolics and flavonoids were identified from the chloroform extract of leaves using UAE, followed by the chloroform extract of leaves using SE. Meanwhile, extraction yield was produced the most from ethanol extract for all plant parts, and SE was identified to be the most efficient in producing a higher yield. In this study, hexane yielded the lowest, which is unsuitable for this study because a higher yield is needed to proceed with antibacterial activity using the agar well diffusion assay.

The bacterial isolates in this study, *Enterococcus* sp. and *Mammaliicoccus* sp. were identified and confirmed using 16s rRNA sequencing. Despite of isolated from the environment, *Enterococcus* sp. is one of the major concerns in healthcare because of the limited number of antibiotics that could be used to treat vancomycin-resistant *Enterococcus* infections. Meanwhile, *Mammaliicoccus* sp. caused mastitis in animals, which recently transferred as a novel genus *Mammaliicoccus*, and thus, limited studies have been performed on it. Most importantly, both bacteria showed resistance towards more than one antibiotic tested from each class of antibiotic (MAR value = 0.83 and 0.40), which makes them significant to evaluate the antibacterial effectiveness of *S. alata* extracts from our study.

From this research, ethanol and chloroform extracts of *S. alata* exhibited a potential antibacterial activity against *Mammaliicoccus* sp., while poor antibacterial activities were identified from ethanol extracts towards *Enterococcus* sp. The lowest MIC value was

identified from the chloroform extract of roots using SE against *Mammaliicoccus* sp., while the highest MIC was identified from most of the extracts against *Enterococcus* sp. (UAE-EtOH-L, UAE-Chlo-L, SE-Chlo-L). Compared to the ultrasonic assisted extraction, soxhlet extraction exhibited a higher antimicrobial activity, especially using chloroform. All of the extracts in this study can be utilised as bactericidal agents.

Using a solvent system Hex: EtOAc: Chlo (8:2:1), 4 spots from SE-Chlo-L, 7 spots from SE-Chlo-S and 5 spots from SE-Chlo-R were managed to separate using the thin layer chromatography method. Among these spots, L2 from SE-Chlo-L, S2 from SE-Chlo-S, and R1 from SE-Chlo-R were identified with strong antibacterial activity, especially against *Mammaliicoccus* sp., and further identified using GC-MS analysis. A few of the bioactive compounds with the highest area was Phenol, 3,5-bis(1,1-dimethyl ethyl)-, Cholest-5-en-3-ol (3.beta.)-, carbonochloridate, Neophytadiene, Decane, 3,7-dimethyl-, Dodecane, 2,6,11-trimethyl-, and 2-Pentadecanone, 6,10,14-trimethyl-. These bioactive compounds possibly contributed to the antibacterial properties identified in extracts. Thus, these discoveries are expected to be significant and provide valuable insights for various industries, particularly pharmaceutical and medicinal ones. It is also expected to discover novel bioactive molecules that can be used to produce new medications for combating diseases, especially involving multiple antibiotic resistant bacteria.

## 5.2 Recommendations

In future studies, it is recommended that various amounts of the solid-to-solvent ratio in ultrasonic assisted extraction should be studied and optimised to achieve good antibacterial properties. Apart from ethanol and chloroform, other solvents such as ethyl acetate, methanol, and water should be incorporated as solvent extraction. Furthermore, other biological studies, such as antifungal and anticancer, are recommended to learn more about their other potential therapeutic properties. Additional research must also be conducted on purified *S. alata* extracts to determine their bioactivity and toxicity thoroughly. Thus, a deeper comprehension of the bioactive compounds and their biological properties can be attained.

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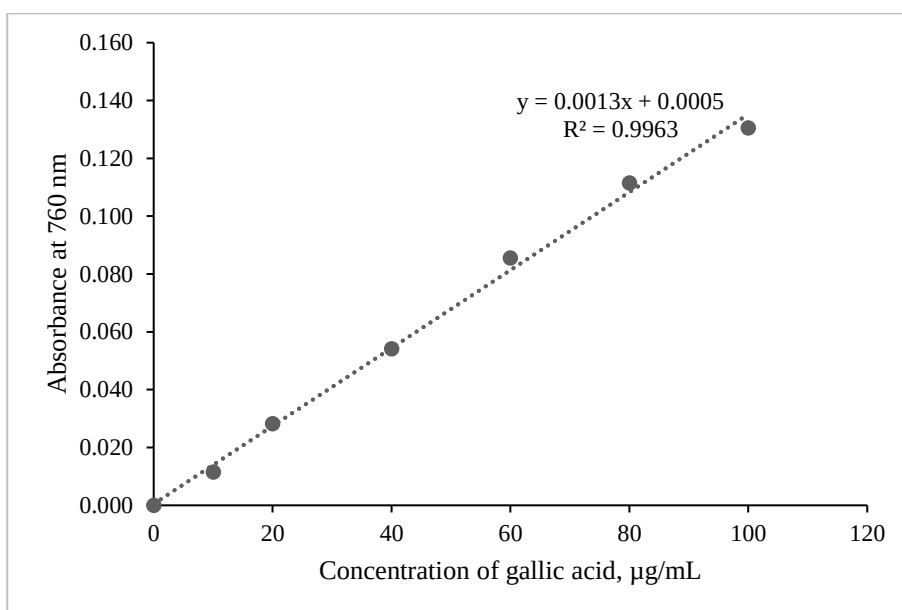
## APPENDICES

### Appendix A: Yield of Extraction

#### Appendix A1: Raw data of weight of *S. alata* extracts

| Extract    | Extract obtained (g) |       |       | Extraction yield (%) |       |       | Average | Standard deviation |
|------------|----------------------|-------|-------|----------------------|-------|-------|---------|--------------------|
| SE-EtOH-L  | 4.287                | 4.193 | 4.399 | 28.58                | 27.95 | 29.33 | 28.62   | 0.69               |
| SE-Chlo-L  | 1.255                | 1.236 | 1.332 | 8.37                 | 8.24  | 8.88  | 8.50    | 0.34               |
| SE-Hex-L   | 0.775                | 0.725 | 0.688 | 5.17                 | 4.83  | 4.59  | 4.86    | 0.29               |
| SE-EtOH-S  | 1.515                | 1.511 | 1.502 | 10.10                | 10.07 | 10.01 | 10.06   | 0.05               |
| SE-Chlo-S  | 0.133                | 0.230 | 0.148 | 0.89                 | 1.53  | 0.99  | 1.14    | 0.34               |
| SE-Hex-S   | 0.094                | 0.123 | 0.121 | 0.63                 | 0.82  | 0.81  | 0.75    | 0.11               |
| SE-EtOH-R  | 1.47                 | 1.490 | 1.447 | 9.80                 | 9.93  | 9.65  | 9.79    | 0.14               |
| SE-Chlo-R  | 0.131                | 0.165 | 0.291 | 0.87                 | 1.10  | 1.94  | 1.30    | 0.56               |
| SE-Hex-R   | 0.090                | 0.089 | 0.087 | 0.60                 | 0.59  | 0.58  | 0.59    | 0.01               |
| UAE-EtOH-L | 0.108                | 0.112 | 0.134 | 10.80                | 11.20 | 13.40 | 10.80   | 1.40               |
| UAE-Chlo-L | 0.048                | 0.054 | 0.046 | 4.80                 | 5.40  | 4.60  | 4.93    | 0.42               |
| UAE-Hex-L  | 0.032                | 0.031 | 0.034 | 3.20                 | 3.10  | 3.40  | 3.23    | 0.15               |
| UAE-EtOH-S | 0.038                | 0.029 | 0.040 | 3.80                 | 2.90  | 4.00  | 3.57    | 0.59               |
| UAE-Chlo-S | 0.009                | 0.009 | 0.007 | 0.90                 | 0.90  | 0.70  | 0.90    | 0.12               |
| UAE-Hex-S  | 0.004                | 0.005 | 0.007 | 0.40                 | 0.50  | 0.70  | 0.53    | 0.15               |
| UAE-EtOH-R | 0.023                | 0.015 | 0.022 | 2.30                 | 1.50  | 2.20  | 2.30    | 0.44               |
| UAE-Chlo-R | 0.008                | 0.008 | 0.006 | 0.80                 | 0.80  | 0.60  | 0.80    | 0.12               |
| UAE-Hex-R  | 0.003                | 0.005 | 0.003 | 0.30                 | 0.50  | 0.30  | 0.37    | 0.12               |

## Appendix B: Total Phenolic Content

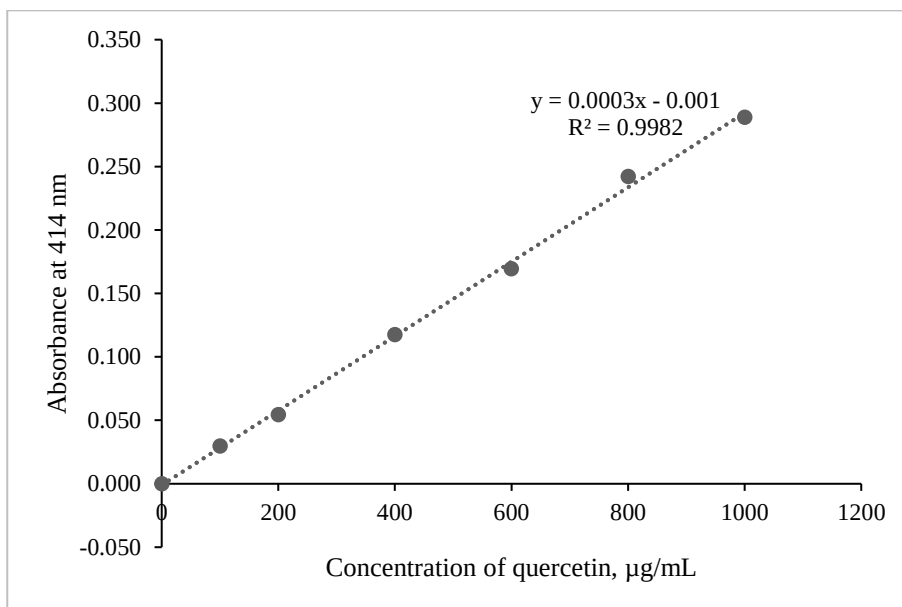


Appendix B1: Gallic acid calibration curve measured at a wavelength of 760 nm for total phenolic content analysis of *S. alata* extracts

Appendix B2: Raw data of total phenolic content of *S. alata* extracts measured at a wavelength of 760 nm

| Extract    | Absorbance (760 nm) |       |       | Total phenolic content<br>(mg GAE/g) |        |        | Average | Standard deviation |
|------------|---------------------|-------|-------|--------------------------------------|--------|--------|---------|--------------------|
|            |                     |       |       |                                      |        |        |         |                    |
| SE-EtOH-L  | 0.091               | 0.091 | 0.091 | 69.54                                | 66.00  | 75.92  | 70.49   | 5.03               |
| SE-Chlo-L  | 0.095               | 0.095 | 0.095 | 72.77                                | 84.46  | 83.92  | 80.39   | 6.60               |
| SE-EtOH-S  | 0.048               | 0.048 | 0.048 | 36.46                                | 40.62  | 39.15  | 38.74   | 2.11               |
| SE-Chlo-S  | 0.079               | 0.079 | 0.079 | 60.15                                | 63.92  | 55.77  | 59.95   | 4.08               |
| SE-EtOH-R  | 0.082               | 0.082 | 0.082 | 62.85                                | 62.54  | 59.62  | 61.67   | 1.78               |
| SE-Chlo-R  | 0.088               | 0.088 | 0.088 | 67.00                                | 64.46  | 66.15  | 65.87   | 1.29               |
| UAE-EtOH-L | 0.097               | 0.097 | 0.097 | 74.23                                | 78.23  | 74.92  | 75.79   | 2.14               |
| UAE-Chlo-L | 0.152               | 0.152 | 0.152 | 116.23                               | 120.62 | 115.46 | 117.44  | 2.78               |
| UAE-EtOH-S | 0.050               | 0.050 | 0.050 | 38.23                                | 41.62  | 43.15  | 41.00   | 2.52               |
| UAE-Chlo-S | 0.077               | 0.077 | 0.077 | 58.46                                | 71.08  | 63.54  | 64.36   | 6.35               |
| UAE-EtOH-R | 0.068               | 0.068 | 0.068 | 52.00                                | 61.00  | 61.23  | 58.08   | 5.26               |
| UAE-Chlo-R | 0.073               | 0.073 | 0.073 | 56.08                                | 62.31  | 59.23  | 59.21   | 3.12               |

## Appendix C: Total Flavonoid Content

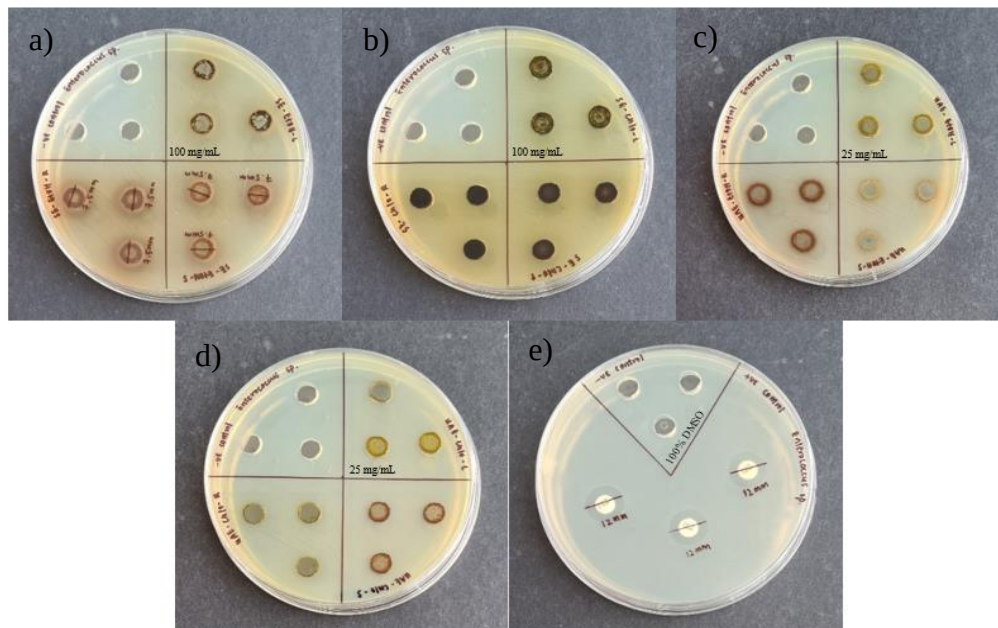


Appendix C1: Quercetin calibration curve measured at a wavelength of 414 nm for total flavonoid content analysis of *S. alata* extracts

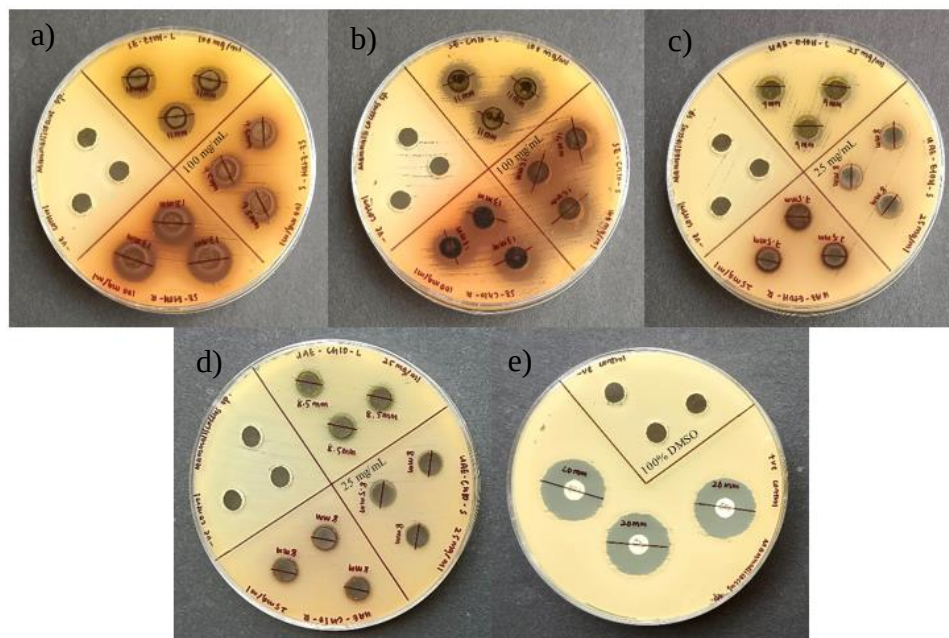
Appendix C2: Raw data of total flavonoid content of *S. alata* extracts measured at a wavelength of 414 nm

| Extract    | Absorbance (414 nm) |       |       | Total phenolic content<br>(mg QE/g) |        |        | Average | Standard deviation |
|------------|---------------------|-------|-------|-------------------------------------|--------|--------|---------|--------------------|
| SE-EtOH-L  | 0.095               | 0.093 | 0.094 | 320.33                              | 312.00 | 316.67 | 316.33  | 4.18               |
| SE-Chlo-L  | 0.120               | 0.120 | 0.124 | 402.00                              | 402.33 | 415.00 | 406.44  | 7.41               |
| SE-EtOH-S  | 0.056               | 0.055 | 0.054 | 191.33                              | 187.67 | 182.67 | 187.22  | 4.35               |
| SE-Chlo-S  | 0.084               | 0.078 | 0.078 | 281.67                              | 263.33 | 262.33 | 269.11  | 10.88              |
| SE-EtOH-R  | 0.086               | 0.084 | 0.081 | 290.00                              | 282.33 | 273.67 | 282.00  | 8.17               |
| SE-Chlo-R  | 0.113               | 0.105 | 0.110 | 380.33                              | 354.67 | 370.00 | 368.33  | 12.91              |
| UAE-EtOH-L | 0.089               | 0.086 | 0.088 | 298.33                              | 289.67 | 298.00 | 295.33  | 4.91               |
| UAE-Chlo-L | 0.175               | 0.161 | 0.173 | 587.00                              | 541.00 | 578.33 | 568.78  | 24.44              |
| UAE-EtOH-S | 0.047               | 0.050 | 0.048 | 159.67                              | 169.67 | 164.33 | 164.56  | 5.00               |
| UAE-Chlo-S | 0.076               | 0.070 | 0.073 | 255.67                              | 237.67 | 246.33 | 246.56  | 9.00               |
| UAE-EtOH-R | 0.067               | 0.069 | 0.069 | 227.33                              | 231.67 | 233.00 | 230.67  | 2.96               |
| UAE-Chlo-R | 0.068               | 0.065 | 0.067 | 230.00                              | 220.33 | 225.33 | 225.22  | 4.83               |

## Appendix D: Antimicrobial Activity of *S. alata* Extracts



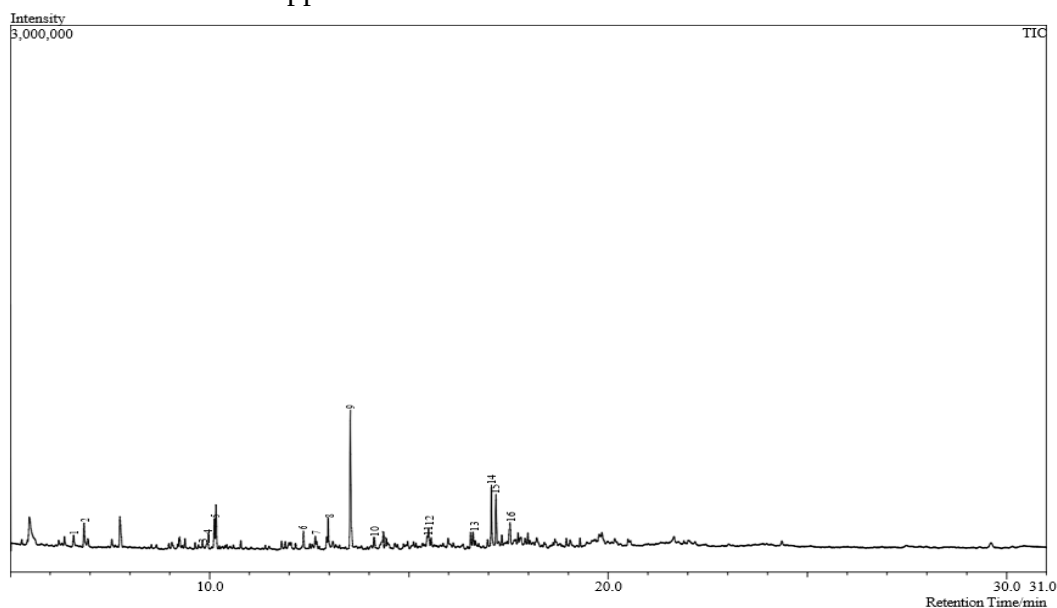
Appendix D1: Agar well diffusion assays of *S. alata* extracts against *Enterococcus* sp. a) SE-EtOH of L, S, R at 100 mg/mL. b) SE-Chlo of L, S, R at 100 mg/mL. c) UAE-EtOH of L, S, R at 25 mg/mL. d) UAE-Chlo of L, S, R at 25 mg/mL. e) Positive control- Vancomycin



Appendix D2: Agar well diffusion assays of *S. alata* extracts against *Mammaliicoccus* sp. a) SE-EtOH of L, S, R at 100 mg/mL. b) SE-Chlo of L, S, R at 100 mg/mL. c) UAE-EtOH of L, S, R at 25 mg/mL. d) UAE-Chlo of L, S, R at 25 mg/mL. e) Positive control- Gentamicin

## Appendix E: Gas Chromatography – Mass Spectrometry (GC-MS) Analysis

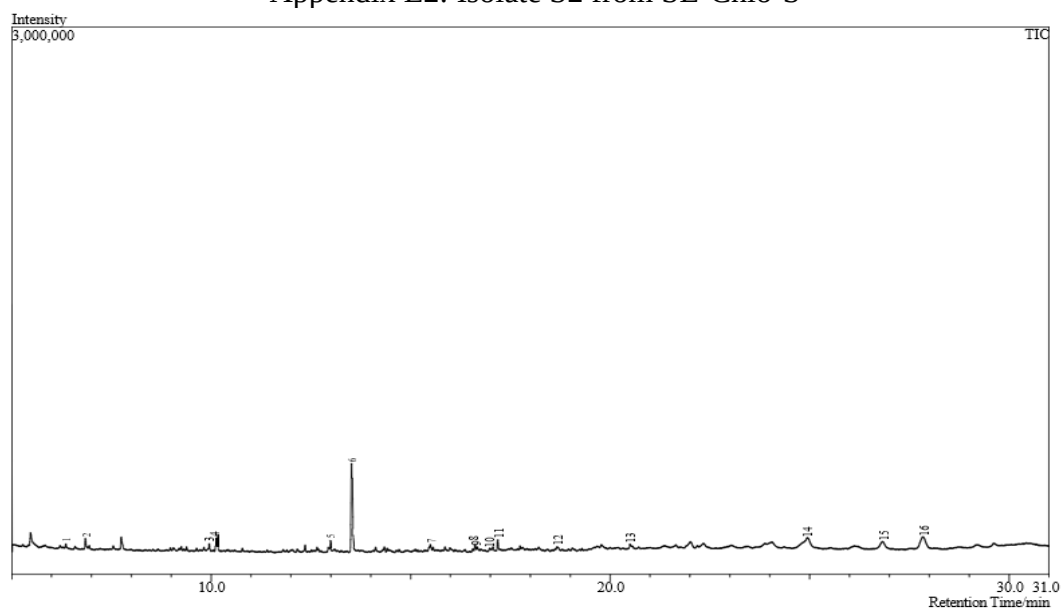
### Appendix E1: Isolate L2 from SE-Chlo-L



Peak Report TIC

| Peak # | R. time | Area    | Height  | Name                                          |
|--------|---------|---------|---------|-----------------------------------------------|
| 1      | 6.586   | 120756  | 60188   | 1-Hexanol, 2-ethyl-                           |
| 2      | 6.848   | 261911  | 131564  | Dodecane, 2,6,10-trimethyl-                   |
| 3      | 9.810   | 135544  | 34054   | Dodecane, 4,6-dimethyl-                       |
| 4      | 9.952   | 130010  | 86223   | Undecane, 2,4-dimethyl-                       |
| 5      | 10.118  | 281932  | 165723  | Dodecane, 2,6,11-trimethyl-                   |
| 6      | 12.356  | 137399  | 90053   | 3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tri |
| 7      | 12.652  | 118182  | 65807   | 2,6,10-Trimethyltridecane                     |
| 8      | 12.975  | 270746  | 165302  | Dodecane, 2,6,11-trimethyl-                   |
| 9      | 13.515  | 1509758 | 782472  | Phenol, 3,5-bis(1,1-dimethylethyl)-           |
| 10     | 14.118  | 115347  | 60322   | 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4  |
| 11     | 15.455  | 120020  | 50204   | 2-Methyltetracosane                           |
| 12     | 15.495  | 158473  | 97376   | Eicosane                                      |
| 13     | 16.613  | 140374  | 79178   | 1-Tricosene                                   |
| 14     | 17.065  | 558667  | 329829  | Neophytadiene                                 |
| 15     | 17.180  | 458235  | 282316  | 2-Pentadecanone, 6,10,14-trimethyl-           |
| 16     | 17.525  | 190511  | 116009  | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol        |
|        |         | 4707865 | 2596620 | 2596620                                       |

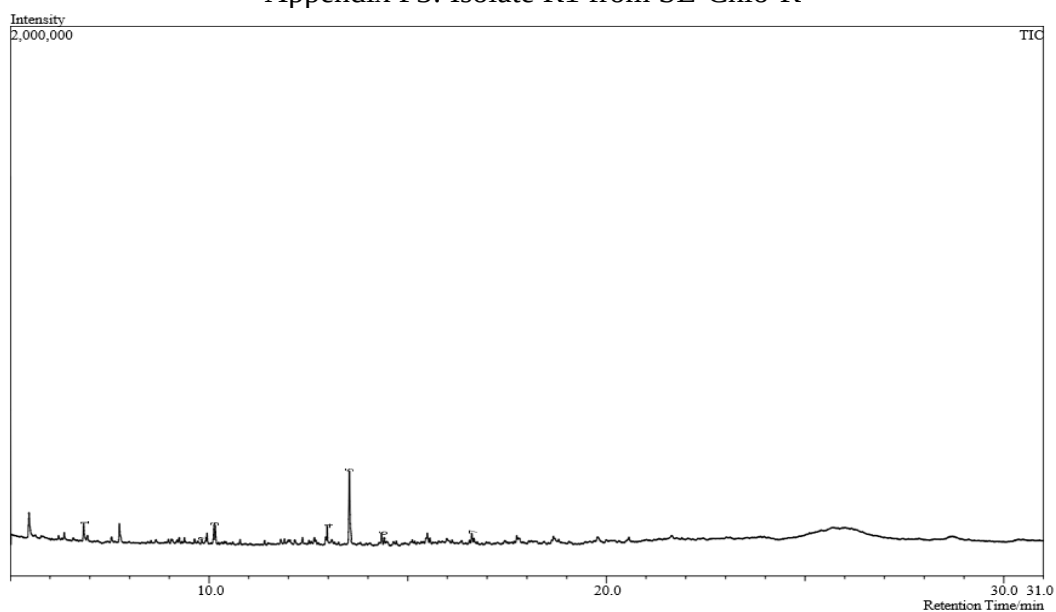
## Appendix E2: Isolate S2 from SE-Chlo-S



Peak Report TIC

| Peak # | R. time | Area    | Height  | Name                                            |
|--------|---------|---------|---------|-------------------------------------------------|
| 1      | 6.359   | 50109   | 24984   | Octane, 3,3-dimethyl-                           |
| 2      | 6.849   | 136735  | 62118   | Decane, 3,7-dimethyl-                           |
| 3      | 9.954   | 92461   | 41440   | Undecane, 2,4-dimethyl-                         |
| 4      | 10.119  | 117167  | 72002   | Dodecane, 2,6,11-trimethyl-                     |
| 5      | 12.976  | 101454  | 61540   | Dodecane, 2,6,11-trimethyl-                     |
| 6      | 13.518  | 1004712 | 499036  | Phenol, 3,5-bis(1,1-dimethylethyl)-             |
| 7      | 15.496  | 72208   | 37845   | Dodecane, 2,6,11-trimethyl-                     |
| 8      | 16.617  | 77964   | 39682   | 9-Eicosene, (E)-                                |
| 9      | 16.671  | 55840   | 24433   | Heptadecane                                     |
| 10     | 16.975  | 50965   | 14171   | Tetrapentacontane, 1,54-dibromo-                |
| 11     | 17.184  | 111901  | 61519   | 2-Pentadecanone, 6,10,14-trimethyl-             |
| 12     | 18.670  | 54467   | 18002   | 1-Nonadecene                                    |
| 13     | 20.499  | 54893   | 25699   | 1,2-dihydro-8-hydroxylinalool                   |
| 14     | 24.936  | 124894  | 22058   | Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)-   |
| 15     | 26.835  | 211750  | 31615   | Cholesta-4,6-dien-3-ol, (3.beta.)-              |
| 16     | 27.841  | 497931  | 58760   | Cholest-5-en-3-ol (3.beta.)-, carbonochloridate |
|        |         | 2815451 | 1094904 |                                                 |

### Appendix F3: Isolate R1 from SE-Chlo-R



Peak Report TIC

| Peak # | R. time | Area    | Height | Name                                |
|--------|---------|---------|--------|-------------------------------------|
| 1      | 6.847   | 132112  | 65179  | Decane, 3,7-dimethyl-               |
| 2      | 9.810   | 77641   | 17691  | Dodecane, 4,6-dimethyl-             |
| 3      | 10.117  | 117875  | 71789  | Dodecane, 2,6,11-trimethyl-         |
| 4      | 12.974  | 99847   | 66179  | Dodecane, 2,6,11-trimethyl-         |
| 5      | 13.517  | 575866  | 278983 | Phenol, 3,5-bis(1,1-dimethylethyl)- |
| 6      | 14.342  | 60693   | 35824  | 9-Eicosene, (E)-                    |
| 7      | 16.615  | 62648   | 35620  | 1-Hexadecanol                       |
|        |         | 1126682 | 571265 |                                     |