THE STUDY OF MULTIPLE EXTRACTION METHODS OF PHYTOCHEMICAL CONSTITUENTS OF *Senna alata* LEAVES

BY

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ABSTRACT

The demand for “Gelenggang” scientifically named *Senna alata* Linn-based product, is increasing due to the well-known therapeutic properties that can treat various diseases such as skin disease, ringworm, and constipation. Various extraction methods have been used to study the active ingredients and find scientific evidence for their herbal activities. Thus, this study aims to investigate the difference in yield percentage, phytochemicals, antioxidant and antimicrobial activities of different extracts of *S. alata* leaf. The extraction methods used in this study were maceration, Soxhlet, and Subcritical Carbon Dioxide Soxhlet (SCDS), our findings suggested that conventional methods yield higher percentages of extracts compared to SCDS ranging from (1.20 ± 0.417^{e} %) to (25.14 ± 1.017^{a} %) with methanol extraction showed higher yields than hexane extraction, indicating the solvent's influence on extraction efficiency. The study also explores the effect of sample: co-solvent ratio on the yield of extracts using SCDS (1.20 ± 0.417^{e} to 2.45 ± 0.417^{e} %). GCMS analysis reveals the presence of eighteen compounds, with phytol acetate, dihydroactinidiolide, and hexadecenoic acid methyl ester identified as major constituents. The extracts exhibit in vitro antioxidant activity by the DPPH assay with the SCDS extract using methanol as the soaking solvent showing the strongest potency (IC_{50} value = 0.693 ± 0.1725 mg/ml), while the weakest also from SCDS method but ethanol as co-solvent with highest IC_{50} value (55.569 ± 2.29 mg/ml). On the other hand, agar disc diffusion assay resulted that methanolic extracts from Soxhlet showed the most significant inhibition zones against *Staphylococcus aureus* ATCC25923 which ranges from 9 to 11 mm. Furthermore, the resazurin-based microdilution techniques yielded the lowest MIC and MBC value from both methanolic Soxhlet extracts against *K. pneumoniae* (20.75 mg/ml). Overall, this research highlights the potential of SCDS as a promising extraction method for *S. alata* leaves, with potential to preserve bioactive constituents despite lower yield percentages compared to conventional methods.

يزداد الطلب على المنتجات القائمة على عشبية *Senna alata* Linn بسبب خصائصها العلاجية المعروفة في علاج أمراض مختلفة مثل أمراض الجلد والدودة الحلقية والإمساك. تم استخدام طرق استخراج مختلفة لدراسة المكونات النشطة واستكشاف الأدلة العلمية للأنشطة العشبية لأوراق *S. alata*. بحثت هذه الدراسة الاختلافات في إجمالي نسبة العائد، والمواد الكيميائية النباتية، والأنشطة المضادة للأكسدة والميكروبات لأوراق *S. alata* المستخرجة بواسطة طرق النقع، Soxhlet، وSoxhlet ثاني أكسيد الكربون دون الحرجة (SCDS). أظهرت النتائج أن جميع طرق الاستخراج الثلاثة أسفرت عن نسب مختلفة من المستخلصات، مع الطرق التقليدية، مما أدى بشكل عام إلى إنتاج غلات أعلى مقارنة بـ SCDS. تراوحت النسب المئوية للعائد من (1.20 ± 0.417^{e}٪) إلى (25.14 ± 1.017^{a}٪) بين الطرق. بالإضافة إلى ذلك، لوحظ أن اختيار المذيب أثر بشكل كبير على كفاءة الاستخراج، حيث يوفر استخلاص الميثانول بشكل عام غلات أعلى مقارنة باستخراج الهكسان. علاوة على ذلك، حددت هذه الدراسة ثمانية عشر مركبًا في المستخلصات، مع أسيتات phytol و dihydroactinidiolide و hexadecenoic acid methyl ester التي تم تحديدها كمكونات رئيسية عبر جميع طرق الاستخراج الثلاثة. علاوة على ذلك، جميع المستخلصات المعروضة في نشاط مضاد للأكسدة في المختبر كما هو محدد بواسطة مقايسة DPPH حيث يتم استخراج SCDS باستخدام الميثانول حيث أظهر المذيب النقع أقوى قيمة (IC_{50} = 0.693 ± 0.1725 ملغم / مل)، في حين أن أضعف نشاط مضاد للأكسدة كان أيضًا من طريقة SCDS، ولكن تم استخدام الإيثانول كمذيب النقع، مما أدى إلى أعلى قيمة IC_{50} (55.569 ± 2.29 مجم / مل). من ناحية أخرى، أظهرت المستخلصات مستويات مختلفة من مناطق التثبيط ضد البكتيريا والخميرة المختلفة. تظهر هذه الدراسة أن المستخلص أظهر نشاطًا مثبطًا كبيرًا ضد البكتيريا إيجابية الجرام مثل *S. aureus* (9.33 ± 0.577 مم) و S. البشرة (1.85 ± 0.2887 مم)، بالإضافة إلى البكتيريا سالبة الجرام، بما في ذلك *K. pneumoniae* (6.67 ± 0.577 مم) و *P. aeruginosa* (2.67 ± 0.5 مم). أظهرت هذه الدراسة الخصائص المضادة للميكروبات المحتملة للمستخلصات ضد الكائنات الحية الدقيقة المختبرة. بشكل عام، يسلط هذا البحث الضوء على أهمية النظر في اختيار طريقة الاستخراج والمذيبات في الحصول على النتائج املختلفة و املكوانت الكيميائية النباتية واليت قد تساهم في الخصائص العلاجية لأوراق *S. alata*. في حين أن الطرق التقليدية أدت بشكل عام إلى ارتفاع نسب العائد الإجمالي، فقد أثبتت SCDS أيضًا إمكانية الحفاظ على المواد الكيميائية النباتية الأساسية.

APPROVAL PAGE

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DECLARATION

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This dissertation is lovingly dedicated to my parents, cherished husband, and esteemed supervisors for their unwavering and invaluable support.

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LIST OF SYMBOLS

±	Plus minus
%	Percentage
°C	Degree Celsius
α	Alpha
β	Beta
µg/ml	Microgram per millilitre
µm	Microgram per meter
ANOVA	Analysis of Variance
g	Gram
IC_{50}	Half maximal inhibitory concentration
Kg	Kilogram
mbar	millibar
mg	Milligram
mg/ml	Milligram per millilitre
min	Minute
ml	Millilitre
ml/min	Millilitre per minute
mm	Millimetre
m/z	Mass per charge ratio
n	Number of sample size

CHAPTER ONE:

INTRODUCTION

1.1 RESEARCH BACKGROUND

The growing preference for herbal medicines, nutraceuticals, and natural products has resulted in an increased need for top-notch extracts capable of providing the intended therapeutic or nutritional advantages. This is in line with the finding from World Health Organization (WHO) where they found that approximately 80% of individuals in developing nations primarily depend on herbal medicines (Oladeji et al., 2020). Many commonly used medications are derived from natural sources. In fact, as stated in a study by Stone et al. (2021), from 1981 to 2019, natural products have maintained their significant contribution to drug discovery, as approximately half of the newly developed chemical entities are derived from natural sources. The revitalized scientific curiosity and current research directions in the exploration of natural products for drug discovery strongly suggest that these substances will have a crucial role in the future development of therapeutic drugs (Najmi et al., 2022).

The advancements in extraction technology have enabled the acquisition of more potent and pure extracts from plants, leading to the enhancement of the effectiveness and safety of these products. *Senna alata* Linn is becoming increasingly popular for extraction in the pharmaceutical, skincare, and cosmetic industries based on natural ingredients (Oladeji et al., 2021; Clement et al., 2021; Ceylan et al., 2019). Multiple extraction techniques, including solvent extraction, supercritical fluid extraction, and pressurized liquid extraction, have been developed and improved to maximize the yield, purity, and bioactivity of plant extracts (Menshutina et al., 2021; Zhang et al.,2018). Moreover, these methods can be customized according to the specific chemical properties of the desired compounds while minimizing solvent usage and reducing environmental impact (Zhang et al., 2018). Furthermore, the use of advanced analytical techniques, such as high-performance liquid chromatography

(HPLC), gas chromatography-mass spectrometry (GCMS), and nuclear magnetic resonance (NMR), can help ensure the quality, consistency, and standardization of herbal extracts and nutraceuticals. Overall, the adoption of cutting-edge extraction technology and analytical methods can help meet the growing demand for natural products with enhanced bioactivity and safety.

S. alata, a member of the Fabaceae or Leguminosae family, is a widely recognized medicinal plant that has been extensively utilized in traditional medicine worldwide to address various health ailments. It has gained a reputation for its therapeutic properties in treating skin conditions, constipation, burns, diarrhea, and gastroenteritis (Goh et al., 2017). Additionally, research by Fatmawati et al. (2020) has highlighted its potential in alleviating stomach pain during pregnancy, headaches, and paralysis. In Malaysia, *S. alata* is notably employed in the treatment of fungal infections and skin diseases associated with ringworm (Yon et al., 2022; Kulip, 2003; Ong & Nordiana, 1999). The medicinal and laxative properties of *S. alata* leaves have gained significant popularity, leading researchers to explore the bioactive compounds present in this plant. These compounds include anthraquinones, tannins, saponins, alkaloids, flavonoids, terpenes, steroids, as well as cardiac glycosides and phenolics (Channa et al., 2020; Yakubu et al., 2010). Numerous studies have documented a wide range of biological activities associated with *S. alata*, including antimicrobial, antifungal, antitumor, antioxidant, cytotoxic, and hypoglycemic effects (Rahim et al., 2023; Chahardehi et al., 2021; Oladeji et al., 2020; Fernand et al., 2008).

Researchers have employed various extraction methods, ranging from conventional method such as maceration, percolation, Soxhlet extraction (Rahim et al., 2023; Fatmawati et al. 2020; Subuki et al., 2018; Dhanani et al., 2017) to non-conventional method that offer several advantages, including automation, shorter extraction times, and reduced use of organic solvents (Rahim et al., 2023, Zhang et al., 2018) such as supercritical fluid extraction (SFE), and microwave-assisted extraction to extract the bioactive compounds from *S. alata* (Rahim et al., 2023; Fatmawati et al. 2020; Subuki et al., 2018). An innovative extraction method known as Subcritical Carbon Dioxide Soxhlet (SCDS) extraction has emerged as a promising option,

particularly for preserving the quality of extracts (Ghafoor et al., 2022; Easmin et al., 2017; Chia et al., 2015). Although it may yield a lower quantity compared to SFE, it effectively safeguards delicate constituents such as essential oils, terpenes, and other sensitive chemicals within the plant, making it highly desirable for producing comprehensive plant products (Chia et al., 2015). Notably, no previous study has investigated the use of the SCDS extraction method specifically for *S. alata*. Therefore, the objective of this project is to extract bioactive compounds from *S. alata* leaves using SCDS extraction, conventional maceration and Soxhlet extraction methods for comparative analysis. The resulting extracts were thoroughly examined, and the antimicrobial and antioxidant properties of the different *S. alata* leaf extracts were assessed as part of this research endeavour.

1.2 PROBLEM STATEMENT

There is a pressing and ongoing need to explore and develop more efficient extraction techniques for retrieving compounds from *S. alata* leaves. Conventional extraction methods, such as Soxhlet extraction and maceration involve the use of organic solvents and prolonged extraction durations, which may lead to solvent residue, thermal degradation of sensitive compounds, and low extraction efficiency. In contrast, non-conventional extraction techniques, such as supercritical fluid extraction, subcritical carbon dioxide Soxhlet (SCDS) extraction, offer several advantages, including reduced extraction time, selective extraction compounds, the potential to preserve bioactive components and environmentally friendly techniques. Despite the availability of different extraction techniques, there is a significant knowledge gap between conventional and SCDS methods regarding their efficacy in extracting phytochemicals from *S. alata* leaves and the subsequent impact on the antioxidant and antimicrobial properties of the obtained extracts. Understanding the variations in extraction efficiency, phytochemicals profiles, and bioactivity of the extracts obtained using conventional (Soxhlet and maceration) and non-conventional (SCDS) methods is essential for maximizing the therapeutic potential of *S. alata* leaves. By comparing the conventional and non-conventional extraction methods, this study seeks to provide

insights into a more sustainable approach for extracting phytochemicals from *S. alata* leaves while preserving their bioactivity. The findings will contribute to enhance the understanding of the phytochemical profile and biological potential of *S. alata* extracts and facilitate their utilization in the development of natural antioxidants and antimicrobial agents.

1.3 RESEARCH OBJECTIVES

The aim of this research is to explore a sustainable extraction approach for obtaining phytochemicals from *S. alata* leaves while preserving their bioactivity. This study compares three different extraction methods which are Soxhlet, maceration and SCDS extraction methods.

1. To compare the total yield percentage of *S. alata* leaf extracts obtained from SCDS, Soxhlet and maceration methods.
2. To identify the phytochemical constituents from different extracts of *S. alata* leaves.
3. To evaluate antioxidant activity of different extracts of *S. alata* leaves.
4. To examine antimicrobial activity of *S. alata* leaf extracts against *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Salmonella enterica, Klebsiella pneumoniae, Enterococcus faecalis* and *Candida albicans* using disc diffusion method.

CHAPTER TWO:

LITERATURE REVIEW

2.1 ETHNOBOTANICAL DESCRIPTION OF *S. ALATA*

2.1.1 Taxonomy of *S. alata*

The plant commonly known as Gelenggang in Malaysia is scientifically named *Senna alata*, previously referred to as *Cassia alata* (Rahim et al., 2023). Additionally, this plant has various other names across different regions, including 'Ewe Asunwon Oyinbo' in the Southwest of Nigeria (Adelowo & Oladeji, 2017), 'Chumhetthet' in Thailand (Oladeji et al., 2021), 'Ketepeng China' in Indonesia (Fatmawati et al., 2020), and 'Akalpuko' in the Philippines (Tababa et al., 2020). In English, it is commonly referred to as candle bush or ringworm bush. Besides, *S. alata* is a shrub that blooms annually or biannually and belongs to the Fabaceae family. It thrives in tropical regions and has the ability to grow naturally (Fatmawati et al., 2020). Oladeji et al. (2021) described *S. alata* as a branching shrub with a maximum height of 1.8 meters. The plant features pinnate leaves with a parallel arrangement of leaflets ranging from thirty to sixty centimeters in length. Each leaf typically consists of eight to twenty pairs of leaflets. Furthermore, each leaflet is oblong or elliptic in shape, with rounded ends on both ends, and the flowers are dense in axillary racemes that are twenty to fifty cm long and three to four cm wide (Rahim et al., 2023). The common name ‘candle bush’ is due to the flowering inflorescence mimicking the candle (Fatmawati et al., 2020). As seen in Figure 1.0, the inflorescence resembles a yellow candle, and they have thick, flattened wings. Taxonomically, *S. alata* is classified as in Table 2.1.

Table 2.1 Taxonomy Classical of *S. alata* Linn

Kingdom	: Plantae
Subkingdom	: Viridiplantae
Infrakingdom	: Streptophyta
Superdivision	: Embryophyta
Class	: Magnoliopsida
Superorder	: Rosanae
Order	: Fabales
Family	: Fabaceae
Genus	: *Senna*
Species	: *Senna alata* Linn

(a)

(b)

Figure 2.1 (a) *Senna alata* plant, (b) Leaves of *S. alata*

2.1.2 Habitat of *S. alata*

According to Norlizah (2017), *S. alata*, also known as the Candle Bush, exhibits vigorous growth when exposed to full sun and cultivated in soils that retain adequate moisture. It can thrive in a wide range of soil types, including heavy and sandy soils, and it can tolerate soil pH levels ranging from slightly acidic to slightly alkaline, with a pH range of 5.6 to 7.8, as mentioned by Rahim et al. (2023). Norlizah (2017) further explains that *S. alata* prefers regions with an annual rainfall range of 600 to 4300 mm. It is also adapted to average annual temperatures between 15 and 30°C (Oladeji et al., 2021; Rahim et al., 2023).

2.1.3 Traditional uses of *S. alata*

S. alata has been widely employed as a traditional remedy by individuals across the globe due to its therapeutic properties. Over the years, people have commonly utilized the leaves of this plant by preparing decoctions or simply boiling them (Rahim et al., 2023). Additionally, raw *S. alata* leaves have been applied topically (Fatmawati et al., 2020). However, among the plant parts of *S. alata*, the leaves are the most frequently utilized in traditional applications compared to stems, bulbs, roots, and seeds (Oladeji et al., 2020). In addition to its therapeutic attributes, *S. alata* is processed into various forms such as tablets, pellets, and tea in certain countries (Rahim et al., 2023; Oladeji et al., 2020). These products are intended to support overall well-being and maintain good health (Oladeji et al., 2020). Thus, *S. alata* not only serves as a traditional medicinal resource but is also incorporated into supplements for its beneficial properties.

S. alata, a plant renowned for its medicinal properties, is utilized in traditional medicine through its different plant parts. The leaves of *S. alata* are commonly used in traditional medicinal practices. They are prepared as a decoction in the southwestern region to relieve abdominal pain, toothaches, convulsions, dermal infections, and stress,

while also acting as a purgative and antidote (Oladeji et al., 2020). In Egypt, the leaves are brewed into decoctions to stimulate bowel movement and prevent constipation. Additionally, leaf extracts have been employed to address constipation, syphilis, intestinal parasitosis, hernia, and diabetes (Rahim et al., 2023; Tcheghebe et al., 2017). *S. alata* leaf decoctions have shown effectiveness in treating various skin conditions such as ringworm, eczema, itching and rashes (Rahim et al., 2023). The leaves are used in tropical Africa to treat stomach pain in pregnant women as well as haemorrhoids, dysentery, jaundice, diarrhoea, hernia, and paralysis. Furthermore, a topically applied mixture of powdered leaves and vegetable oil is traditionally used to treat a variety of skin problems (Tcheghebe et al., 2017).

Aside of that, *S. alata* seeds possess notable medicinal properties and are specifically utilized in traditional Chinese medicine for addressing asthma, improving visual health, and serving as aperients and diuretic agents (Tcheghebe et al., 2017). Similarly, the flowers of *S. alata* hold medicinal significance in selected regions, particularly in India, where they are employed as immune stimulants and are believed to possess curative properties for conditions such as ringworms, scabies, gonorrhoea, and urinary tract infections (Rahim et al., 2023).

Furthermore, the combination of different plant parts in therapeutic applications is evident. In Malaysia, a synergistic mixture of *S. alata* leaves and barks are employed to effectively inhibit superficial fungal growth (Rahim et al., 2023). In northern Nigeria, a decoction prepared from the leaves, stems, and roots of the plant are utilized for treating diverse ailments including burns, respiratory tract issues, wounds, and constipation. Additionally, in Cameroon, *S. alata* finds application in the treatment of gastroenteritis, dermal infections, ringworm, and hepatitis (Tcheghebe et al., 2017). Therefore, the extensive utilization of distinct components of the *S. alata* plant in traditional medicinal practices serves as a testament to its significant therapeutic potential and the diverse range of traditional applications associated with it.

2.2 EXTRACTION METHODS OF *S. alata*

The choice of extraction methods and solvents is crucial in determining both the quantity and quality of extracts obtained from *S. alata* (Rahim et al., 2023). However, the existing scientific research on *S. alata* is limited in identifying the specific chemical constituents and their associated bioactivities. This lack of comprehensive knowledge makes it challenging to develop a standardized method to effectively extract and determine which compounds in *S. alata* provide human health benefits.

The process of extracting phytochemicals from *S. alata* typically involves several sequential steps. First, the solvent is introduced to the plant matrix, allowing it to permeate and interact with the plant material. Second, the solute present in the plant dissolves into the solvent, facilitating the extraction of desired compounds. Third, the solute is diffused from the plant tissue, allowing the bioactive compounds to be released and collected. This final step involves gathering the extracted compounds, which can then be further studied and utilized (Zhang et al., 2018).

To better understand the selectivity of plant extraction, it is recommended to employ different extraction techniques under specific conditions. This approach, as proposed by Azmir et al. (2013), acknowledges that different plant materials may require tailored extraction methods to maximize the yield and specificity of the desired bioactive compounds. Researchers can explore the most effective ways to extract the beneficial compounds from *S. alata* by utilizing various extraction techniques. These techniques can involve variations in factors such as temperature, pressure, solvent composition, and extraction duration (Zhang et al., 2018). Table 2.2 summarizes extraction methods, solvents used, yield percentages, locations, and corresponding references for previous findings of the leaves of *S. alata*.

Table 2.2. Extraction methods, solvents used, locations of sample collection, and yield obtained from *S. alata* leaves

Extraction Method	**Location**	**Type of Solvent**	**Yield Percentage (%)**	**Reference**
Reflux	Vietnam	Methanol	20.9	Pham et al. (2021)
Percolation	Malaysia	Hexane	4.023	Adiana & Mazura (2011)
		Dichloromethane	5.044	
		Ethyl acetate	4.256	
		Methanol	8.051	
	Nigeria	Ethanol	1.89	Igwe & Onwu (2015)
	Thailand	80% ethanol	22.6	Gritsanapan & Mangmeesri (2009)
	Nigeria	Water	46	Doughari and Okafor (2007)
		Acetone	30	
		Methanol	24	
	Nigeria	Hot water	46	Ehiowemwenguan et al. (2014)
Hydrodistillation	Brunei	Distilled water	<1	Aminuddin et al. (2016)
	Nigeria	Distilled water	0.1	Ogunwande et al. (2010)
	Thailand	Distilled water	15.58	Naowaboot & Wannasiri (2016)
	Japan	Distilled water	19.9	Moriyama et al. (2003)
Soxhlet	Malaysia	Distilled Water	19	Somchit et al. (2003)
		Ethanol	27	
	Thailand	70% ethanol	34.94	Wuthi-Udomlert et al. (2010)
	Thailand	80% ethanol	25.6	Gritsanapan & Mangmeesri, (2009)
	India	95% ethanol	15	Anandan et al. (2016)
	Thailand	90% ethanol	25.4	Wuthi-Udomlert et al. (2003)
	Nigeria	80% methanol	23.12	Kingsley et al. (2011)
Microwave-assisted extraction	Vietnam	Ethanol	8.16	Le (2019)

Supercritical Fluid extraction	Malaysia	Carbon dioxide	3.62	Subuki et al. (2018)

2.2.1 Conventional extraction method

Prior to the emergence of new extraction technologies, researchers commonly employed conventional methods for plant sample extraction. These conventional approaches encompassed techniques such as maceration, percolation, Soxhlet extraction, hydrodistillation and reflux (Rahim et al., 2023; Dhanani et al., 2017; Adelowo and Oladeji, 2017; Gritsanapan and Mangmeesri, 2009).

The Soxhlet extraction method, a widely recognized conventional approach, utilizes heat and solvent to extract plant samples. In Soxhlet extraction, the size of the sample plays a crucial role, with smaller particles being preferred as they offer a larger surface area for greater phytochemical extraction (Gopalasatheeskumar, 2018). While Soxhlet extraction is known for its efficiency and the requirement of only a small amount of solvent compared to maceration and percolation, it has a significant drawback. The method exposes researchers to hazardous and flammable solvents, some of which may be toxic. During the heating process, vaporized solvents may escape from the system, posing risks to the surrounding environment (Azwanida, 2015).

In a study conducted by Somchit et al. (2003) on the extraction of *S. alata* using the Soxhlet method, it was observed that a higher crude yield could be obtained from leaves compared to stem barks. The researchers used 95% ethanol as the solvent and performed continuous hot percolation for 12 hours to extract the leaves and bark. The ethanolic extract yielded 27% for leaves and 25% for barks. Another study focused on anthraquinone aglycones from the crude ethanolic extract of *S. alata* leaves, which reported a yield of 34.94% using 70% ethanol. The crude extract was subsequently further extracted with an ethanolic aqueous mixture to obtain anthraquinone aglycones extracts (Wuthi-Udomlert et al., 2010).

In addition to ethanol, methanol is frequently used in Soxhlet extraction of *S. alata*. Kingsley et al. (2011) conducted a study using 80% methanol, extracting it for

24 hours. After removing excess methanol through drying with a rotary evaporator, a yield of 23.12% was obtained. The sample was then stored and used for toxicity testing. In an in vitro antioxidant study that focused on phenolic compounds of *S. alata* leaves, a mixture of methanol and water in a 9:1 ratio was used for extraction. The extract was shaken with n-hexane, resulting in a hexane fraction yield of 31%. The remaining extract was diluted with water and subjected to fractionation using solvent extractions with chloroform, ethyl acetate, and n-butanol, yielding 1.38 g, 0.84 g, and 0.82 g, respectively (Okpuzor et al., 2009). These findings portray the potential of conventional extraction methods to obtain bioactive compounds from *S. alata*. Further research and optimization of extraction parameters may lead to improved extraction yields and enhanced utilization of this plant species for various applications.

2.2.2 Non-conventional Subcritical Carbon Dioxide Soxhlet (SCDS) method

The SCDS method represents an advanced extraction technique combining the strengths of Soxhlet and carbon dioxide extraction. This innovative method utilizes liquid carbon dioxide to extract phytoconstituents from natural products, benefiting from continuous carbon dioxide reformulation through boiling and condensation during extraction (Easmin et al., 2017). Notably, the SCDS method operates without the need for a pump, as the solvent circulates naturally (Chia et al., 2015). Subcritical fluids, which are compressed below their critical temperature but maintained in a liquid state and used above their boiling points with the application of pressure (Chia et al., 2015), play a crucial role in this method. These fluids exhibit distinct behaviour, demonstrating characteristics of both liquids and gases. Their unique properties, such as liquid-like density and gas-like diffusivity, enable rapid equilibrium and efficient permeation in micropores. As a result, subcritical fluid extraction finds extensive applications in various fields, including extractions, controlled drug delivery systems, food processing, remediation, pollution prevention, powder processing, crystallization, bio separations, and polymerization (Gu et al., 2019; Easmin et al., 2017; Chia et al., 2015).

The SCDS method utilizes an intriguing phenomenon known as superheating, allowing a liquid to be heated above its boiling point without undergoing boiling

(Easmin et al., 2017). This process involves uniformly heating a pure substance in a smooth container, eliminating nucleation sites that could trap air and form vapor bubbles (Chia et al., 2015). By exceeding the critical point through increased pressure and temperature, the fluid enters a supercritical state where the gas and liquid phases coexist without a clear phase boundary. This transition occurs because thermal expansion reduces liquid density while pressure increments enhance gas density. These advanced supercritical and subcritical fluid extraction methods have proven instrumental in reducing reliance on hazardous organic chemicals and promoting eco-friendly practices within the field of green technology (Chia et al., 2015).

Co-solvents are introduced alongside the main solvent to enhance carbon dioxide's solvent power for dissolving polar compounds. Commonly used co-solvents include short-chain alcohols, esters, or ketones, with ethanol being a popular choice. Ethanol offers advantages such as widespread availability, affordability, and environmental safety in its pure forms (food and pharmacopeia grades) (Souza et al., 2017). Carbon dioxide is considered an optimal choice for subcritical fluid extraction due to its ability to produce extracts with reduced levels of pigments, waxes, and resins, while effectively preserving significant bioactive compounds such as phenolics and terpenoids (Easmin et al., 2017; Chia et al., 2015). The acceptance and application of carbon dioxide in both supercritical and subcritical fluid states are driven by its desirable characteristics, including non-toxicity, recyclability, non-flammability, cost-effectiveness, easy availability, and straightforward removal from extracted materials (Gu et al., 2019; Chatterjee & Bhattacharjee, 2012). Although the SCDS method offers several advantages, there has been a lack of studies investigating its application to *S. alata* leaves. As a result, this research presents a valuable opportunity to explore new findings and insights in this area.

2.3 PHYTOCHEMICAL CONSTITUENTS OF *S. alata*

Plants contain a wide range of chemical compounds, including primary metabolites and secondary metabolites. Primary metabolites are essential for plant growth and are commonly found in most plant species. On the other hand, secondary metabolites are bioactive compounds that plants produce in response to specific environmental stresses, and they are believed to offer additional health benefits. *S. alata* has long been recognized for its therapeutic properties, and various parts of the plant, such as leaves, barks, flowers, roots, and seeds, have been traditionally used in medicine (Rahim et al., 2023). This indicates that all parts of the *S. alata* plant possess beneficial properties and contain functional compounds.

Numerous studies have focused on identifying the active compounds present in different parts of *S. alata*. Phytochemical screening tests have revealed the presence of tannins, saponins, anthraquinones, and flavonoids in the leaves and seeds of *S. alata*. Furthermore, the stem barks of the plant contain similar compounds found in the leaves and seeds, along with alkaloids, flavonoids, terpenes, and steroids (Fatmawati et al., 2020). Previous studies conducted by Rahim et al. (2023) and Fatmawati et al. (2020) have provided a comprehensive overview of the phytoconstituents present in different extracts of *S. alata*. The analysis revealed that the ethanol extract displayed positive results for alkaloids, flavonoids, carbohydrates, proteins, tannins, saponins, anthraquinones, and cardiac glycosides. Similarly, the methanol extract demonstrated the presence of alkaloids, flavonoids, carbohydrates, and proteins.

According to Fatmawati et al. (2020), the investigation revealed positive results for alkaloids, flavonoids, carbohydrates, and saponins in the chloroform extract. The acetone extracts similarly exhibited positive results, with the presence of tannins also detected. Moreover, the benzene extract demonstrated positive outcomes for alkaloids, carbohydrates, tannins, saponins, anthraquinones, and cardiac glycosides. Conversely, the petroleum ether extracts solely showed positive results for carbohydrates. Besides, the hexane extract did not indicate any positive indications of the tested phytochemical constituents, except for proteins. On the other hand, the aqueous extract of *S. alata*

exhibited the presence of alkaloids, flavonoids, carbohydrates, and proteins. The only solvent extract that displayed positive results for terpenoids was the benzene extract. Additionally, phlobatannins were not detected in any of the solvent extracts examined (Fatmawati et al., 2020).

To further investigate the specific compounds, present in *S. alata* extracts, various analytical chemistry methods are commonly employed. Techniques such as thin-layer chromatography (TLC), column chromatography, gas chromatography-mass spectrometry (GCMS), High-Performance Liquid Chromatography (HPLC) and Liquid chromatography mass spectrometry (LCMS) are used to identify and quantify the compounds in the extracts. According to a study by Dhanani et al. (2017), the primary bioactive phytochemicals present in Senna leaves and pods are dianthraquinone glucosides.

In another investigation, Adelowo and Oladeji (2017) found several compounds in the same plant, including phenolic compounds, flavonoids, alkaloids, tannins, steroids, quercetin, anthocyanin, and coumarins. The leaves oil of *S. alata* from Nigeria, as reported by Agnaniet et al. (2005), contained 1,8-cineole, β-caryophyllene, caryophyllene oxide, limonene, germacrene D, and α-selinene. In Bangladesh, the methanolic extract of *S. alata* leaves was found to contain tannin, specifically an ellagic acid derivative known as 2,3,7-tri-O-methylellagic acid (Sugita et al., 2014). Additionally, the ethanolic extract of *S. alata* leaves from Nigeria was found to contain flavonol glycosides, particularly 5,7-dihydroxy-2-(4-hydroxyphenyl)-3-oxy-chromene-4-one (Sugita et al., 2014). Anthraquinone derivatives have received significant attention due to their laxative effects found in *S. alata* leaves, as highlighted by Dhanani et al. (2017).

Extensive research by Fatmawati et al. (2020) and Rahim et al. (2023) has revealed the presence of more than 50 chemical constituents in different parts of the *S. alata* plant. A comprehensive analysis of the chemical constituents in different parts of the *S. alata* plant has revealed a diverse array of compounds. Notably, the leaf of *S.*

alata contains significant quantities of kaempferol and kaempferol-3-O-β-D-glucopyranoside, identified through NMR and HPLC methods, respectively (Panichayupakarant & Kaewsuwan, 2004; Moriyama et al., 2003). Flavone and 2,5,7,40-tetrahydroxy isoflavones have also been detected in the leaf using PTLC analysis (Fatmawati et al., 2020). Furthermore, the leaf exhibits the presence of anthraquinone, kaempferol 3-O-gentiobioside, (6Z)-7,11-dimethyl-3-methylidenedodeca-1,6,10-triene, 4a,8-dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,8a-octahydronaphthalene, 4,4,7a-trimethyl-5,6,7,7a-tetrahydro-1-benzofuran-2(4H)-one, 3,7-dimethylocta-1,6-diene, hexadecanoic acid methyl ester, hexadecanoic acid, octadecanoic acid methyl ester, adenine, chrysoeriol, quercetin, 5,7,40-trihydroflavanone, kaempferol-3-O-beta-D-glucopyranosyl-(1-6)-beta-D-glucopyranoside, n-dotriacontanol, n-triacontanol, stearic acid, palmitic acid, diomestin, luteolin, and 1,3,5-trihydroxy-7-methylanthracene-9,10-dione, as identified through various analytical techniques (Vandebroek & Picking (2020); Adiana & Mazura, 2011; Igwe & Onwu, 2015; Moriyama et al., 2003; Promgool et al., 2014; Tatsimo et al., 2017; Prasenjit et al., 2016). *S. alata* contains a variety of phytochemical compounds with varying distribution among different solvent extracts. These compounds include alkaloids, flavonoids, carbohydrates, proteins, tannins, saponins, anthraquinones, and cardiac glycosides, which are known for their potential bioactive properties. These findings provide valuable insights into the chemical composition of *S. alata* and highlight its potential for diverse applications in the field of natural product research.

2.4 BIOLOGICAL ACTIVITIES OF *S. alata*

Bioactive compounds play crucial roles in plant growth, development, and defense mechanisms, protecting predators (Zhang et al., 2018). However, their effects on humans and animals can range from beneficial pharmacological effects to potentially harmful toxicological effects (Azmir et al., 2013). Senna species, including *S. alata*, have gained significant attention in phytochemical and pharmacological research due to their therapeutic value (Fatmawati et al., 2020). Recent studies by Rahim et al. (2023) have highlighted the diverse range of beneficial properties exhibited by *S. alata*, including anti-inflammatory, antioxidant, antiplasmodial, antimicrobial, antifungal, antimutagenic, and potentially anticancer effects. Traditionally, *S. alata* has been used for treating wounds and various skin ailments due to its pharmacological properties (Rahim et al., 2023; Fatmawati et al., 2020).

Antibacterial activity in *S. alata* has been associated with compounds such as steroids, anthraquinones, glycosides, volatile oils, and tannins (Adedayo et al., 2001). Pieme et al. (2009) found potential antileukemic effects of *S. alata* attributed to flavonoids, polyphenols, tannins, steroids, and glycosides. Sule et al. (2011) reported antifungal properties in *S. alata* leaves, linked to various compounds such as anthraquinones, tannins, saponins, alkaloids, flavonoids, terpenes, and steroids. Naowaboot and Wannasiri (2016) evaluated the antilipogenic activity of *S. alata*, associated with flavonoids and polyphenols. Oladeji et al. (2016) observed the antimicrobial activity of *S. alata* and identified the presence of anthraquinones, flavonoids, and saponins. Furthermore, Moriyama et al. (2003) demonstrated the anti-inflammatory effects of *S. alata* in in vivo tests on mice, linked to the flavonoid glycoside kaempferol 3-O-gentiobioside. Chua et al. (2019) identified volatile oils and phytosterol in *S. alata*, which contribute to its antioxidant activity. These studies collectively provide evidence for the various bioactive compounds present in *S. alata* and their diverse pharmacological properties, supporting their traditional use in wound healing and skin ailments.

2.4.1 Antioxidant activity of *S. alata*

Antioxidants play a crucial role in maintaining overall health and well-being by counteracting the harmful effects of oxidative stress (Ranjit et. al., 2021). One of their most important functions is neutralizing free radicals, unstable molecules that can cause oxidative damage to cells, including DNA, proteins, and lipids (Chaves et al., 2020). By donating electrons or hydrogen atoms, antioxidants stabilize free radicals, preventing them from causing cellular damage. Oxidative stress, which occurs when there is an imbalance between the production of free radicals or reactive oxygen species (ROS) and the body's antioxidant defences, is responsible for the development of numerous diseases (Chua et al., 2020). This imbalance leads to cellular and tissue damage caused by the accumulation of free radicals. The deficiency of antioxidants or excessive production of ROS disrupts the equilibrium between ROS formation and elimination, leading to an increase in ROS levels and the consequent onset of oxidative stress (Chua et al., 2020). Therefore, it is essential to explore plants that possess antioxidant properties as potential sources of antioxidants to counteract this oxidative stress.

In addition to their ability to neutralize free radicals, antioxidants help restore the balance between the production of free radicals and the body's antioxidant defense mechanisms, thereby reducing oxidative stress (Ballester et al., 2023) By scavenging excessive free radicals, antioxidants protect cells and tissues from damage, preserving their optimal function and contributing to overall health. Furthermore, antioxidants play a significant role in supporting immune function. The immune system relies on a well-functioning network of cells and molecules to defend against pathogens and maintain immune health (Kurutas., 2016). Oxidative stress can impair immune function, making the body more susceptible to infections and diseases (Kurutas., 2016). Antioxidants help protect immune cells from oxidative damage, ensuring their proper functioning and maintaining a robust immune response (Kurutas., 2016). Besides that, antioxidants have anti-inflammatory properties. Chronic inflammation is a major contributor to the onset of many diseases, including cardiovascular disease, diabetes, and autoimmune disorders (Arulselven et al., 2016).

Plant extracts can be a valuable source of antioxidants. Many plants contain compounds with antioxidant properties, such as polyphenols, flavonoids, carotenoids, and vitamin C. Extracts obtained from these plants can concentrate these antioxidant compounds and provide a higher antioxidant activity compared to consuming the whole plant (Chaves et al., 2020). One commonly used method is the measurement of total antioxidant capacity, which evaluates the ability of the extract to scavenge free radicals or inhibit oxidative reactions (Chua et al., 2020). Other specific assays may focus on measuring the activity of individual antioxidants or the inhibition of specific oxidative markers. It is important to note that the antioxidant activity of plant extracts can vary depending on several factors, including the specific plant species, growing conditions, extraction methods, and formulation (Chaves et al., 2020). Therefore, it is crucial to choose standardized extracts from reputable sources to ensure consistent quality and potency.

Several bioassays have been developed to evaluate the antioxidant activities of compounds from plant samples. These include the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Oladeji et al., 2020), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Priya et al., 2022), Ferric Reducing Antioxidant Power Assay (FRAP) (Oladeji et al., 2020), Oxygen Radical Absorbance Capacity (ORAC) (Monteiro et al., 2018), and Hydroxyl radical scavenging activity (HRSA) (Oladeji et al., 2020). These bioassays provide different methods to measure the antioxidant potential of compounds, allowing researchers to assess their ability to scavenge free radicals or reduce oxidative stress. Each assay has its own advantages and limitations, and the choice of assay depends on the specific requirements of the study and the type of antioxidant being evaluated.

The plant extracts derived from *S. alata* leaves have demonstrated significant antioxidant potential through different assays (Rahim et al., 2023; Fatmawati et al., 2020). The DPPH radical scavenging activity assay has revealed the ability of *S. alata* leaves extracts to neutralize free radicals and reduce oxidative stress (Rahim et al., 2023). Furthermore, the high total phenolic content found in *S. alata* leaves contributes to their antioxidant properties, as phenolic compounds are known to possess strong

antioxidant activity (Fatmawati et al., 2020). Additionally, *S. alata* leaves extracts have exhibited notable ferric reducing antioxidant power (FRAP), indicating their capacity to donate electrons and scavenge free radicals (Oladeji et al., 2020). Studies have also shown that *S. alata* leaf extracts can inhibit lipid peroxidation, preventing the generation of reactive oxygen species and protecting against cellular damage (Rahim et al., 2023; Oladeji et al., 2020). Moreover, the extracts have been found to enhance the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), further reinforcing their antioxidant defense system (Sagnia et al., 2014). However, it is important to consider that the antioxidant activities of *S. alata* can vary depending on factors such as extraction methods, plant parts used, and geographical location (Angelina et al., 2021).

A study reported by Vedekoi & Selestin (2020) demonstrated remarkable antioxidant activity as evidenced by its superior performance in inhibiting the DPPH radical with an IC_{50} value of 12.05 mg/ml. In addition, the *S. alata* leaves extract exhibited a potent ferric reducing antioxidant power (FRAP) with an IC_{50} value of 2.79 mg/ml. Notably, these antioxidant activities surpassed those of the positive control, ascorbic acid, which had an IC_{50} value of 17.69 mg/ml. These findings indicate that the leaves of *S. alata* contain a significant number of secondary metabolites, which are responsible for its robust antioxidant capabilities. The presence of these secondary metabolites justifies the traditional use of *S. alata* as a natural source of antioxidants in folkloric medicine.

In addition, a study conducted by Oyebade et al. (2021), different extracts of *S. alata* leaves, including hexane, ethyl acetate, ethanol, and distilled water extracts, were tested for their antioxidant activity. Among these extracts, the aqueous extract exhibited the highest antioxidant activity, as indicated by a low IC_{50} value of 12.89 μg/ml. This finding suggests that the water extract of *S. alata* leaves contains potent antioxidant compounds, further highlighting its potential as a natural source of antioxidants (Oyebade et al., 2021).

2.4.2 Antimicrobial activity of *S. alata*

Antimicrobial activity refers to the ability to inhibit the reproduction and growth of microorganisms. The efficacy of antimicrobial agents is influenced by various factors, including the status of bacteria such as susceptibility, resistance, tolerance, persistence, and biofilm formation and the size of the initial bacterial population (Li et al., 2017). Additionally, the concentration of the antimicrobial agent, including the mutant selection window and sub-inhibitory concentrations, as well as host factors like the effects of serum and the impact on the gut microbiota, can impact the efficacy of treatment (Li et al., 2017). Susceptibility testing is critical for discovering new compounds with antimicrobial properties, especially given the rapid emergence of antimicrobial resistance, which could lead to a post-antibiotic era in which existing antibiotics are rendered ineffective (Mercer et al., 2020). The broth dilution test and the disc diffusion test are two common antimicrobial susceptibility tests (Rahim et al., 2023).

Doughari and Okafor (2007) conducted a study using the cup plate agar diffusion technique to evaluate the antimicrobial activity of aqueous and organic extracts of *S. alata* roots and leaves. The results showed significant antimicrobial effects against both gram-negative and gram-positive bacteria, as well as several fungi. The organic extracts exhibited superior antimicrobial activity compared to the aqueous extracts. The minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of the methanol extracts were reported to be 6-20 mg/ml and 25-100 mg/ml, respectively, for bacteria and fungi. Furthermore, preliminary phytochemical investigations revealed the presence of tannins, saponins, glycosides, flavonoids, and phenols in the extracts, supporting the antimicrobial usage of *S. alata*.

In a study using the agar disc diffusion assay, the aqueous and ethanolic extracts of *S. alata* showed excellent inhibition against *Aspergillus niger* and *Aspergillus flavus* (Ogunjobi & Abiala, 2013). Another study utilizing the paper disc diffusion method found that the leaf extract exhibited the highest inhibitory effect against *Staphylococcus aureus* and *Escherichia coli*. Additionally, the seeds of *S. alata* demonstrated

antimicrobial activity with a minimum inhibition concentration (MIC) of 200 mg/ml against *S. aureus* (Le, 2019). More recent studies of antimicrobial activities of *S, alata* are summarized in Table 2.3. While these studies provide evidence of the antimicrobial potential of *S. alata* leaves, it is important to note that further research is necessary. Future studies should focus on determining the optimal extraction methods, identifying the active compounds responsible for the antimicrobial activity, and conducting safety and efficacy evaluations of *S. alata* extracts in human clinical trials.

Table 2.3 *S. alata* antimicrobial activities and methods

Plant parts	Method	Antimicrobial activities	References
Leave extracts	Agar well diffusion	*S. alata* extract showed an inhibition zone ranging from 1.15 to 1.59 mm against *Pseudomonas aeruginosa*	Halim, (2019)
Leave extracts	Agar well diffusion	The herbal gel showed a larger zone of inhibition compared to the standard antibacterial and antifungal formulations against *S. aureus* and *C. albicans*. The MIC value of the herbal gel was lower than that of the marketed formulations	Iraqui et al., (2019)
Leave extracts	In vitro (microdilution) In vivo antifungal efficacy (whole plant method)	Compounds such as kaempferol, (-)-epiafzelechin, rhein, kaempferol-3-O-glycoside, kaempferol-3-O-gentiobiside, and aloe-emodin-8-O-β-D-glucoside displayed in vitro antifungal activity against *Magnaporthe oryzae* and Phytophthora species. Rhein showed the best antifungal activity, inhibiting the growth of Phytophthora species and effectively suppressing tomato late blight in vivo	Pham et al., (2021)
Leaves extracts	Broth microdilution	The MIC of all samples *against S. aureus* was 250 µg/mL, while for other bacteria, the MIC was 125 µg/ml. The geographical origin of the plant extracts affected their antibacterial activity, with the	Angelina et al., (2021)

		lowest MBC observed in extracts from South Tangerang and Kalimantan	
Leaves extract	Broth dilution	Both aqueous and ethanolic leaf extracts showed antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Salmonella Typhi,* and *Klebsiella pneumoniae* at the tested concentrations based on the MIC values	Zakaria et al., (2022)
Root and leaves extracts	Agar well diffusion	Extraction using ethyl acetate showed the largest inhibition zone against *S. aureus* with an average diameter of 15.30 mm (root Soxhlet extract), followed by 14.70 mm (leaf Soxhlet extract) and 13.70 mm (root maceration extract)	Toh et al., (2023)
Leaves and seeds extract	Agar disc diffusion	*S. aureus* and *E. coli* were inhibited at MIC of 400 mg/ml, while *S. enteritidis* and *B. subtilis* were inhibited at MIC of 800 mg/ml. Seed extracts also inhibited *S. aureus* at a MIC of 200 mg/ml and *E. coli, S. enteritidis,* and *B. subtilis* at a MIC of 400 mg/ml. Both extracts had no effect on the growth of *A. niger.*	Le (2019)
Leaves	Agar disc diffusion	Ethanolic extract inhibited *S. aureus, B. cereus, Klebsiella* spp., *A. niger*, and *C. albicans* better than a methanolic extract, with inhibition of 21, 20, 22, 29, and 28 mm, respectively.	Oladeji et al., (2016)
Leaves	Agar well diffusion	Inhibition zones for *A. niger* was the highest for ethanolic and aqueous plant extracts, followed by *A. flavus* and *S. aureus*, which were 25.2, 27.2, 22.1, 20.1, 20.1, and 18.2 mm, respectively.	Ogunjobi & Abiala (2013)

CHAPTER THREE

METHODOLOGY

3.1 MATERIALS

All the materials and organisms utilized in this study, including those involved in the preparation, extraction, analysis of phytochemical constituents, and biological investigations, are comprehensively listed in this section.

3.1.1 Chemicals

The chemicals utilized in the study were sourced from reputable suppliers and manufacturers. Methanol and ethanol were obtained from HMBG Chemical, while hexane was sourced from EMSURE®. The culture media components, including Mueller Hinton Agar and Nutrient Agar, were procured from Oxoid, while Mueller Hinton Broth and Nutrient Broth were obtained from Sigma Aldrich and Oxoid, respectively. Antibiotics such as Streptomycin and Nystatin were purchased from Sigma Aldrich and Oxoid, respectively, for antimicrobial assays. Glycerol, used for bacterial stocks preparation was supplied by Sigma Aldrich. Additionally, the antioxidants DPPH reagent and Resazurin Dye were sourced from Sigma Aldrich for antioxidant assays. Ascorbic Acid, serving as a standard reference, was acquired from SYSTERM for comparative analysis. These chemicals were carefully selected to ensure the accuracy and reliability of the experimental procedures conducted in this study.

3.1.2 Glassware, Disposable and Consumable items

Table 3.1 List of glassware, disposable and consumable items

Material	Supplier/ Brand
Plastic cuvette	Biofil
Whatman filter No. 1	GmbH
1.5 ml centrifuge tube	Eppendorf
3 mL luer lock syringe	Terumo
0.45 µL nylon syringe filter	Agilent
28 mL universal bottle	-
Plastic Petri Dish (90 mm x 15 mm)	Brandon
Pipette tips yellow	Eppendorf
Pipette tips blue	Eppendorf
96-well microplate	BRANDplates®
Beaker	Brandson
Conical flask	Brandson
Schott bottle	Thermo Scientific
Measuring cylinder	Brandson
Inoculating loop	-
Toothpick	-

3.1.3 Apparatus and Equipment

Table 3.2 List of apparatus and equipment

Apparatus/ Equipment	Model/ Manufacturer
Oven dryer	Memmert
Subcritical Carbon Dioxide Soxhlet Unit	FeyeCon BV, Netherlands
Laboratory blender	Panasonic
Weighing balance	Mettler Toledo
Vortex	Thermo Fisher
Water bath	Memmert
Soxhlet apparatus	GMBH
Rotary Evaporator	BÜCHI
Multichannel Micropipette (100 µL)	Eppendorf
Micropipette (10 µL, 100 µL, 1000 µL)	Thermo Fisher
Autoclave Machine	Hirayama
-80 °C Freezer	Sanyo, Medfrez
-20 °C Freezer	Samsung
4 °C Refrigerator	Samsung
Biosafety Cabinet Class II	Esco
Laminar Flow	Esco
Incubator Shaker	Binder
Incubator	Binder
Spectrophotometer	Perkin Elmer
GCMS	Perkin Elmer
Microplate reader	Tecan

3.1.4 Organisms

Table 3.3 List of tested microorganisms

Organism	Strain	Media
Staphylococcus aureus	ATCC25923	Schaedler Sheep Blood Agar
Staphylococcus epidermidis	ATCC12224	Schaedler Sheep Blood Agar
Enterococcus faecalis	ATCC29212	Schaedler Sheep Blood Agar
Pseudomonas aeruginosa	ATCC27353	Schaedler Sheep Blood Agar
Salmonella enterica	ATCC1402	Schaedler Sheep Blood Agar
Klebsiella pneumoniae	ATCC700603	Schaedler Sheep Blood Agar
Candida albicans	ATCC14053	Sabouraud Dextrose Agar (SDA)

3.2 FLOWCHART OF THE STUDY

This study involved five main parts that address the central methodology of this project as illustrated in Figure 3.1. The first part was the authentication of the plant sample used; the second was the determination of moisture content by the gravimetric analysis method. The third part covers the different extraction methods used for the determination of their total yield percentage and statistical data analysis. Then the next part covers phytochemical constituents' analysis by GCMS and lastly was, the biological activities, which were antimicrobial and antioxidant assays.

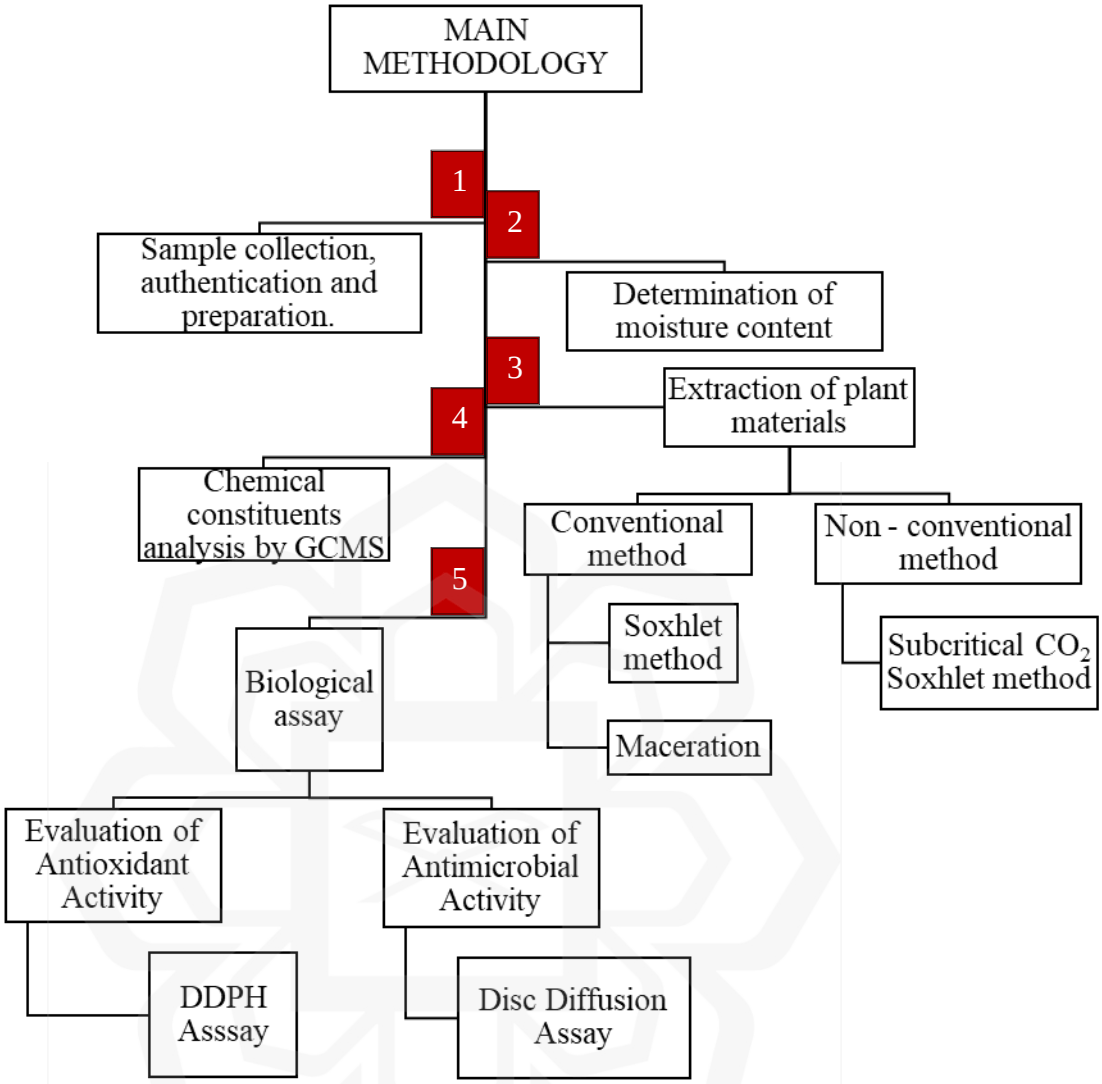

Figure 3.1 Flowchart of the main methodology used in this study

3.3 SAMPLE COLLECTION AND AUTHENTICATION

About 10 kg of *S. alata* plant were collected from Felda Sungai Panching Selatan, Kuantan, Pahang. The specimen sample consists of a stem with attached leaves were pressed in a plant press and deposited at Kulliyyah of Pharmacy Herbarium, IIUM Kuantan for voucher specimen. The specimen was morphologically verified by a botanist, Dr. Shamsul Khamis, from Universiti Kebangsaan Malaysia (UKM). Around 5 kg of *S. alata* leaves were collected and washed with tap water for debris removal. The leaves were oven dried at 40°C for seven days and then the dried leaves were ground into smaller particles as in Figure 3.2 with an electrical grinder and stored at 4°C for further analysis.

Figure 3.2 Ground-dried leaves of *S. alata*

3.4 DETERMINATION OF MOISTURE COMPOSITION OF *S. alata LEAVES*

The sample's moisture content was determined using the method used by Onyegeme-Okerenta et al. (2017). One gram of fresh leaves of *S. alata* was transferred into an empty pre-weight silica dish. Then it was left in the oven at 105 °C for one day. The samples were cooled in the desiccator and reweighed before being dried in the oven for another day. The process of cooling was done repeatedly until a consistent weight was reached. This procedure was done in triplicate. The composition of moisture of *S. alata* leaves was then determined using the equation below.

$$\text{Moisture Content (\%)} = \frac{\text{Fresh wt} - \text{Dry wt}}{\text{Dry weight}} \times 100 \qquad \text{(Equation 1)}$$

3.5 EXTRACTION OF PHYTOCHEMICALS FROM *S. alata* LEAVES

3.5.1 Soxhlet

The extraction of *S. alata* leaves was performed using the Soxhlet extraction method, with some modifications based on the procedure outlined by Dhanani et al. (2017). An amount of 20 g of dry powdered leaves was placed in a cellulose thimble and subjected to extraction using two different solvents which were n-hexane and methanol at a 250 ml volume separately. The extraction process was conducted for 12 hours at a temperature not exceeding 70 °C. The setup of the extraction process is illustrated in Figure 3.3.

Following the extraction, the solvents were removed from the extracts using a rotary evaporator at 60 °C under vacuum pressure. The vacuum pressure applied varied depending on the solvent used, with 335 millibar (mbar) for n-hexane and 373 mbar for methanol. Subsequently, the crude extracts were further dried for three days under fume chambers to obtain the dried yield of the extracts. Each solvent extraction was performed in triplicate to ensure consistency, and the obtained dried extracts were stored in screw cap sample bottles at 4 °C for subsequent analysis.

Figure 3.3 Soxhlet apparatus setup for the extraction of *S. alata* leaves.

3.5.2 Maceration

Maceration was performed according to Gritsanapan and Mangmeesri (2009) and Cacique et al. (2020) with modifications. 20 g of dried powdered leaves of *S. alata* were placed in each conical flask containing 250 mL of solvent. Two different solvents were used in this extraction process, which were methanol and hexane. Then all conical flasks containing dried powdered leaves and solvent were subjected to the shaker water bath at a temperature of 60 ^{o}C and shaken at a speed of 120 rpm for 12 hours. After this, the macerated samples were filtered using Whatman No. 1 filter paper to remove any solid particles. The resulting filtered extracts were concentrated using the rotary evaporator and dried further under a fume chamber for three days. The crude extracts were weighed and stored in 4 ^{o}C for further use. The extraction was performed in triplicate to ensure reproducibility.

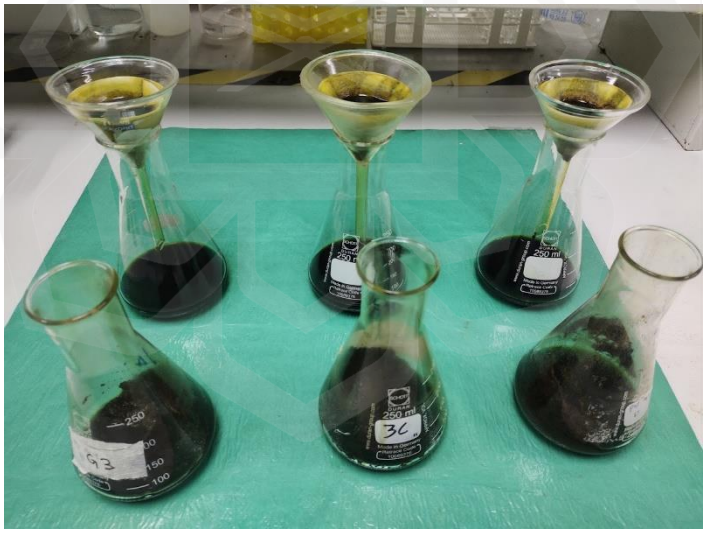

Figure 3.4 Filtration process for the separation of solid particles from the macerated extracts.

3.5.3 Subcritical Carbon Dioxide Soxhlet (SCDS)

The SCDS extraction process was conducted according to the method described by Easmin et al. (2017) and Chia et al. (2015) with some modifications. A 100 g sample of dry powdered leaves was soaked in the extraction vessel in three different soaking solvent-to-sample ratios; 1:1, 1:2, and 2:1. Two soaking solvents, methanol and ethanol were experimentalized in this process. The extraction was carried out in triplicate at 65 bar, 28 °C, 2 g/min of solvent flow rate for 60 minutes of extraction time as summarized in Table 3.4.

In this system, the extractor was filled with 100 g of rice *S. alata* leaves. The schematic diagram of SCDS extraction is illustrated in Figure 3.5. Carbon dioxide was supplied to the system and collected in the condenser as liquid carbon dioxide. Once the liquid carbon dioxide entered the extractor, the extraction process commenced. The liquid carbon dioxide accumulated in the extractor until it reached the desired fill level. The solvent to feed ratio in the entire system was estimated to be 24:1. When the fill level was reached, a level sensor (S1) sent a signal to the pneumatic valve (V1) to open, allowing the carbon dioxide to flow into the reboiler.

After 2 minutes, the pneumatic valve closed, ensuring that all the liquid carbon dioxide had been transferred to the reboiler. In the reboiler, the liquid carbon dioxide transformed into carbon dioxide vapor, which then flowed back to the condenser, completing an extraction cycle. Throughout the cycle, the extract remained at the bottom of the reboiler. The collected extract was directed to the cyclone separator via a controlling valve (V2) for further separation and collection. The blue cap Schott bottle was used to collect the extract from the cyclone separator. After collection, the co-solvents were removed by rotary evaporator and further dry in the fume chamber for three days. The crude extracts were weighted and stored in 4 ^{o}C for further use.

To maintain control over the temperature during the extraction process, three temperature sensors (TS1, TS2, and TS3) were utilized. These sensors ensured that the system maintained the desired temperature levels for optimal extraction efficiency and quality. In summary, the extraction process involved loading *S. alata* sample into the extractor, introducing subcritical carbon dioxide into the system, transferring it to the reboiler, and collecting the extracted sample. The temperature was carefully monitored throughout the process to ensure optimal extraction conditions.

Table 3.4 Subcritical Carbon Dioxide Soxhlet Parameters Setup

Main Solvent	Co-solvent/ Soaking Solvent	Ratio Co-solvent to Sample	Label
Carbon Dioxide	Ethanol	1:1	F1
	Ethanol	1:2	F2
	Ethanol	2:1	F3
	Methanol	1:1	F4
	Methanol	1:2	F5
	Methanol	2:1	F6

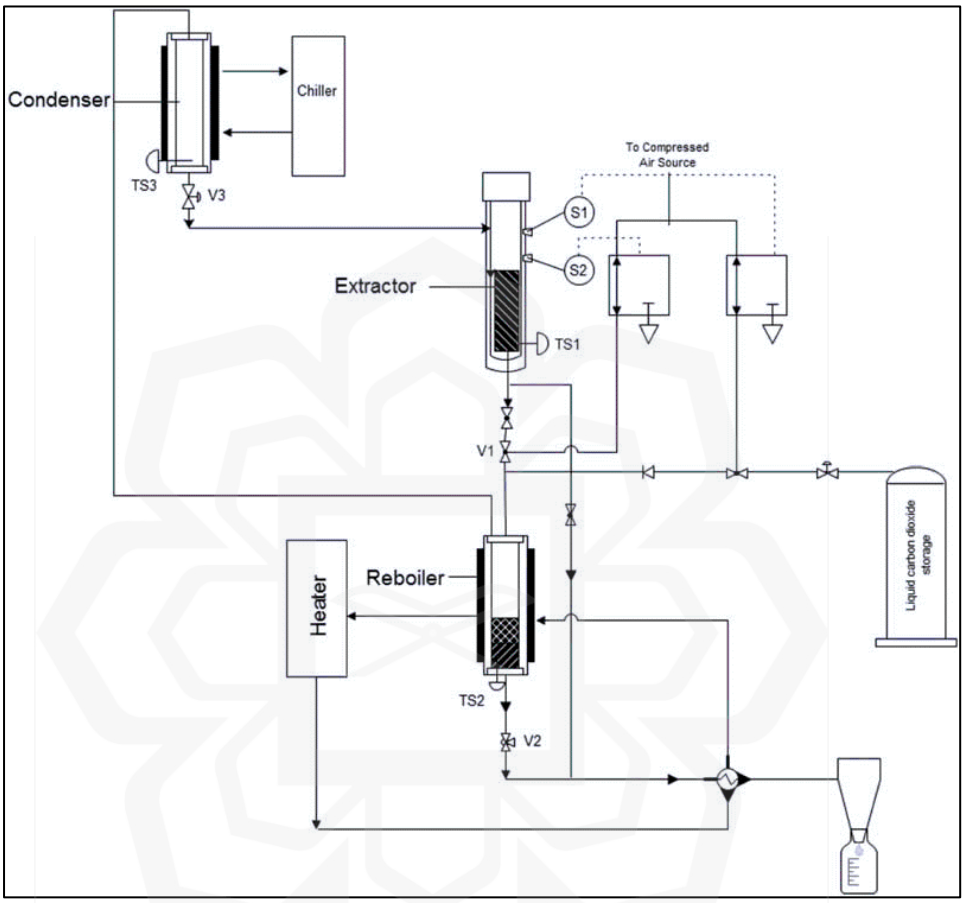

Figure 3.5 Schematic diagram of the Sub CO_2 Soxhlet method extraction process (Chia et al., 2015)

Figure 3.6 Subcritical Carbon Dioxide Soxhlet System at Supercritical Fluid Centre, Universiti Putra Malaysia

3.5.4 Summary of the Extraction Methods

Table 3.5 summarizes the key parameters for three extraction methods: Subcritical Carbon Dioxide (SCDS), Soxhlet extraction, and maceration. SCDS uses a sample size of 100 g, carbon dioxide as the main solvent, and includes methanol and ethanol as co-solvents. The extraction time is 60 minutes at a temperature of 28 °C and a pressure of 65 bar. Soxhlet and maceration methods use a sample size of 20 g, with different solvents and longer extraction times of 12 hours. Co-solvent removal is done using a rotary evaporator, and the extracted samples are dried for 3 days at 4 °C for storage.

Table 3.5 Extraction Parameters for SCDS, Soxhlet Extraction, and Maceration Methods

Parameter	**Non-Conventional Method**	**Conventional Method**	
	SCDS	Soxhlet	Maceration
Sample size	100 g	20 g	20 g
Main Solvent	Carbon Dioxide	250 ml Methanol or hexane	250ml Methanol or hexane
Co-Solvents	Methanol, Ethanol	No co-solvent	No co-solvent
Soaking solvent to sample ratios	1:1, 1:2, 2:1	-	-
Extraction pressure	65 bar	-	-
Extraction temperature	28 °C	<70 °C	60 °C
Solvent flow rate	2 g/min	-	-
Extraction time	60 minutes	12 hours	12 hours
Co-solvent removal	Rotary evaporator	Rotary evaporator	Rotary evaporator
Drying time	3 days	3 days	3 days
Storage temperature	4 °C	4 °C	4 °C

3.5.5 Determination of total yield percentage (%)

The final yield percentage of Soxhlet, maceration and SCDS extracts were calculated which includes the determination of weight of raw *S. alata* leaves, weight of the extract after evaporated. The final weight of yield was written in percentage. The percentage yield of each extract was calculated according to the formula below.

$$\text{Percentage of yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of raw sample}} \times 100 \qquad \text{(Equation 2)}$$

3.6 PHYTOCHEMICAL CONSTITUENT ANALYSIS BY GCMS

The determination of phytochemical compounds in the extracts was conducted using Perkin Elmer Gas Chromatograph (ClarusTM 680) according to the method described by Vanitha et. al. (2019) with slight modifications. The samples were diluted to a concentration of 1 mg/ml with absolute methanol and microfiltered into an analysis vial with a total volume of 1.5 mL and placed onto the autosampler. GCMS analysis was performed using an Elite-5MS Capillary Column, with helium used as the carrier gas at a flow rate of 1.5 mL/min throughout the program. The oven temperature program consisted of an initial hold at 50 °C for 1 minute, followed by a ramping up of 5 °C/min until reaching 250 °C, which was held for 5 minutes. The total run time for the program was 45 minutes. Subsequently, 1µL of the filtered solution was injected into the GCMS instrument.

The identification of components was accomplished by comparing their retention indices and interpreting the mass spectrum. The National Institute of Standards and Technology (NIST) database, which contains over 62,000 patterns of known compounds, was utilized for this purpose. The mass spectra of the unidentified components in the *S. alata* fraction were compared to the standard mass spectra of known components stored in the NIST Library (NISTII). This comparison aided in the identification and characterization of the compounds present in the sample. The percentage concentration of each compound identified was calculated using the formula as below.

Concentration of compound (%):

$$\frac{\text{Peak area}}{\text{Total peak area}} \times 100 \qquad \text{(Equation 3)}$$

Figure 3.7 Perkin Almer Gas chromatography mass spectrometry used in the experiment

3.7 ANTIOXIDANT STUDY OF *S. alata* LEAVES EXTRACTS

3.7.1 Sample Preparation

Different dilutions of extracts were prepared at concentrations of 1000, 800, 600, 400, and 200 mg/ml. The extracts were dissolved in methanol from dried crude forms. Additionally, Ascorbic Acid (ABA) was prepared using the same concentrations to serve as a reference substance. The purpose of these dilutions was to evaluate the properties and effects of the extracts compared to the reference (Azlim Almey et al., 2010).

3.7.2 DPPH Solution Preparation

The working standard was made by dissolving 6.0 mg of DPPH in 100 mL of methanol. Because DPPH is light sensitive, it is critical to prepare in the dark and cover the storing bottle with aluminium to prevent oxidation.

3.7.3 DPPH Radical Scavenging Activity Assay

Free radical scavenging ability of each extract was evaluated according to the method described by Halim (2019) and Azlim Almey et al. (2010) with slight modifications. In this assay, the ability of *S. alata* leaves extracts to donate hydrogen atoms was determined by decolorizing a methanol solution of 2-2-diphenyl-1-picrylhydrazyl (DPPH). In the presence of antioxidants, DPPH produces purple or violet colour in methanol solution and fades to shades of yellow to colourless (Bunya et al., 2022).

100 µL of sample solution was mixed thoroughly with 100 µL of DPPH solution in the microplate well. Methanol and ABA were prepared the same to serve as the control and standard respectively. All the mixtures were incubated in the dark for 30 minutes to allow the reaction. The absorbance of the mixture including the replicates

(triplicate) was measured spectrophotometrically at 517 nm using TECAN microplate reader. The percentage of DPPH radical scavenging activity was calculated by the following Equation 4. The percentage of radical scavenging activity was then plotted against concentration, and the IC_{50} was calculated from the graph. In this study, the antioxidant properties were expressed as the half maximal inhibitory concentration (IC_{50}) values, which indicated the concentration of extracts required for scavenging 50 % of DPPH free radicals.

Percentage of DPPH radical scavenging activity (%) =

$$\frac{A0 - A1}{A0} \times 100 \qquad \text{(Equation 4)}$$

where A0 is the absorbance of the control, and A1 is the absorbance of the extracts/standard.

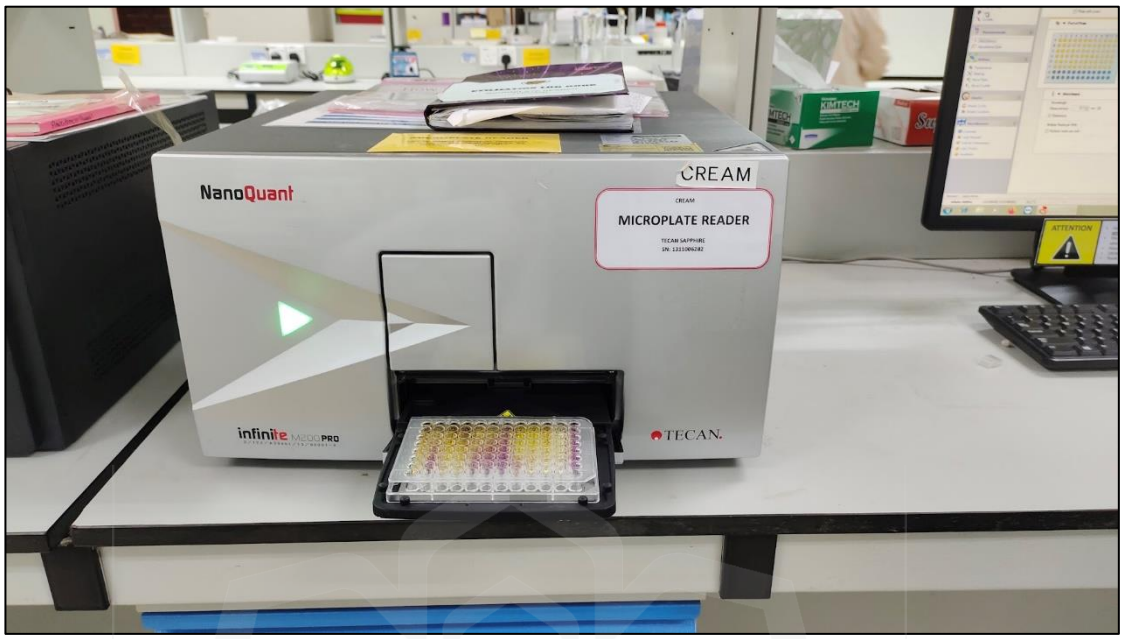

Figure 3.8 TECAN microplate reader used to measure absorbance

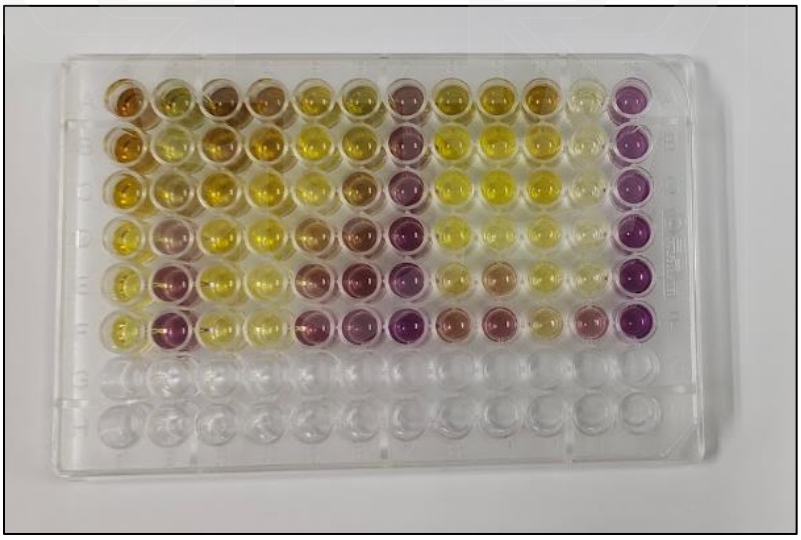

Figure 3.9 Colour changes of DPPH reaction from purple /violet to shades of yellow

3.8 ANTIBACTERIAL STUDY OF *S. alata* LEAVES EXTRACTS

In this study, the antibacterial properties of different extracts of *S. alata* leaves were conducted according to the method described by Paiva et al. (2013) with some modifications. The diameter of zones of inhibitions was determined by the Kirby-Bauer agar disc diffusion method. Meanwhile, the minimum inhibitory concentration (MIC) was evaluated using the resazurin-based microdilution method. The MIC values were furthered confirmed by the minimum bactericidal concentration (MBC) values.

3.8.1 Aseptic Technique

The aseptic technique is a crucial aspect of laboratory research, particularly during this antimicrobial assay. Specific protocols were done to prevent contamination and maintain sterility throughout experiments. This includes practices such as sterilizing the work area, instruments, and materials, using flame sterilization, autoclaving, and appropriate disinfectants. Proper hand hygiene, the use of sterile gloves, and working within a Class II Biosafety Cabinet (Figure 3.11) when necessary were essential to maintain aseptic conditions.

3.8.2 Organisms Stocks

All pure isolates were obtained from the Department of Pathology & Laboratory Medicine, Sultan Ahmad Shah Medical Centre (SASMEC). Table 3.6 shows the microorganisms used in this study, including Gram-positive bacteria, Gram-negative bacteria, and Gram-positive fungus, along with their corresponding ATCC (American Type Culture Collection) numbers.

30 % of glycerol stocks were prepared for all strains and kept at -80 °C for future use. Glycerol stocks are essential in microbiology as they allow for long-term storage of microbial cultures. By adding glycerol, cultures can be preserved for months or even years without loss of viability. In this study, glycerol stocks serve as backups, mitigating the risk of culture loss or contamination. On the other hand, nutrient agar plate stocks were also prepared for all organisms' strains and stored at 4 °C for regular use.

Table 3.6 Microorganisms used and their ATCC number

Category	**Species**	**ATCC Number**
Gram-positive bacteria	*Staphylococcus aureus*	ATCC25923
Gram-positive bacteria	*Staphylococcus epidermidis*	ATCC12224
Gram-positive bacteria	*Enterococcus faecalis*	ATCC29212
Gram-negative bacteria	*Pseudomonas aeruginosa*	ATCC24353
Gram-negative bacteria	*Salmonella enterica*	ATCC1402
Gram-negative bacteria	*Klebsiella pneumoniae*	ATCC700603
Gram-positive fungus	*Candida albicans*	ATCC14053

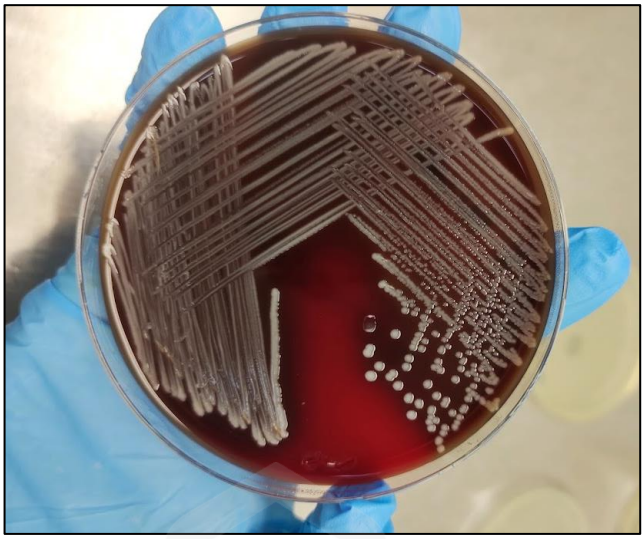

Figure 3.10 *Staphylococcus aureus* on Sheep Blood Agar obtained from SASMEC

Figure 3.11 Class II Biosafety Cabinet (BSC)

Figure 3.12 Glycerol stocks prepared in 1.5 mL centrifuges

3.8.3 Media Preparation

The culture media used in this study, namely Nutrient Agar (NA), Nutrient Broth (NB), Mueller Hinton Agar (MHA), and Mueller Hinton Broth (MHB), were prepared following the standard method outlined by the American Society for Microbiology 2016 with slight modifications (Hudzicki, J., 2009). To prepare the media, the appropriate amounts of media powders were mixed with distilled water as per the standard protocol and subsequently autoclaved. For agar media, the liquid mixture was poured into sterile Petri dishes to achieve a depth of approximately 4 mm and allowed to cool until the agar solidified completely. It is important to maintain the proper depth of agar in the plates, as shallow plates can lead to larger zones of inhibition and inaccurate susceptibility results, while plates poured to a depth greater than 4 mm can yield false-resistant outcomes. As for the broth media, approximately 10 mL was poured into universal bottles. All the prepared media were sealed using parafilm and stored at 4 °C for regular use.

3.8.4 Disc Diffusion Assay

The procedure began by streaking the bacterial strains stock on Mueller Hinton Agar (MHA) and incubating them at 37°C for 24 hours. Subsequently, sub-culturing was performed to obtain isolated single colonies. After another 24 hours of incubation, a single colony was transferred to 10 ml of Mueller Hinton Broth (MHB) using a sterile inoculating loop. The broth culture was vigorously shaken and then incubated at 37°C, with agitation at 150 rpm, for 18 hours.

To prepare the bacterial culture for testing, the density was adjusted spectrophotometrically to an optical density of 0.8-1 at a wavelength of 600nm (OD_{600}), which is equivalent to a 0.5 McFarland standard, corresponding to a concentration of approximately 1.0×10^8 CFU/ml. The culture suspensions were swabbed evenly onto Mueller Hinton Agar plates using sterile cotton swabs.

Afterward, the discs impregnated with the test samples were placed on the surface of the MHA plates accordingly. Each test plate included three discs, as shown in Figure 3.13, with a positive control represented by a standard commercial antibiotic disc, a negative control, and a disc containing the tested sample. For *S. aureus, S. epidermidis, E. faecalis, P. aeruginosa, S. enterica,* and *K. pneumoniae*, the positive control used was Streptomycin (30 µg/ml), while Nystatin (10 µg/ml) served as the positive control for *C. albicans*.

The plates were then incubated at 37°C for 18 to 24 hours, depending on the bacterial species being tested. After the incubation period, the diameter of the inhibition zone was measured in millimetres (mm), as depicted in Figure 3.14. To ensure reliability, the experiment was repeated three times independently.

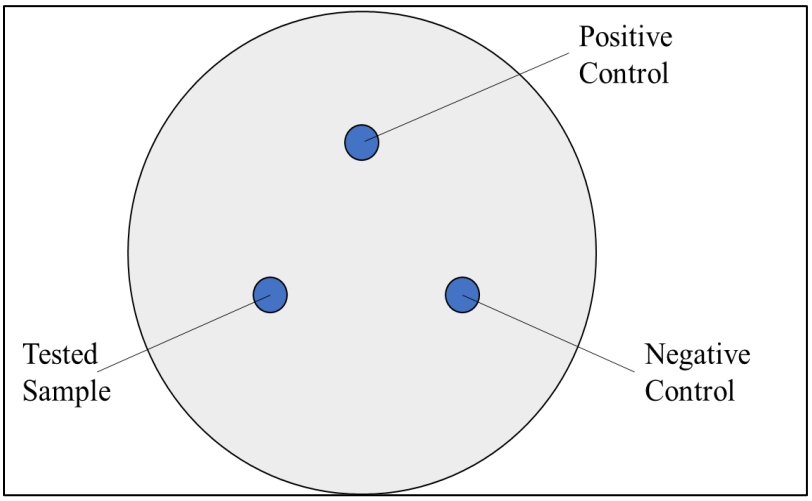

Figure 3.13 Disk Diffusion Assay Illustration

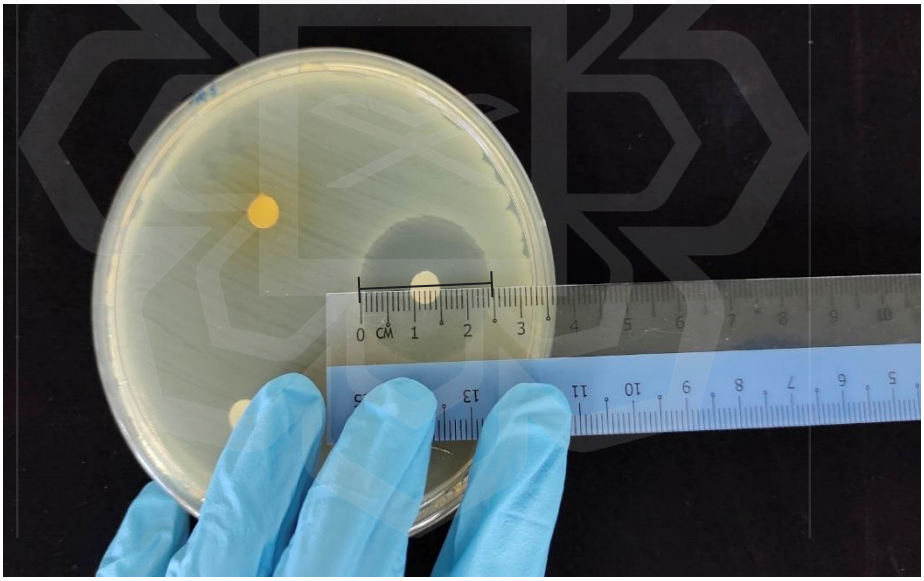

Figure 3.14 Measuring the diameter of inhibition zones in millimetre (mm)

3.8.5 Minimum Inhibitory Concentration (MIC)

The extracts that exhibited positive results in the disc diffusion assay against specific organisms were chosen for further evaluation of their minimum inhibitory concentration (MIC). This evaluation followed a modified version of the method described by Elshikh et al. (2016).

In this study, 500 mg/ml of extracts stocks were prepared in hexane, ethanol and methanol accordingly before the solvent was used during the extraction process. For the determination of MIC, 96-well microplates were used in this study to minimalize the usage of extracts. Approximately 100 µl of MHB was dispensed in each well of column 1, while columns 2-12 contained 50 µl of MHB only. A multichannel pipette was then used to transfer and mix the extracts from columns 1–12, resulting in 50 µl extracts per well as illustrated in Figure 3.15. The tested concentrations of the different extracts achieved through double serial dilutions from columns 12-1 were as follows; 166–0.0813 mg/ml (extracts) and 5–0.0024 µg/mL of Streptomycin. The next row contained 100 µl of diluted standardized inoculum, and another row contained 100 µl of the medium broth to serve as sterility control.

The standardized suspension of microorganisms was diluted 1:100 in MHB broth. Subsequently, 50 µl of the adjusted bacterial suspension, measured using OD_{600}, was added to each well containing extracts and the control wells. This resulted in an approximate concentration of 5×10^5 CFU ml^{-1}. The preparation and dispensing of the OD of the adjusted bacteria were completed within a maximum of 15 minutes. Following an 18-hour incubation at 37 °C, resazurin (0.015%) was introduced to all wells in a volume of 30 µl per well. The plate was then further incubated for 4 hours to observe any colour changes. On completion of the incubation, columns with no colour change (blue resazurin colour remained unchanged) were scored as above the MIC value.

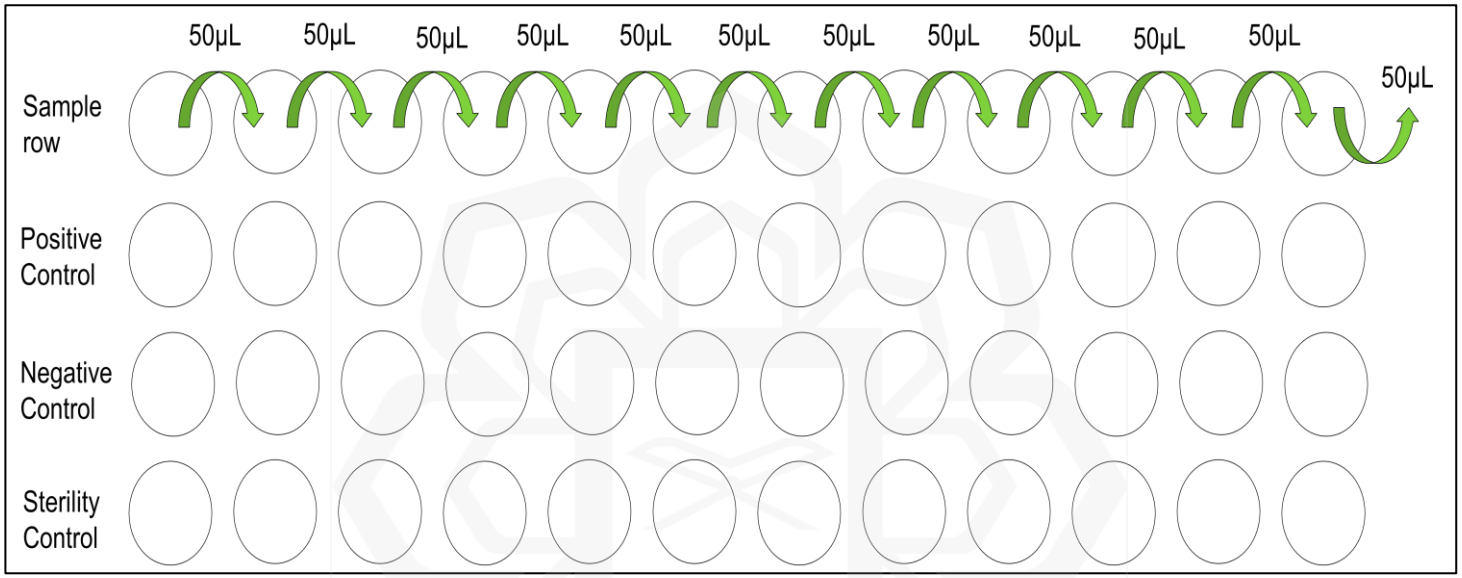

Figure 3.15 Illustration of resazurin-based microdilution method

3.8.6 Minimum Bactericidal Concentration (MBC)

The determination of minimum bactericidal (MBC) involved several steps. First, after the incubation period for the MIC determination, the wells that exhibited growth inhibition were identified. The contents of these wells, indicating potential antibacterial activity, were transferred onto separate culture plates using a sterile transfer loop. These contents were directly streaked onto the culture plates without any further dilution. The plates were then incubated for 18 hrs at 37 °C. After the incubation, the plates were carefully examined for the presence or absence of visible bacterial colonies. The MBC value was determined as the lowest concentration of the extract at which no visible growth of bacterial colonies was observed on the plates.

3.9 STATISTICAL ANALYSIS

The results of the study were presented as means ± standard deviation (SD), and all analyses were conducted in triplicate to ensure accuracy and reliability. To analyze the data, SPSS version 22.0 software was utilized, employing a one-way analysis of variance (ANOVA) test. Following the ANOVA, a multiple comparison procedure of the treatment means was conducted using the Duncan Test. The significance of the differences between treatments was considered statistically significant if the p-value was less than 0.05 ($P < 0.05$). This statistical analysis allowed for the evaluation of significant variations among the treatment groups and provided insights into the efficacy and potential differences between the tested extracts.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 AUTHENTICATION AND OBSERVATION OF PLANT MATERIAL

The plant specimen was identified and confirmed as *Senna alata* (L.) Roxb, which belongs to the Fabaceae family. The local name for the plant is "Gelenggang besar." The voucher number for the identification is PIIUM 0320-1 (Appendix A). The starting materials of *S. alata* were obtained from oven-dried leaves after grinding them into powder. It is essential in herbal medicine to grind plant materials to smaller particles to increase the surface area of the biomass to be exposed to the extraction solvents (Fotsing et al., 2022). These pretreatment steps, such as drying and grinding of *S. alata* leaves were conducted to facilitate the extraction process.

The physical characteristics of the ground samples were brownish green to green with different consistencies, and an approximate 6.333 ± 1.528 % moisture was recorded as in Appendix B. For a variety of reasons, dried plant material is more commonly employed as a source of secondary plant components compared to fresh leaves. Ncube et al. (2008) reported that differences in water content might alter the solubility of later separation, and secondary metabolic plant components should be relatively stable, especially if it is to be utilized as an antimicrobial agent. To maintain the stability of the powdered sample before being processed, it is kept in an airtight container at 4°C.

4.2 COMPARISON OF TOTAL YIELD PERCENTAGE (%) OF *S. alata* LEAVES EXTRACTS FROM MACERATION, SOXHLET AND SCDS EXTRACTION METHOD

The first stage in separating desired natural products from raw plant materials is through the extraction process. According to the extraction principle, extraction processes include solvent extraction, distillation, pressing, and sublimation methods. Among these, the solvent extraction method was the most used (Zhang et al., 2018). Any component that increases the diffusivity and solubility in the preceding processes will aid in extraction. The extraction efficiency might be influenced by the extraction solvent's properties, the particle size of the raw materials, the solvent-to-sample ratio, the extraction temperature, and the extraction duration (Rahim et al., 2023).

In this study, three different extraction methods which were maceration, Soxhlet, and Subcritical Carbon Dioxide Soxhlet (SCDS) were compared in terms of their total yield percentage of *S. alata* leaves extracts. From the statistical data (Appendix C), the results of the F test show that the p-value was smaller than 0.05. Therefore, the model was significant. The statistical analysis was furthered with Duncan's post hoc test to find the difference between the means. From the presented data (Figure 4.1), it was clearly portrayed a significant difference in total yield percentage between the methods of extraction and the type of solvent used.

This study found the highest total yield percentage was obtained from the Soxhlet method (E4) with a value of 25.14 ± 1.017^{a} %, followed by the maceration method (E2) with a value of 20.34 ± 1.136^{b} %. The first two highest yields were extracted from methanol. Meanwhile, the following total yield percentage was E1 (maceration) and E3 (Soxhlet) with values 14 ± 3.094^{c} % and 11.23 ± 1.008^{d} % respectively which were extracted using hexane. Among the lowest total yield were found to be from the SCDS extraction method (F1-F6) which the values were less than 2 %, ranging from 1.20 ± 0.417^{e} % to 2.45 ± 0.417^{e} %. This finding was coherent with a previous study by Easmin et al. (2017), which also obtained approximately 2 % of the

total yield from the SCDS method and much less than the conventional method, which obtained more than 20 % of the total yield of the plant sample extractions. Some other studies also reported a low yield of SCDS extracts, however, the quality of the extracts was promising (Chia et al., 2015; Easmin et al 2017; Sarker et al., 2022). For instance, Chia et al. (2015) reported that oils obtained from the SCDS method were superior to other conventional methods with 10 times higher thermolabile compounds.

The solvent choice might significantly impact the total yield of extracts obtained from a particular sample. Certain chemicals may be extracted more successfully by particular solvents than by others, and some solvents may be more selective about the types of compounds they extract (Zhang et al., 2018). This study shows a significantly higher yield of methanol extracts than extracts from hexane which was in agreement with other reports (Faruq et al.,2010, Easmin et al., 2017). The reason might be that most plants contain a mixture of polar and non-polar compounds (Altemimi et al., 2017). Methanol acts as a polar solvent, possessing a slight positive charge at one end and a corresponding slight negative charge at the other (Ahmadkelayeh & Hawboldt, 2020). This distinguishing characteristic enables it to dissolve polar molecules, which are commonly found in plant extracts. In contrast, hexane functions as a non-polar solvent, lacking an overall charge within its molecular structure (Abubakar & Haque, 2020). Consequently, methanol's tendency to dissolve a wider array of compounds makes it a preferable solvent for extraction purposes compared to other options (Altemimi et al., 2017).

On the other hand, in the SCDS method, subcritical carbon dioxide was employed as the primary solvent, resulting in the extraction of the least amount of total yield percentage. In general, using carbon dioxide as a solvent has some benefits over traditional solvents such as hexane or methanol, including lower toxicity, lower environmental impact, and the ability to extract specific compounds without leaving residual solvents in the final product (Zhang et al., 2018). However, the choice of solvent should be carefully considered depending on the type of sample being extracted and the compounds of interest. Some solvents may also extract unwanted compounds that can interfere with subsequent analysis or affect the quality of the extract (Jones &

Kinghorn, 2012). Moreover, the solvent used in the extraction process may be unwanted, toxic, or contains impurities, and removing it using rotavapor helps to separate the target compound from those solvents as impurities may cause side effects to the user (Fotsing et al., 2022). Abubakar and Haque (2020) also address various factors for choosing a solvent, such as the selectivity, safety, reactivity, recovery, viscosity, and boiling point of the solvent.

Other than that, the ratio of solvent-to-sample could be an important factor that affects the yield of extracts. Coherent to the study by Easmin et al. (2017), this study also showed that a higher solvent-to-sample ratio gave a higher extraction yield (Figure 4.1). However, there was no significant difference between the two polar solvents used in this method. In contrast, Soedirga et al. (2020) reported a significant effect on the interaction between the type of polar solvents and the ratio of sample-to-solvents. Thus, whatever solvent was used, there was a maximum amount that can be dissolved, implying that the sample-to-solvent ratio was an important factor because more solvents may increase the solubility of the sample in the solvent (Easmin et al., 2017). The sample-to-solvent ratio could affect extraction efficiency in sample preparation techniques such as solid-phase extraction like the SCDS method. If the sample was too concentrated, the analytes may not be extracted from the matrix efficiently. If the sample was too dilute, the extraction may be insufficient, resulting in poor analyte recovery. Thus, it was important to optimize the sample-to-solvent ratio to ensure accurate and reliable analytical results.

Overall, conventional methods such as maceration and Soxhlet are still leading in terms of the yield percentage of extracts compared to SCDS method. However, the high temperatures used in Soxhlet extraction can cause thermal degradation of the sample. While both techniques are effective, some differences between them may make one more suitable than the other depending on the desired outcome. Meanwhile, SCDS is a greener method based on the use of subcritical carbon dioxide as a solvent for the extraction of compounds from a solid sample which provides a safer alternative for the extraction of food additives and natural products (Easmin et al., 2017).

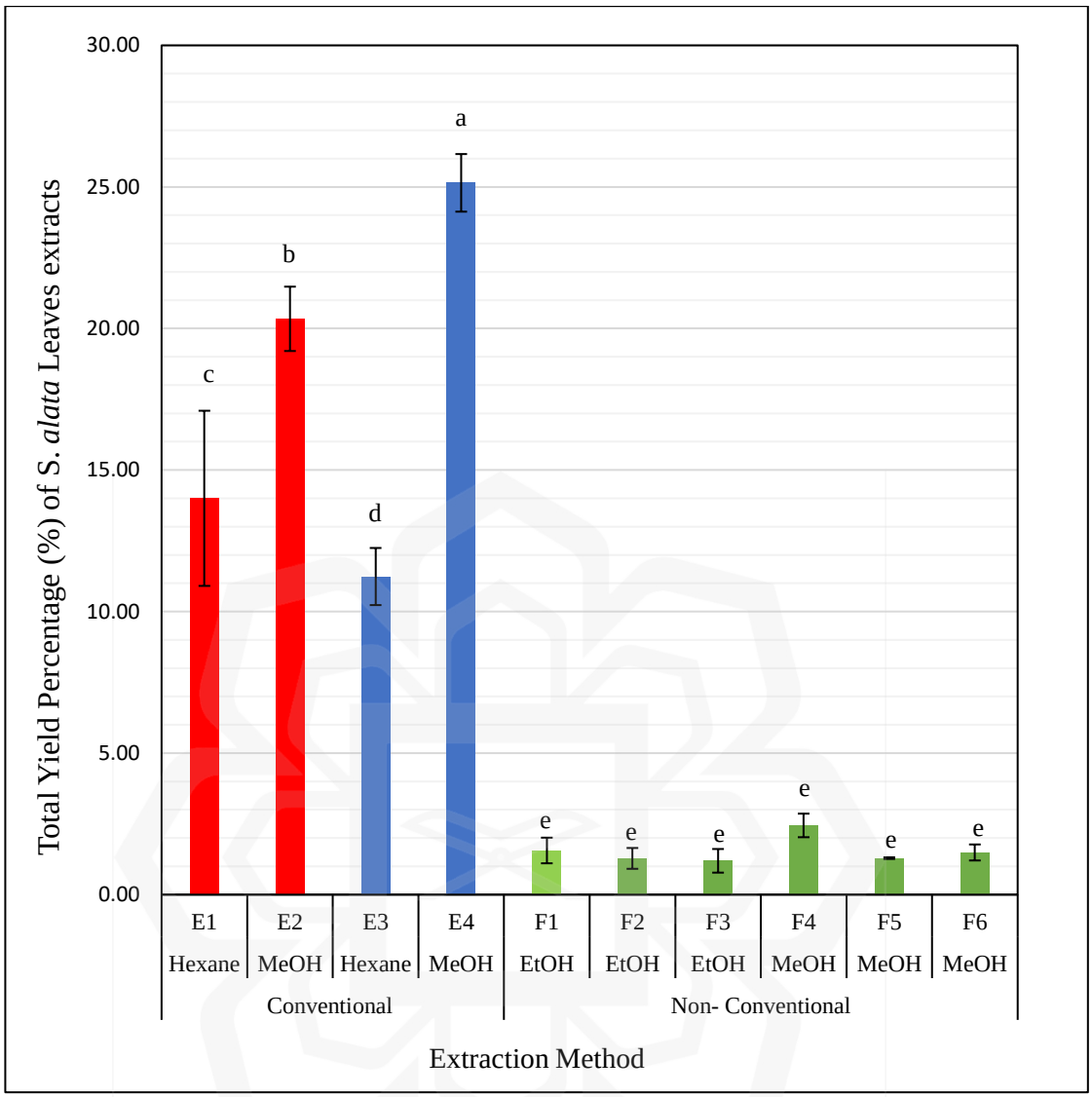

Figure 4.1. Comparison of total yield percentages of *S. alata* leaves using different extraction methods. Red bars for maceration, blue bars for Soxhlet extraction, and green bars for SCDS extraction. The data were presented as the mean and standard deviation derived from three biological replicates. Statistical significance was assessed using a one-way ANOVA Duncan's test (p-value < 0.05).

4.3 DETERMINATION OF CHEMICAL CONSTITUENTS FROM *S. alata* LEAVES EXTRACTS USING GCMS

Using the analytical GCMS technique, the phytochemical constituents in ten *S. alata* leaf extracts were identified and quantified. This study revealed a total of eighteen soluble compounds in these extracts, with their identification primarily based on molecular formulas, retention times, and peak areas. One standout compound that have been encountered in this study was phytol acetate, and it was found in most of the extracts such as in E1, E2, F2, F3, F5, and F6. This suggests that phytol acetate is a prominent phytochemical in the leaves of *S. alata*. Phytol acetate was reported to possess antioxidant properties, which help to neutralize harmful free radicals in the body (Singh et al., 2022). Free radicals are unstable molecules that can damage cells and contribute to various diseases and aging processes (Sharifi-Rad et al., 2020). By acting as an antioxidant, phytol acetate may help protect cells from oxidative stress, reducing the risk of cellular damage and potentially offering health benefits (Singh et al., 2022).

Dihydroactinidiolide and hexadecanoic acid, methyl ester were two other major compounds found in the extracts. Hexadecanoic acid was identified in E2, F1, F4, F5, and F6, while dihydroactinidiolide was confined to the SCDS extraction method, which only appeared in F1, F3, and F6 extracts. In an independent study, Shaaban et al. (2021) reported that an active compound hexadecanoic acid methyl ester had the highest antimicrobial effect against clinical pathogenic bacteria in their study. Hexadecanoic acid methyl ester is a methyl ester derivative of hexadecenoic acid, a fatty acid commonly found in plant oils (Sansone et al., 2013). Fatty acids play essential roles in the body and are involved in various physiological processes. Hexadecanoic acid, methyl ester has been reported to exhibit antimicrobial and anti-inflammatory properties (Shaaban et al., 2021). It may help inhibit the growth of certain microorganisms and reduce inflammation in the body (Shaaban et al., 2021). Additionally, due to its emollient and moisturizing properties, it may have potential applications in skincare products, providing hydration and promoting skin health (Kang et al., 2022).

Another compound found in *S. alata* leaves was dihydroactinidiolide. It is a natural compound found in plant leaves and fruits, that have been reported to function

as a potent plant growth inhibitor and gene expression regulator, while also displaying antioxidant, antibacterial, anticancer, and neuroprotective activities (Das et al., 2018). While its specific role in *S. alata* leaves is not extensively documented, it is known for its characteristic fruity or green aroma as suggested by Dadi et al. (2019), its presence in *S. alata* leaves may contribute to the overall sensory profile and flavour of the plant. However, further research is needed to understand its potential biological activities or benefits in the context of *S. alata.*

On the other hand, E3 extract stood as the sole repository of palmitic acid, linoleic acid, Bis(2-ethylhexyl) phthalate, and propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-y). F3 and E3 extracts were uniquely found to contain 2,4,5,6-Di-O-benzyldene d-glucose. Other than that, an exclusivity was observed in one extract, wherein α-curcumene and propanoic acid were uniquely present in F4 extract. The inconsistency of compounds presents in *S. alata* leaf extracts obtained via various extraction methods suggested that the extraction method and parameters may influence the presence of compounds. Table 4.1 summarises the results of the GCMS analysis, and the chromatograms of each extract were attached Appendix D.

It is important to note that while these compounds have shown certain properties or benefits in other contexts or plant species, their specific roles and benefits in *S. alata* leaves are not extensively studied or well-documented especially extracts from SCDS method. The understanding of their potential effects on *S. alata* leaves is still limited and requires further investigation. Moreover, the concentration and interactions of these compounds with other constituents in *S. alata* leaves can influence their overall impact. Therefore, more research, including pharmacological studies, clinical trials, and in-depth chemical analysis, is necessary to fully explore the roles, benefits, and potential applications of these compounds in the context of *S. alata* leaves.

Table 4.1. Chemical constituents found in *S. alata* leaves extract analysed by GCMS

Peak No.	Retention time	Concentration (%)										Mol. weight	Mol. formula	Expected Element
		Conventional				Non-conventional								
		E1	E2	E3	E4	F1	F2	F3	F4	F5	F6			
1	11.58	0	0	0	0	0	0	0	59	0	0	202	$C_{15}H_{22}$	α- curcumene
2	11.7	0	0	0	0	0	0	0	0	7.71	0	330	$C_{14}H_6Cl_2F_2O_3$	2,4-difluorobenzoic acid, 2formyl-4,6-dichlorophenyl ester
3	11.84	0	21.7	0.9	43	6.0	0	0	0	0	0	206	$C_{14}H_{22}O$	Phenol, 2,4-di-tert-butyl
4	12.27	0	0	0	0	33.3	0	55.3	0	0	9.1	180	$C_{11}H_{16}O_2$	Dihydroactinidiolide
5	13.21	11.8	21.9	0	31	10.3	4.0	0	0	0	8.6	207	$C_{11}H_{13}NO_3$	Corydaldine
6	14.86	0	0	0	26	12.3	5.0	0	0	0	0	353	$C_{12}H_{20}INO_3$	Ethyl 6-iodo-9-oxabicyclo [3,3,1) non-2-yl(methyl) carbamate
7	15.48	49.3	25.9	0	0	0	8.0	19.9	0	39.8	43	338	$C_{22}H_{42}O_2$	Phytol acetate
8	15.84	38.9	0	3.0	0	0	0	0	0	28	0	296	$C_{20}H_{40}O$	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
9	16.37	0	30.5	0	0	11.7	0	0	0	24.5	32.37	270	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester
10	16.73	0	0	15.7	0	0	0	0	0	0	0	256	$C_{16}H_{32}O_2$	Palmitic acid
11	16.8	0	0	0.0	0	0	37.1	0	0	0	0	436	$C_{26}H_{44}O_5$	Ethyl-iso-allocholate
12	17.57	0	0	0.0	0	26.4	28.9	0	0	0	0	284	$C_{18}H_{36}O_2$	Palmitic acid, ethyl ester
13	17.64	0	0	4.2	0	0	0	9.8	0	0	0	356	$C_{20}H_{20}O_6$	2,4,5,6-Di-O-benzyldene d-glucose
14	18.39	0	0	37.9	0	0	0	0	0	0	0	280	$C_{18}H_{32}O_2$	Linoleic acid
15	18.59	0	0	26.9	0	0	17.0	15	0	0	6.90	278	$C_{18}H_{30}O_2$	Gamolenic acid

16	18.87	0	0	5.2	0	0	0	0	0	0	0	430	$C_{27}H_{42}O_4$	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)
17	22	0	0	6.0	0	0	0	0	0	0	0	390	$C_{24}H_{38}O_4$	Bis(2-ethylhexyl) phthalate
18	23.06	0	0	0	0	0	0	0	41	0	0	430	$C_{27}H_{42}O_4$	Propanoic acid, 2-(3-acetoxy-4,4, 14-trimethylandrost-8-en-17-y)

This table summarizes the concentration of phytochemical constituents extracted from various extraction techniques of *S. alata* leaves and analysed by GCMS. The extracts labelled as follow: E1= Maceration (Hexane), E2= Maceration (Methanol), E3= Soxhlet (Hexane), E4= Soxhlet (Methanol). In the SCDS method, the solvent-to-sample ratios were labelled as follows: F1 = Ethanol (1:1), F2 = Ethanol (1:2), F3 = Ethanol (2:1), F4 = Methanol (1:1), F5 = Methanol (1:2), and F6 = Methanol (2:1).

4.4 EVALUATION OF ANTIOXIDANT ACTIVITY OF *S. alata* LEAVES EXTRACTS

The evaluation of antioxidant properties of the *S. alata* leaves extracts in this study were determined by the DPPH radical scavenging activities. The basic concept underlying this test is the reduction of DPPH in the presence of a hydrogen donating antioxidant which that antioxidants can convert the purple-coloured DPPH solution to the yellow-coloured non-radical form DPPH-H in a concentration-dependent manner (Vedekoi & Sélestin, 2020; Olugbami et al., 2014). This colour change can be measured spectrophotometrically at 517 nm as a measure of the antioxidant-containing compound's ability to scavenge DPPH free radicals (Olugbami et al., 2014).

Figure 4.2 depicts the DPPH radical scavenging percentage of different *S. alata* leaves extracts. All extracts tested were found to scavenge DPPH radicals in a concentration-dependent manner. The results show that the antioxidant activity increases when the concentration of extracts increases. It indirectly implies that the bioactive components increased correspondingly with increasing concentrations, and the bioactive components may even be more potent than the standard compounds if the pure form is isolated (Olugbami et al., 2014). These radical scavenging results were supported further by the IC_{50} values and equivalents of standard compounds obtained as shown in Figure 4.3. IC_{50} represents the concentration of a substance containing antioxidants needed to remove 50% of the initial DPPH radicals (Vedekoi & Sélestin, 2020). A lower IC_{50} value indicates a higher effectiveness of the substance in eliminating DPPH, suggesting a stronger antioxidant activity (Olugbami et al., 2014).

These findings show that F6 from SCDS method with the employment of methanol as soaking solvent was the most potent than the other extracts with IC_{50} value = 0.693 ± 0.1725 mg/ml and had the nearest IC_{50} value to the standard antioxidant ABA (IC_{50} value = 0.659 ± 0.0691 mg/ml) that it acts as a primary antioxidant based on its ability to scavenge DPPH-free radicals. Meanwhile, F6 was significantly different with E2, F2 and F3 which had the lowest DPPH scavenging activity. Besides that, F3 has a significantly higher IC_{50} value (55.569 ± 2.29 mg/ml) compared to all other extracts

which indicates it had the lowest potential of antioxidant activities. Among the extracts from conventional extraction methods (E1, E2, E3, E4) there were no significant differences of antioxidant activities were observed. On the other hand, Halim-Lim et al. (2020) reported the highest antioxidant activities was IC_{50} 52.08 mg/ml from ethanolic *S. alata* leaves extract when compared to water extracts. However, our study obtained a higher antioxidant activity for all extracts except F3 extract.

Furthermore, among the ten extracts, six extracts (E1, E3, E4, F1, F4, F5, F6) showed no significant difference of antioxidant potency compared to the standard antioxidant ABA. Therefore, this antioxidant activity of *S. alata* leaves may account for its use in treating a variety of diseases, especially those associated with the generations of free radicals (Vedekoi & Selestin, 2020). However, there were significantly higher IC_{50} values for F2 and highest in F3 wherein they were extracted from SCDS method with the employment of different ratio of ethanol to sample. This may portray that the ethanol or ethanol to sample ratio used may not be suitable in SCDS method for this plant. A notable compound identified in this investigation was phytol acetate, detected across various extracts including E1, E2, F2, F3, F5, and F6 have been reported previously for its antioxidant attributes (Singh et al., 2022). However, deeper investigation regarding the employment of soaking solvent and the ratio is recommended. Other possibilities of the low antioxidant activities by F2 and F3 could be because of the degradation of active compounds in the sample throughout the sample preparation or the storage process (Zhang et al., 2018).

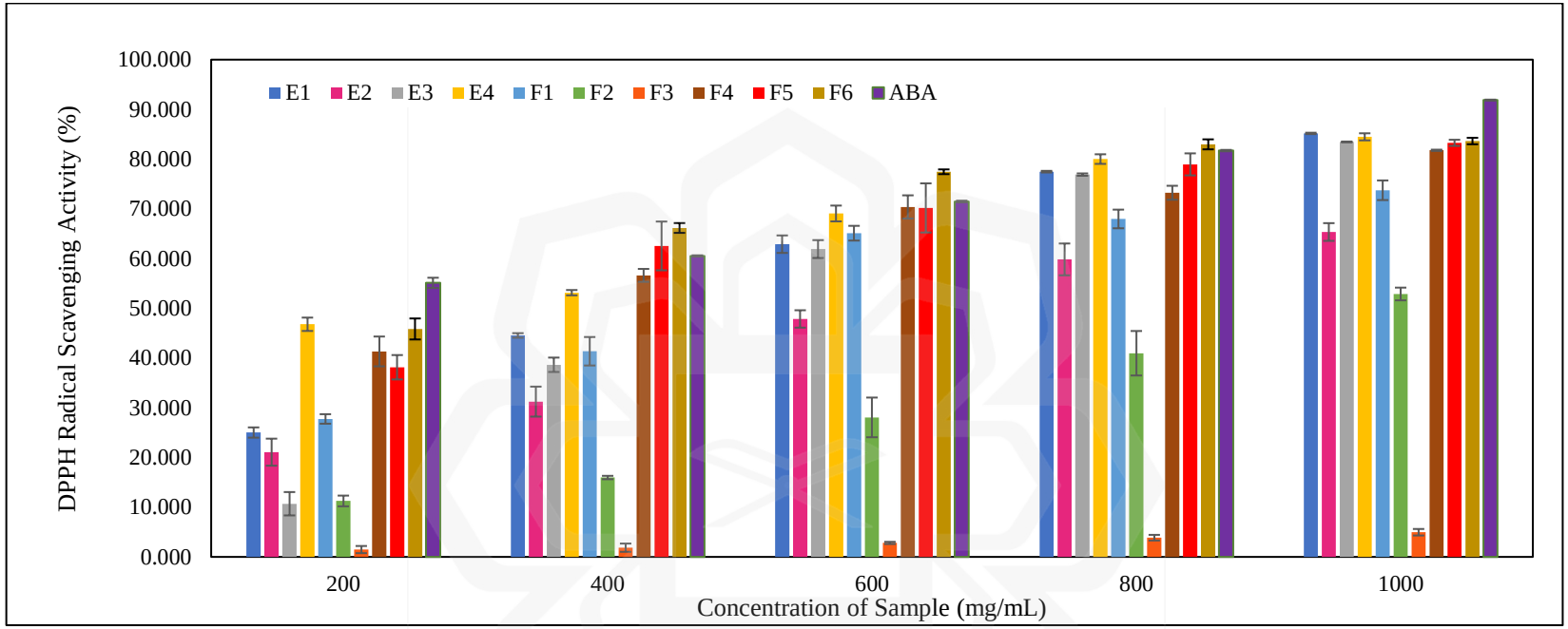

Figure 4.2 Graph of DPPH Radical Scavenging Activity of *S. alata* leaves extracts

Values are means ± Standard deviation values of three replicates. The tested samples are labelled as follow: E1= Maceration (Hexane), E2= Maceration (Methanol), E3= Soxhlet (Hexane), E4= Soxhlet (Methanol). In the SCDS method, the solvent-to-sample ratios were labelled as follows: F1 = Ethanol (1:1), F2 = Ethanol (1:2), F3 = Ethanol (2:1), F4 = Methanol (1:1), F5 = Methanol (1:2), and F6 = Methanol (2:1). Meanwhile, ABA=Ascorbic Acid, as the standard antioxidant.

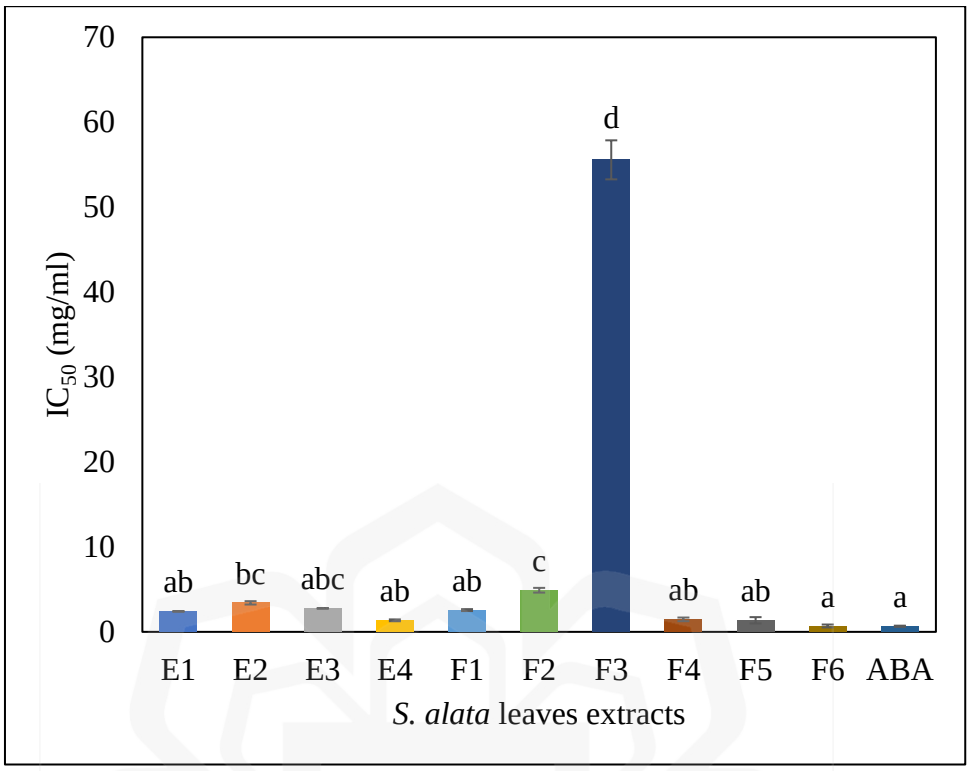

Figure 4.3 Half maximal inhibitory concentration (IC_{50}) of *S. alata* leaves extracts. Values are means ± SEM values of three replicates. Different letters indicate significant difference at $p < 0.05$ by Duncan's test. Means with the same letter are not significantly different.

4.5 EVALUATION OF ANTIMICROBIAL ACTIVTY OF *S. alata* LEAVES EXTRACTS

The extracts were further studied for biological assay study to evaluate the antimicrobial potentials of the methanol, hexane and liquid carbon dioxide extracted samples from the leaves of *S. alata*. The Kirby-Bauer disc diffusion susceptibility test was performed in this study to evaluate the susceptibility or resistance of some pathogenic microbes to various extracts of *S. alata* leaves. The zones of inhibition observed from different extracts of *S. alata* leaves against *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC12224, *Enterococcus faecalis* ATCC29212, *Pseudomonas aeruginosa* ATCC24353, *Salmonella enterica* ATCC1402, *Klebsiella pneumoniae* ATCC700603, and *Candida albicans* ATCC14053 were summarized in Table 4.2.

Based on Table 4.2, five extracts which were E1, E2, E3, E4 and F6 were found to be biologically active wherein the inhibition zones were observed from the tested extracts in this antimicrobial susceptibility test. It was found that E4 showed the largest inhibition zones against *Staphylococcus aureus* ATCC25923 which ranges from 9 to 11 mm. Besides, E4 also showed the most susceptible to the strains such as *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC12224, *Klebsiella pneumoniae* ATCC700603 and was the only extract that was susceptible to *Pseudomonas aeruginosa* ATCC24353 (2-3 mm). Of the five active extracts, the lowest inhibition comes from E1 (0.5 – 1.5 mm) against *Staphylococcus aureus* ATCC25923. However, this study showed that *Enterococcus faecalis* ATCC29212, *Salmonella enterica* ATCC1402, and *Candida albicans* ATCC14053 were resistant to all *S. alata* leaves extracts. The result of this research highlights the fact that the organic solvent (methanol) extracts which are E2 and E4 exhibited greater antimicrobial activity because the antimicrobial principles were either polar or non-polar and they were extracted more or only through the organic solvent medium. Moreover, the results may be also influenced by the different method of extraction used. On the other hand, Table 4.2 showed that the negative control (10 % methanol) showed no inhibition of bacteria occurred. Meanwhile, all the positive control used showed inhibition of bacteria.

From the disc diffusion assay, four organisms which are *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC12224, *Pseudomonas aeruginosa* ATCC24353 and *K. pneumoniae* ATCC700603 that showed their susceptibility to some extracts (E1, E2, E3, E4 and F6) were chosen for the determination of their MIC and MBC values. Resazurin-based microdilution method have been used to determine the MIC due to the presence of various colour of the tested extracts. This method allows for a more precise assessment of growth inhibition by monitoring the color changes of resazurin, which serves as an indicator of metabolic activity in microorganisms (Costa et al., 2021). The changes of colour of the resazurin are demonstrated in Figure 4.8. The MBC is complementary to the MIC wherein the MIC test demonstrates the lowest level of the sample that inhibits growth while the MBC demonstrates the lowest sample that results in microbial death. Based on this study, the MIC of the *S. alata* leaves extracts were found on the range between 20.75 to 166 mg/ml. The lowest MIC and MBC value was found from E2 and E4 extracts against *K. pneumoniae* (20.75 mg/ml). Meanwhile, the MIC and MBC value for Streptomycin ranging from 1.9×10^{-5} to 0.1563×10^{-4} mg/ml as portrayed in Table 4.3. This discrepancy suggests that Streptomycin exhibited higher potency in inhibiting the growth of the tested organisms compared to the *S. alata* extracts.

The variations in antibacterial activity observed among the extracts can be attributed to several factors. Firstly, the composition and concentration of bioactive compounds within the extracts play a crucial role in their antimicrobial efficacy. Álvarez-Martínez et al. (2021) mentioned that different extracts may contain varying amounts and types of bioactive compounds, such as alkaloids, phenolics, flavonoids, or terpenoids, which can contribute to their antibacterial properties. These variations in compound composition can lead to differences in the observed MIC values. Additionally, the choice of solvent for extraction can impact the extraction efficiency and subsequently affect the antimicrobial activity of the extracts (Maqbool et al., 2020). In this study, organic solvents, particularly methanol, ethanol and hexane were used for extraction. It is known that different solvents have different polarities and extraction capabilities, which can influence the extraction of bioactive compounds (Labar et al., 2019). The extracts obtained using the organic solvent (E2 and E4) displayed greater antimicrobial activity, indicating that the antimicrobial compounds may be more

soluble or selectively extracted using the organic solvent as stated by Neiva et al. (2020).

Moreover, the method of extraction employed can also contribute to variations in antimicrobial activity. Factors such as extraction time, temperature, and technique as mentioned in chapter 4.2 can influence the release and extraction efficiency of antimicrobial compounds. These methodological differences may have influenced the composition and concentration of bioactive compounds in the extracts, leading to variations in their antibacterial activity. For instances, this study found a compound which was hexadecanoic acid, methyl ester that has been reported to exhibit antimicrobial and anti-inflammatory properties (Shaaban et al., 2021). Different bacterial strains possess varying susceptibilities to antimicrobial compounds (Reygaert, 2018). The observed variations in inhibition zones against different bacterial strains may be due to inherent differences in the resistance mechanisms, cell wall structures, or efflux pump activity of the strains tested (Reygaert, 2018). Consequently, the extracts may exhibit varying degrees of efficacy against different bacterial species or strains.

It is important to acknowledge the limitations of the study. The presence of synergistic or antagonistic interactions between different compounds within the extracts could have influenced the observed antibacterial activity (Guglielmi et al., 2020). Interactions between compounds can either enhance or inhibit the antimicrobial effects, further contributing to the variations in MIC values. Furthermore, the quality and variability of the plant material used for extraction should be considered. Factors such as the geographic origin, cultivation conditions, harvesting time, and post-harvest handling can affect the concentration and quality of bioactive compounds in the plant material (Mykhailenko et al., 2020). These variations in plant material can impact the overall antimicrobial activity of the extracts. Further studies are warranted to identify and isolate specific bioactive compounds responsible for the observed antimicrobial activity.

Table 4.2 The inhibition zones exhibited by *S. alata* leaves extracts obtained from disc diffusion assay

Tested Sample	Diameter of inhibition zone (mm)						
	Gram positive bacteria			Gram negative bacteria			Yeast
	S. aureus	*S. epidermidis*	*E. faecalis*	*K. pneumoniae*	*P. aeruginosa*	*S. enterica*	*C. albicans*
E1	0.533 ± 0.0577	NA	NA	NA	NA	NA	NA
E2	9.33 ± 0.5774	2.0 ± 0.577	NA	2.167 ± 0.2887	NA	NA	NA
E3	2.0 ± 1.0	NA	NA	NA	NA	NA	NA
E4	9.33 ± 0.577	1.85 ± 0.2887	NA	6.67 ± 0.577	2.67 ± 0.5	NA	NA
F1	NA	NA	NA	NA	NA	NA	NA
F2	NA	NA	NA	NA	NA	NA	NA
F3	NA	NA	NA	NA	NA	NA	NA
F4	NA	NA	NA	NA	NA	NA	NA
F5	NA	NA	NA	NA	NA	NA	NA
F6	2.83 ± 0.288	NA	NA	1.5 ± 0.2887	NA	NA	NA
Positive control (+)	26 ± 4.359[a]	14.0 ± 1.155[a]	10.67 ± 1.155[a]	29.66 ± 0.577[a]	30.66 ± 0.577[a]	12.33 ± 0.577[a]	11.33 ± 1.155[b]

*Positive control [a] = Streptomycin 30 µg/ml, [b] = Nystatin 10 µg/ml. Values expressed as mean ± standard deviation; n=3 in each group. "NA" indicates that inhibition zone was not observed. Inhibition zones were measured in mm including the disc diameter.

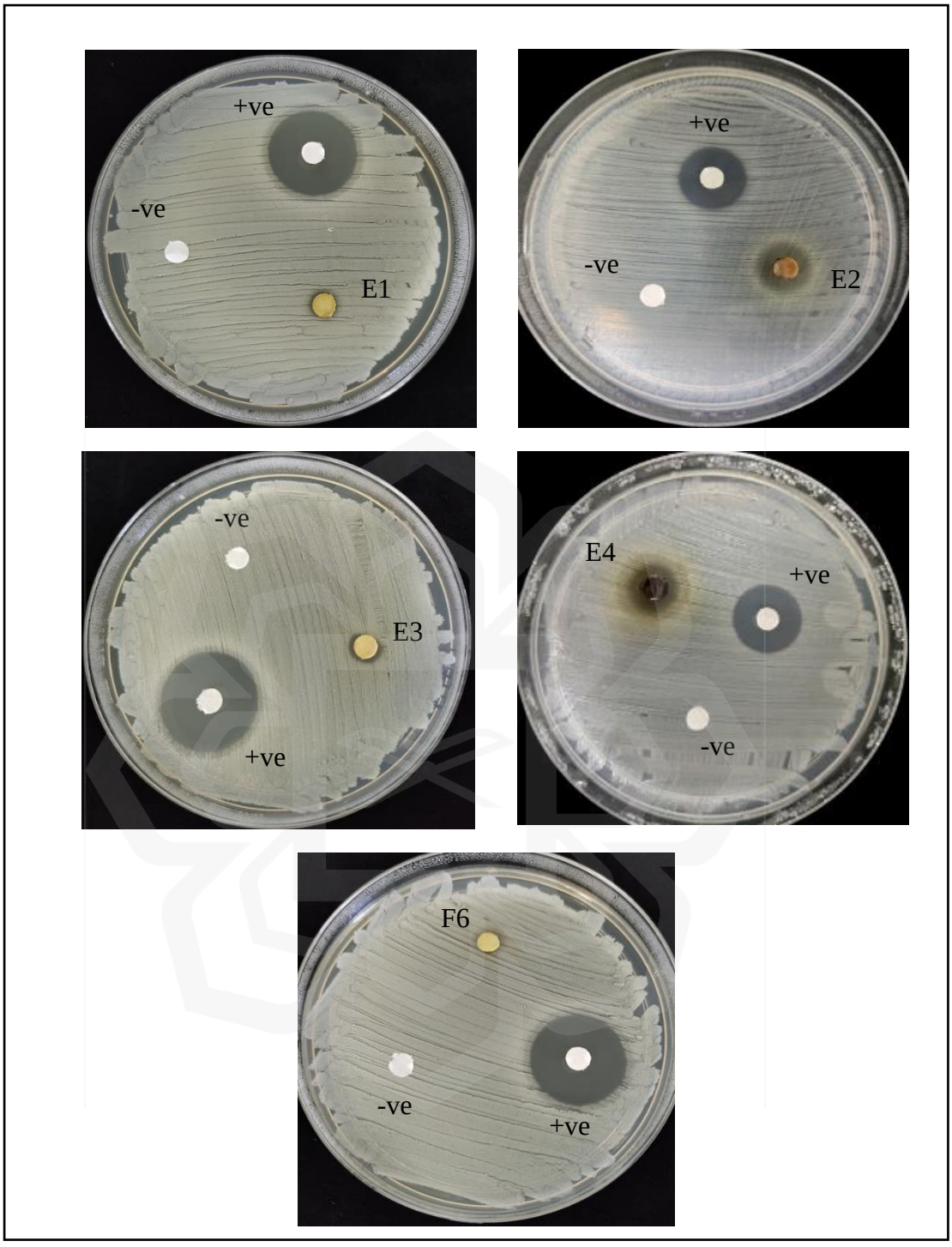

Figure 4.4 Disc diffusion assay of *Senna alata* leaves extracts (E1, E2, E3, E4, F6) against *Staphylococcus aureus* ATCC25923. Negative control (-ve) =10 % MeOH, Positive control(+ve) =30 μg/ml Streptomycin in sterile distilled water.

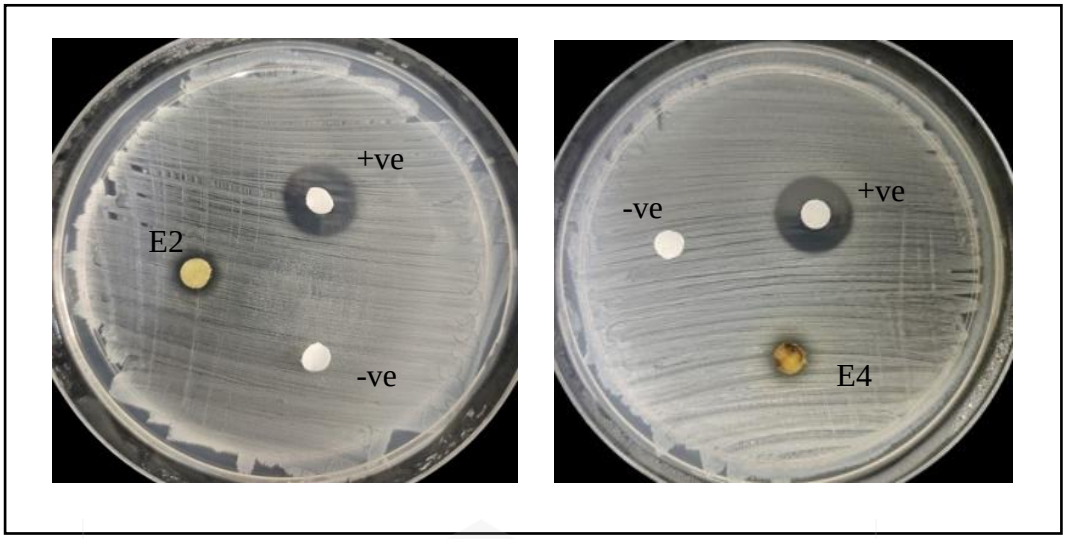

Figure 4.5 Disc diffusion assay of *Senna alata* leaves extracts (E2 and E4) against *Staphylococcus epidermidis* ATCC12224. Negative control (-ve) =10 % MeOH, Positive control(+ve) =30 µg/ml Streptomycin in sterile distilled water.

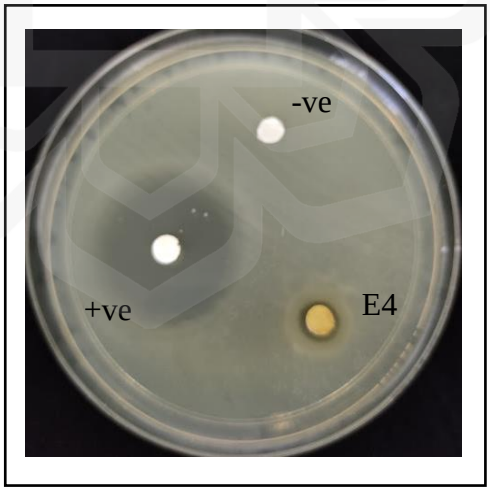

Figure 4.6 Disc diffusion assay of *Senna alata* leaves extract (E4) against *Pseudomonas aeruginosa* ATCC24353. Negative control (-ve) =10 % MeOH, Positive control (+ve) =30 µg/ml Streptomycin in sterile distilled water.

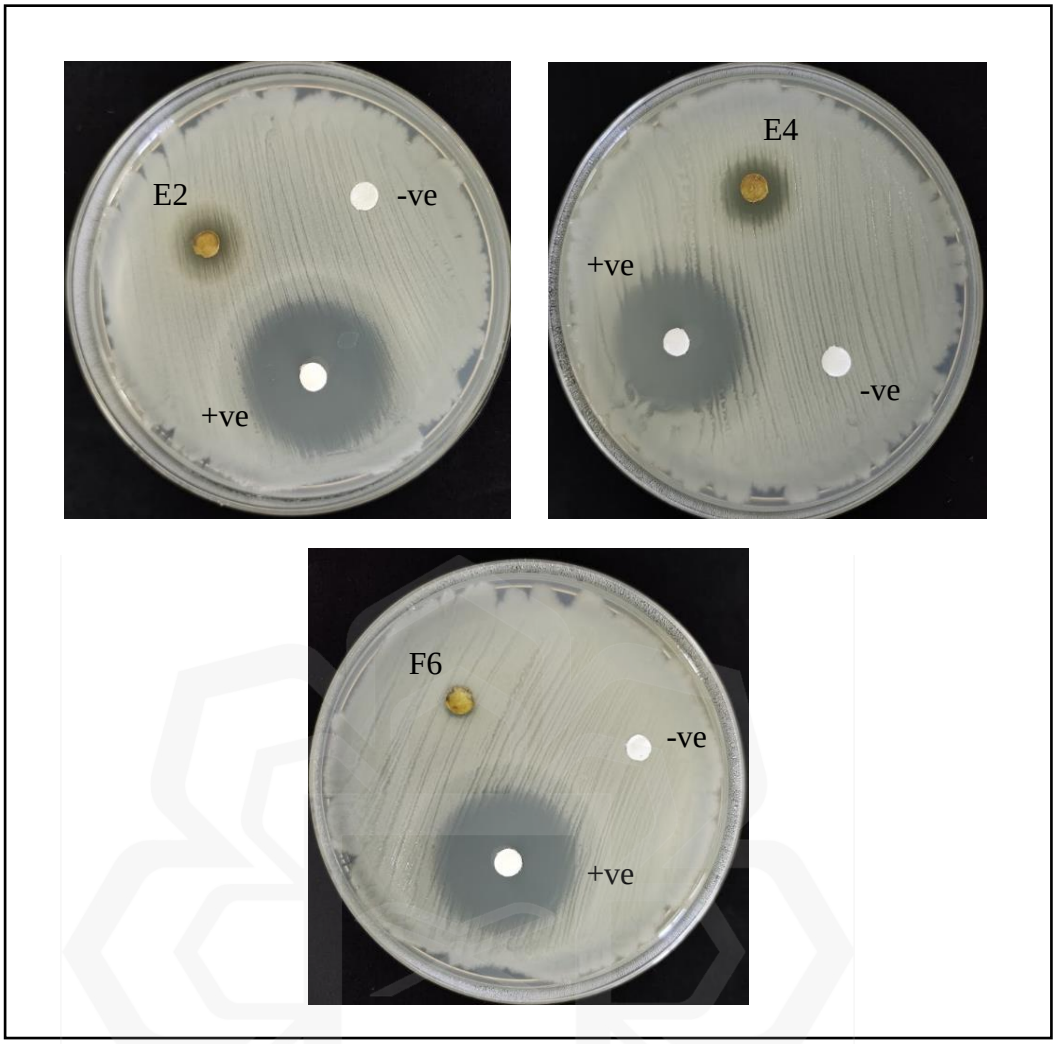

Figure 4.7 Disc diffusion assay of *Senna alata* leaves extracts (E2, E4 and F6) against, *Klebsiella pneumoniae* ATCC700603. Negative control (-ve) =10 % MeOH, Positive control (+ve) =30 µg/ml Streptomycin in sterile distilled water.

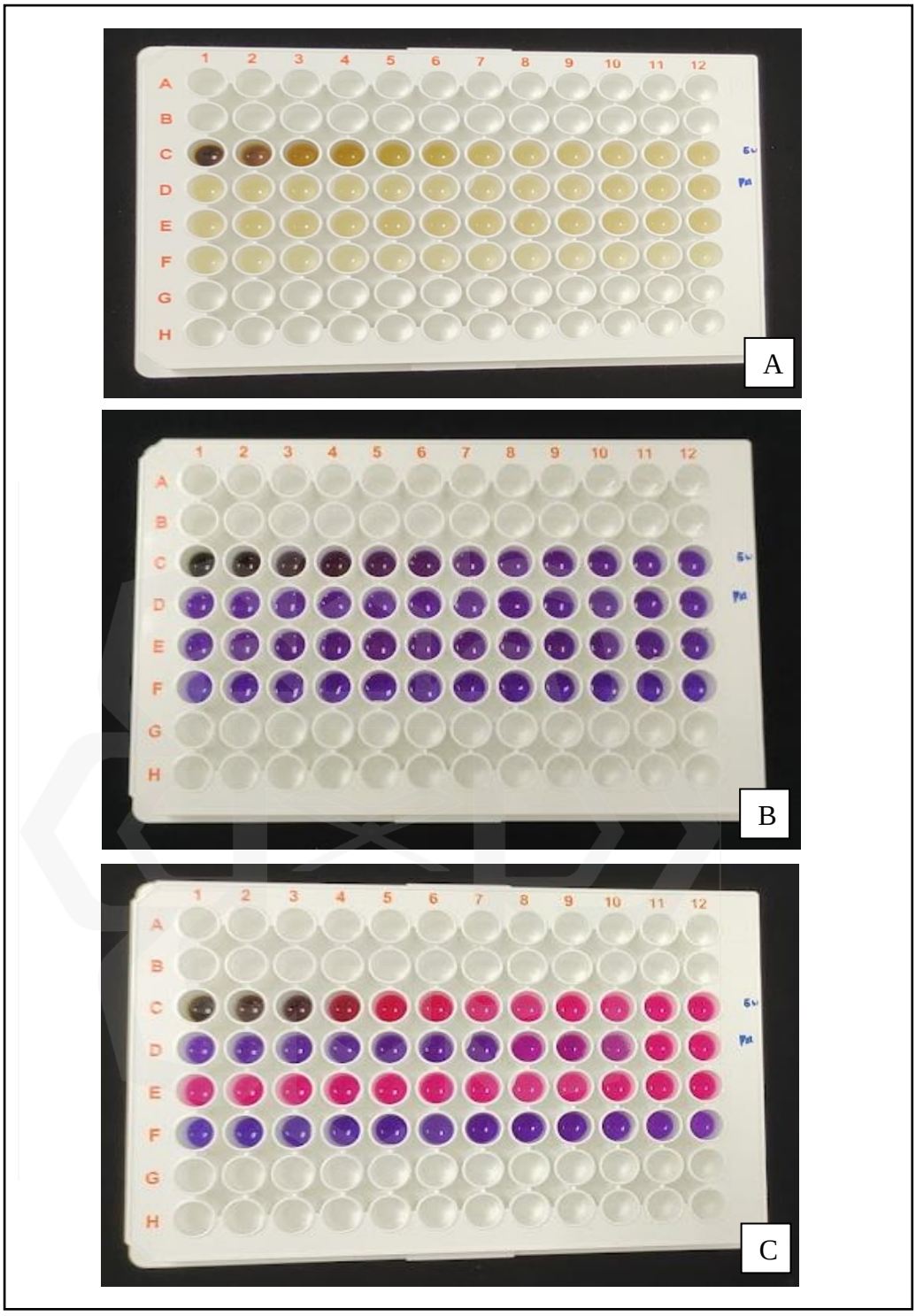

Figure 4.8. The resazurin-based microdilution method. (A)The colour of extracts and controls well before adding the resazurin dye. Column that shows a change of resazurin natural colour (B)(blue/purple) to the reduced form (C)(red/pink) indicates that there was bacterial growth (Elshiekh et al., 2017). The column that shows no colour changes therefore concentration of the extract in that column was taken as the MIC value.

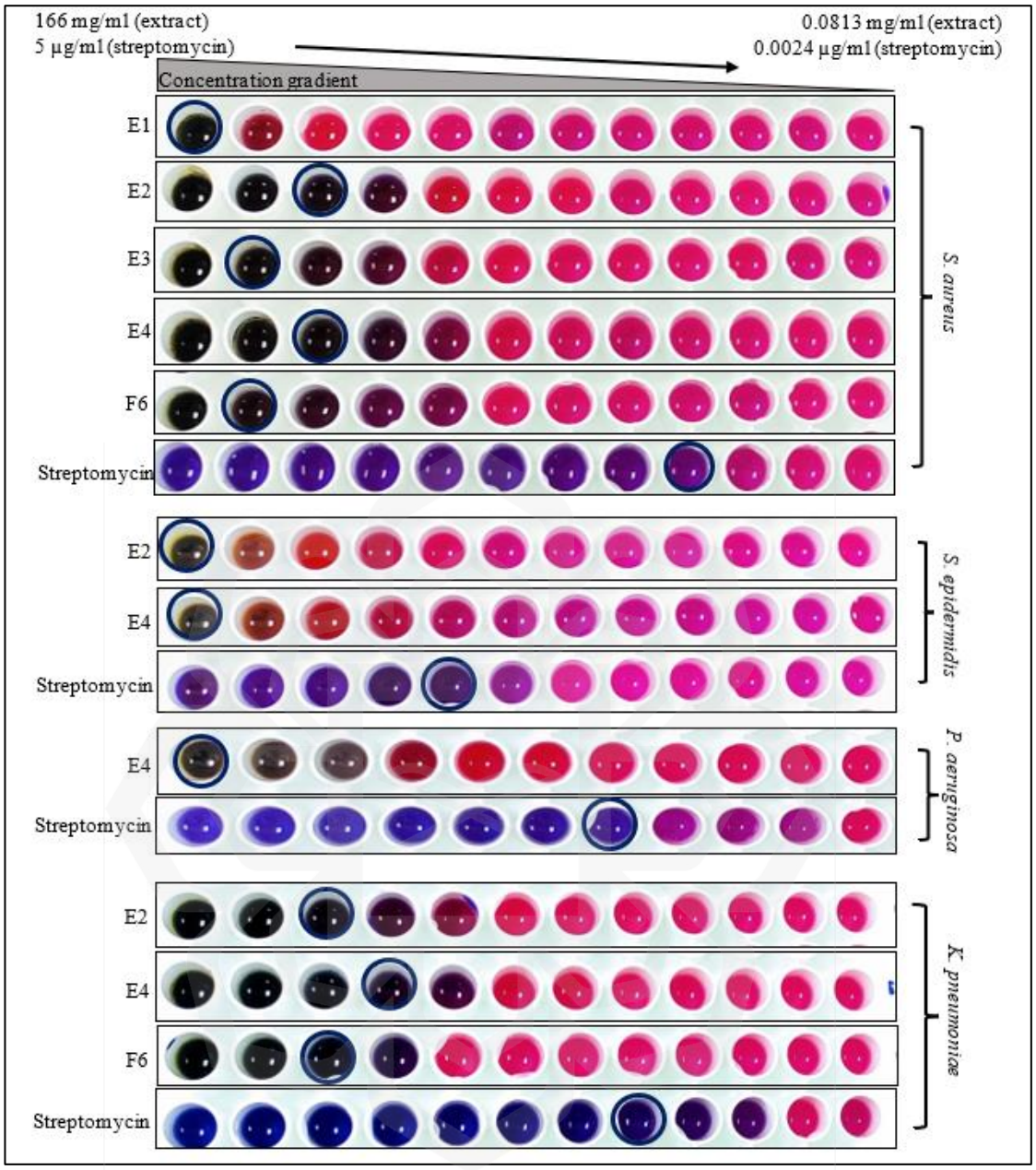

Figure 4.9 Resazurin-based microdilution for the determination of MIC (E1, E2, E3, E4, F6 and Streptomycin) against *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *K. pneumoniae*. The well-marked with blue circle indicates the MIC value. The tested samples are labelled as follow: E1= Maceration (Hexane), E2= Maceration (Methanol), E3= Soxhlet (Hexane), E4= Soxhlet (Methanol), F6 = SCDS (Methanol), and ABA= Ascorbic acid.

Table 4.3 MIC and MBC value of *S. alata* leaves extract againts *S. aureus S. epidermidis P. aeruginosa K. pneumoniae*

Tested sample	MIC value				MBC value			
	S. aureus	*S. epidermidis*	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*K. pneumoniae*
E1	166	ND	ND	ND	166	ND	ND	ND
E2	41.5	166	ND	41.5	41.5	83	ND	20.75
E3	83	ND	ND	ND	83	ND	ND	ND
E4	41.5	166	166	20.75	20.75	83	166	20.75
F6	83	ND	ND	41.5	41.5	ND	ND	41.5
Streptomycin	3.9×10^{-5}	3.125×10^{-4}	7.82×10^{-5}	3.9×10^{-5}	1.9×10^{-5}	1.563×10^{-4}	7.82×10^{-5}	1.9×10^{-5}

*The unit for concentration for E1, E2, E3, E4, F6 and streptomycin are expressed as (mg/ml). ND= Not Determined as there is no inhibition zone observed from disc diffusion assay. The tested samples are labelled as follow: E1= Maceration (Hexane), E2= Maceration (Methanol), E3= Soxhlet (Hexane), E4= Soxhlet (Methanol), F6 = SCDS (Methanol), and ABA= Ascorbic acid.

CHAPTER FIVE

CONCLUSION

5.1 SUMMARY

Among the three extraction methods compared (maceration, Soxhlet, and Subcritical Carbon Dioxide Soxhlet), conventional methods like maceration and Soxhlet extraction showed significantly higher total yield percentage compared to the non-conventional SCDS method. However, SCDS extraction has the potential for extracting high-quality compounds, and further research is needed to optimize its conditions and enhance the yield. The choice of solvent significantly affects the total yield percentage of extracts. Polar solvents like methanol are more effective at extracting polar compounds, while non-polar solvents like hexane are suitable for non-polar compounds. Subcritical carbon dioxide, used in SCDS, has benefits like lower toxicity and environmental impact but requires further optimization for improved yield. The sample-to-soaking solvent ratio affects the total yield percentage, and higher ratios generally result in higher extraction yields. Optimization of this ratio is important for accurate and reliable results. GCMS analysis identified various compounds in the extracts, including phytol acetate, dihydroactinidiolide, and hexadecenoic acid, methyl ester. These compounds may have potential antimicrobial and antioxidant properties, but further research is needed to understand their specific roles and benefits in *S. alata* leaves. The extracts showed antioxidant activity, with higher concentrations exhibiting stronger scavenging effects on DPPH radicals. The SCDS extract (F6) using methanol as the solvent showed the highest antioxidant potency among the tested extracts.

5.2 FUTURE RECOMMENDATIONS

Based on the aforementioned findings, several future recommendations can be proposed. Firstly, it is crucial to further optimize the SCDS extraction method by thoroughly investigating and adjusting various parameters such as temperature, pressure, and extraction time. This optimization process aims to enhance both the yield and quality of the extracts obtained. Secondly, it is essential to delve into the potential biological activities and benefits offered by the identified compounds, namely phytol acetate, dihydroactinidiolide, and hexadecenoic acid, methyl ester. This can be accomplished through comprehensive pharmacological studies and subsequent clinical trials. Additionally, conducting in-depth chemical analysis utilizing advanced instruments will enable the identification and quantification of specific compounds of interest within *S. alata* leaves. Furthermore, it is recommended to investigate potential synergistic effects and interactions between the identified compounds and other constituents present in *S. alata* leaves. This exploration can provide valuable insights into the complex chemical composition of the plant. Moreover, further studies on the antioxidant activity of *S. alata* extracts are warranted, including in vivo experiments and testing against various oxidative stress models. Lastly, exploring alternative extraction methods or techniques may uncover approaches that yield higher quantities or different types of bioactive compounds from *S. alata* leaves. By pursuing these future recommendations, a more comprehensive understanding of *S. alata's* potential applications in herbal medicine can be achieved.

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APPENDIX A: AUTHENTICATION OF PLANT SAMPLE CERTIFICATE

الجامعة الإسلامية العالمية ماليزيا
INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA
Garden of Knowledge and Virtue

LEADING THE WAY
KHALĪFAH - AMĀNAH - IQRA' - RAHMATAN LIL-ĀLAMĪN | SUSTAINABILITY INSTITUTION OF THE YEAR

KULLIYYAH OF PHARMACY

Our Ref. : IIUM/308/15/2/1/NMPC22-2/1
Date : 22 June 2022

Nadirah binti Abd Rahim
Kulliyyah of Science
International Islamic University Malaysia
25200 Kuantan
Pahang

UKMB
VERIFIED
28 JUN 2022
Universiti Kebangsaan Malaysia
Herbarium

Assalamu'alaikum Warahmatullahi Wabarakatuh

Dear Sr. Nadirah,

PLANT SPECIES IDENTIFICATION

Kindly be informed that the plant specimen submitted for identification is confirmed as follows:

Voucher No.	Family Name	Scientific Name	Local Name
PIIUM 0230-1	Fabaceae	*Senna alata* (L.) Roxb.	Gelenggang besar

Student's name : Nadirah binti Abd Rahim
Supervisor's name : Asst. Prof. Dr. Sahena Ferdosh
Herbarium : Kulliyyah of Pharmacy
Person in-charged : Assoc. Prof. Dr. Norazian Mohd Hassan
Identified by : Dr. Shamsul Khamis

It should be noted that we are not involved with anything as a result of research conducted by the host.

Thank you and wassalam.

Sincerely,

ASSOC. PROF. DR. NORAZIAN BINTI MOHD HASSAN
Kulliyyah of Pharmacy
International Islamic University Malaysia
Jalan Sultan Ahmad Shah
Bandar Indera Mahkota
25200 Kuantan
Pahang

DR. SHAMSUL KHAMIS
Botanist
Herbarium UKMB
Faculty of Science and Technology
The National University of Malaysia (UKM)
43600 Bangi
Selangor

Kulliyyah of Pharmacy
International Islamic University Malaysia
Jalan Sultan Haji Ahmad Shah
Bandar Indera Mahkota, 25200 Kuantan
Pahang, Malaysia
Telephone: (+609) 570 4818 / 4816 / 4802 | Fax: (+609) 571 6548

APPENDIX B: DATA AND CALCULATION OF MOISTURE CONTENT

Moisture Content (%) =

$$\frac{\text{Fresh weight of sample} - \text{Dry weight of sample}}{\text{Fresh weight of sample}} \times 100 \quad \text{(Equation 1)}$$

Moisture composition of *S. alata* leaves.

Replicate	**Fresh Weight (g)**	**Dry Weight (g)**	**Moisture Composition (%)**
Rep 1	1	0.95	5
Rep 2	1	0.92	8
Rep 3	1	0.94	6

APPENDIX C: STATISTICAL DATA FOR YIELD PERCENTAGE OF DIFFERENT EXTRACTION METHOD

Percentage of yield (%) =

$$\frac{\text{Weight of extract}}{\text{Weight of sample}} \times 100$$

(Equation 2)

Method of extraction	Label of sample	Weight of sample before extraction (g)	Weight of extract (g)	Yield percentage (%)
Soxhlet	E1			
R1		20	2.25	11.25
R2		20	3.47	17.35
R3		20	2.68	13.4
Soxhlet	E2			
R1		20	3.89	19.45
R2		20	3.99	19.95
R3		20	4.325	21.62
Maceration	E3			
R1		20	2.145	10.7
R2		20	2.480	12.4
R3		20	2.123	10.61
Maceration	E4			
R1		20	5.250	26.25
R2		20	4.985	24.93
R3		20	4.850	24.25
Subcritical	E5	100		
R1			1.8787	1.88
R2			1.2378	1.24
Subcritical	E6	100		
R1			1.5357	1.54
R2			1.0245	1.02
Subcritical	E7	100		
R1			0.9	0.9
R2			1.486	1.49
Subcritical	E8	100		
R1			2.1483	2.15
R2			2.7406	2.74
Subcritical	E9	100		
R1			1.2709	1.27
R2			1.3064	1.31
Subcritical	E10	100		
R1			1.2865	1.29
R2			1.6897	1.69

Univariate analysis of variance

Descriptive Statistics

Dependent Variable: Total yield percentage

Extraction Method	Mean	Std. Deviation	N
E1	14.0000	3.09395	3
E2	20.3400	1.13635	3
E3	11.2367	1.00848	3
E4	25.1433	1.01692	3
E5	1.5600	.45255	2
E6	1.2800	.36770	2
E7	1.1950	.41719	2
E8	2.4450	.41719	2
E9	1.2900	.02828	2
E10	1.4900	.28284	2
Total	9.6117	9.19499	24

Tests of Between-Subjects Effects

Dependent Variable: Total yield percentage

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1918.002[a]	9	213.111	112.169	<0.001
Intercept	1476.185	1	1476.185	776.973	<0.001
Extraction Method	1918.002	9	213.111	112.169	<0.001
Error	26.599	14	1.900		
Total	4161.820	24			
Corrected Total	1944.601	23			

a. R Squared = .986 (Adjusted R Squared = .978)

Post hoc Test

Homogenous subsets

Total Yield Percentage

Duncan[a,b,c]

		Subset				
Extraction Method	N	e	d	c	b	a
E1	3			14.0000		
E2	3				20.3400	
E3	3		11.2367			
E4	3					25.1433
E5	2	1.5600				
E6	2	1.2800				
E7	2	1.1950				
E8	2	2.4450				
E9	2	1.2900				
E10	2	1.4900				
Sig.		.395	1.000	1.000	1.000	1.000

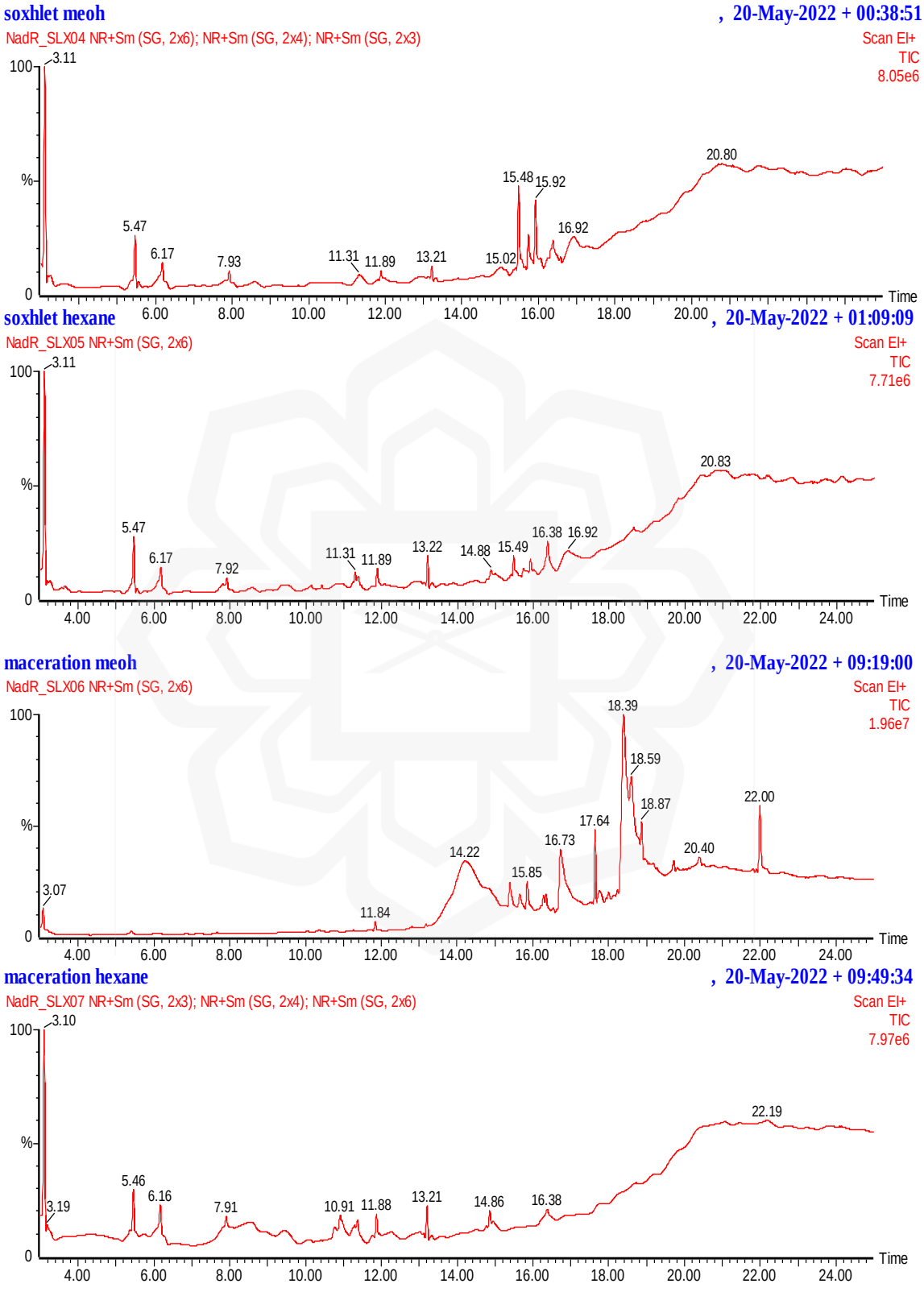
Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = 1.900.
a. Uses Harmonic Mean Sample Size = 2.308.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = 0.05.

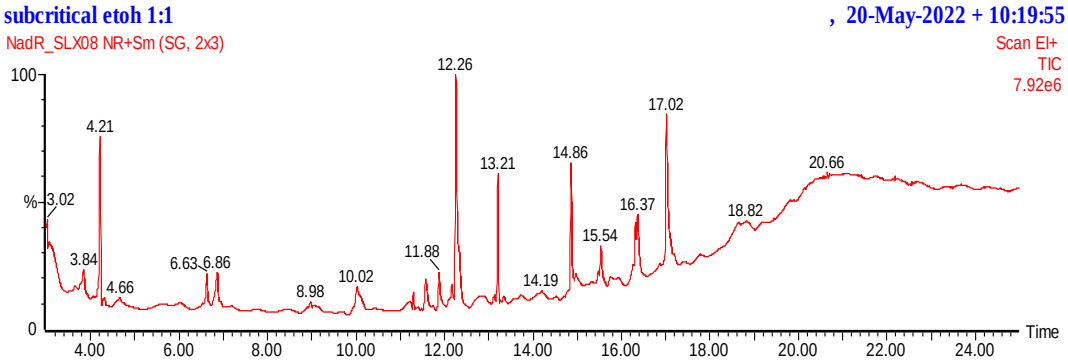
APPENDIX D: SUMMARY OF TOTAL YIELD PERCENTAGE OF EXTRACTION METHODS

Extraction mode		Label of sample	Mean values of total yield percentage (%)
Conventional Maceration Extraction			
Solvent:			
Hexane		E1	14 ± 3.094^{c}
Methanol		E2	20.34 ± 1.136^{b}
Conventional Soxhlet Extraction			
Hexane		E3	11.23 ± 1.008^{d}
Methanol		E4	25.14 ± 1.017^{a}
Non- Conventional Subcritical Extraction			
Solvent: Carbon dioxide			
Co- solvent:	Ratio of (sample: co-solvent)		
Ethanol	1:2	F1	1.56 ± 0.453^{e}
	1:1	F2	1.28 ± 0.368^{e}
	2:1	F3	1.20 ± 0.417^{e}
Methanol	1:2	F4	2.45 ± 0.417^{e}
	1:1	F5	1.29 ± 0.283^{e}
	2:1	F6	1.49 ± 0.283^{e}

Values in the yield column followed by superscript letters are significantly different (p-value < 0.05) resulted from one-way ANOVA Duncan's test. Values are stated as mean ± standard deviation of duplicates.

APPENDIX E: CHROMATOGRAM OF *S. ALATA* LEAVES EXTRACT ANALYZED BY GCMS




subcritical etoh 1:1
, 20-May-2022 + 10:19:55
NadR_SLX08 NR+Sm (SG, 2x3)
Scan EI+
TIC
7.92e6
100
%
0
Time
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
3.02
3.84
4.21
4.66
6.63
6.86
8.98
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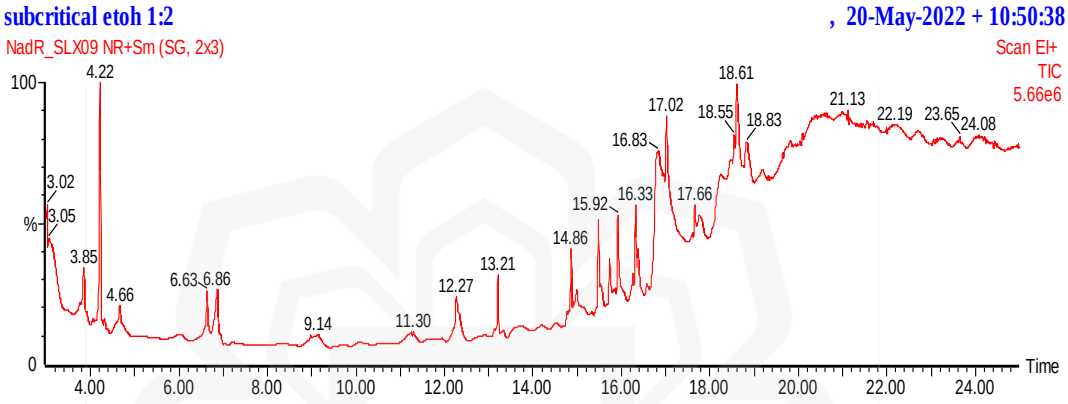
14.86
15.54
16.37
17.02
18.82
20.66


subcritical etoh 1:2
, 20-May-2022 + 10:50:38
NadR_SLX09 NR+Sm (SG, 2x3)
Scan EI+
TIC
5.66e6
100
%
0
Time
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
3.02
3.05
3.85
4.22
4.66
6.63
6.86
9.14
11.30
12.27
13.21
14.86
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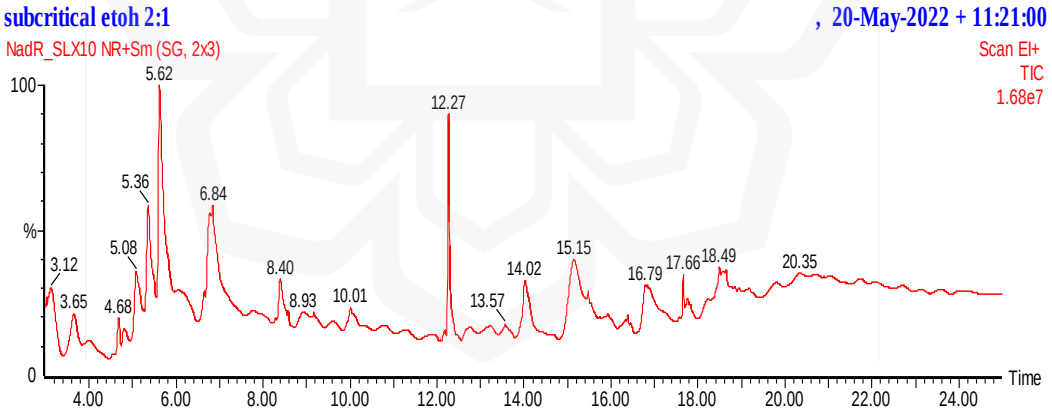
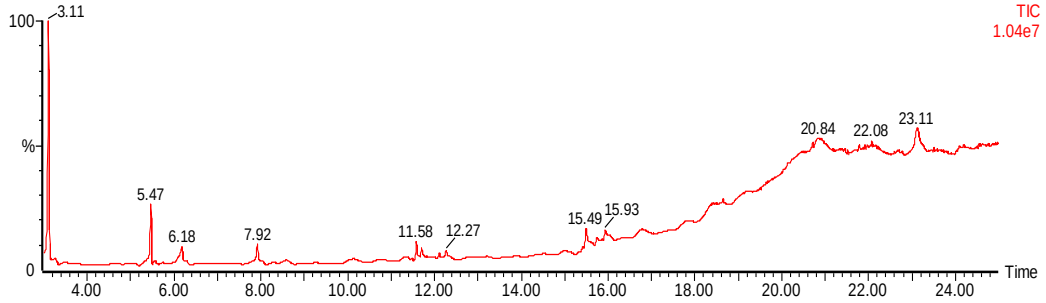
18.61
18.83
21.13
22.19
23.65
24.08


subcritical etoh 2:1
, 20-May-2022 + 11:21:00
NadR_SLX10 NR+Sm (SG, 2x3)
Scan EI+
TIC
1.68e7
100
%
0
Time
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
3.12
3.65
4.68
5.08
5.36
5.62
6.84
8.40
8.93
10.01
12.27
13.57
14.02
15.15
16.79
17.66
18.49
20.35

subcritical meoh 1:1 , 19-May-2022 + 23:08:10

NadR_SLX01 NR+Sm (SG, 2x3)

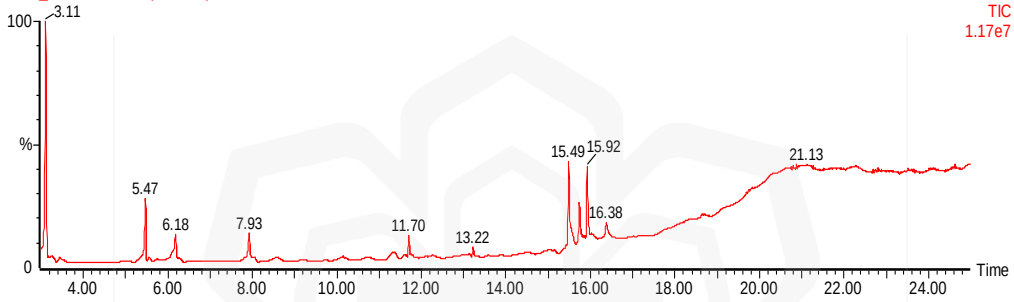
Scan EI+
TIC
1.04e7



subcritical meoh 1:2 , 19-May-2022 + 23:38:22

NadR_SLX02 NR+Sm (SG, 2x3)

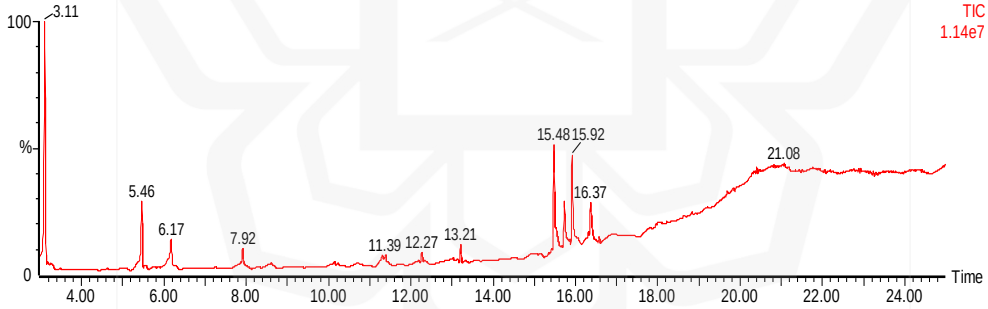
Scan EI+
TIC
1.17e7



subcritical meoh 2:1 , 20-May-2022 + 00:08:37

NadR_SLX03 NR+Sm (SG, 2x3)

Scan EI+
TIC
1.14e7

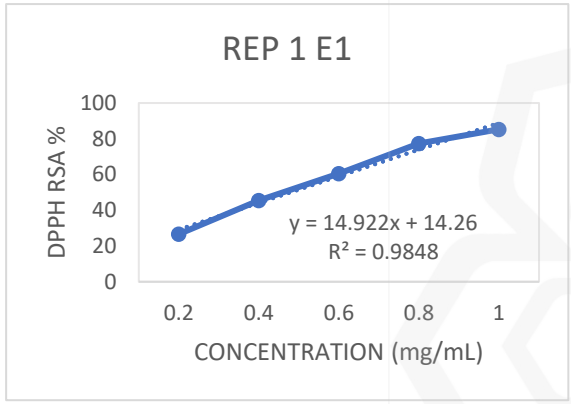


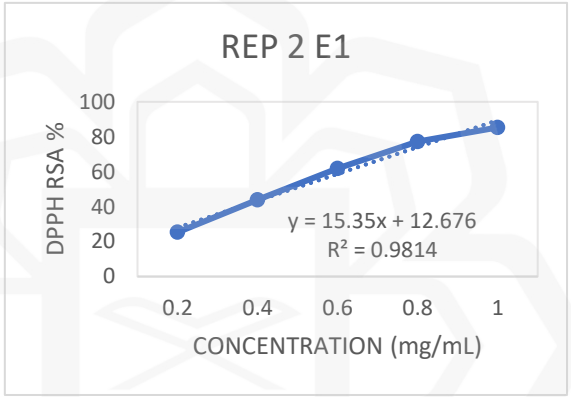
APPENDIX F: RADICAL SCAVENGING ACTIVITY PERCENTAGE OF *S. alata* LEAVES EXTRACTS

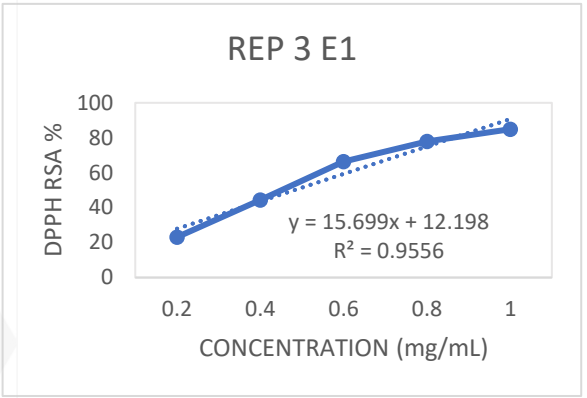
Extracts	Mean	Std. Deviation	Std. Error of Mean	Statistical significance
E1	2.412	0.0188	0.0108	ab
E2	3.415	0.3414	0.1971	bc
E3	2.765	0.0372	0.0215	abc
E4	1.359	0.1873	0.1081	ab
F1	2.562	0.1961	0.1132	ab
F2	4.901	0.4833	0.2790	c
F3	55.569	3.9803	2.2980	d
F4	1.472	0.3721	0.2148	ab
F5	1.365	0.6371	0.3678	ab
F6	0.693	0.2987	0.1725	a
ABA (control)	0.659	0.1197	0.0691	a

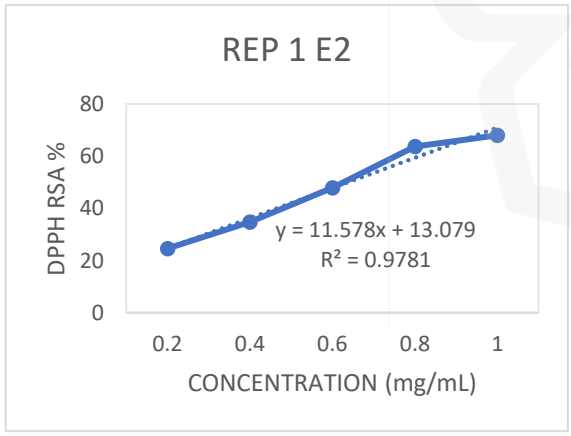
APPENDIX G: GRAPH OF DPPH RSA % FOR EACH EXTRACT AND THEIR REPLICATES

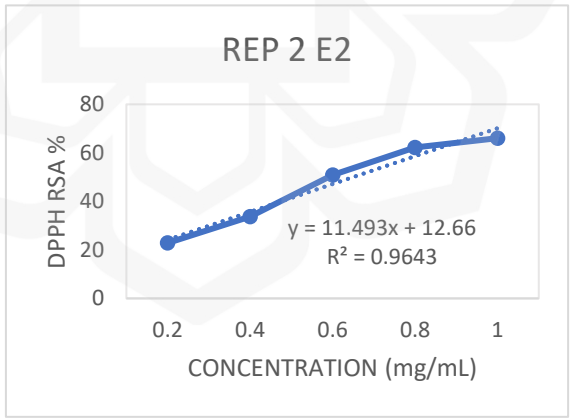
Appendix: Radical Scavenging Activity of E1 and E2 extracts

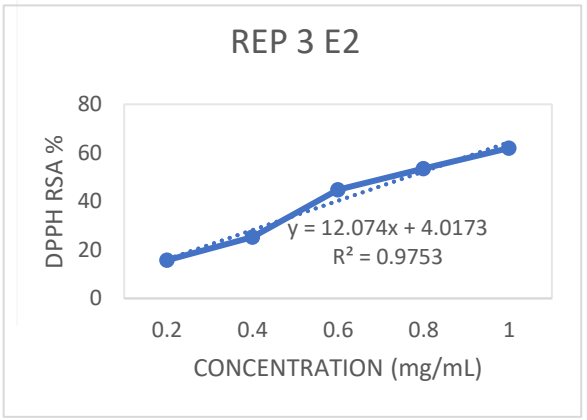




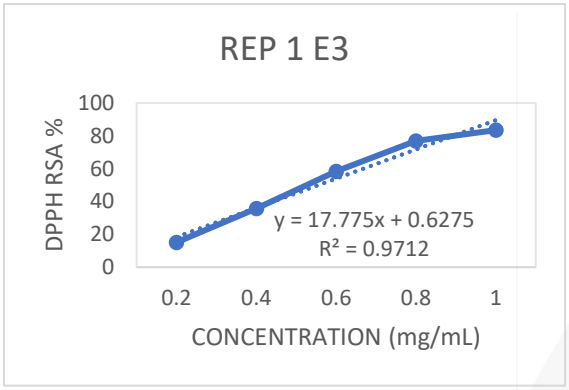


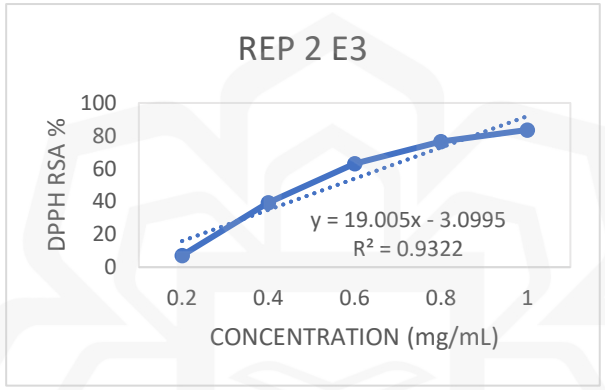


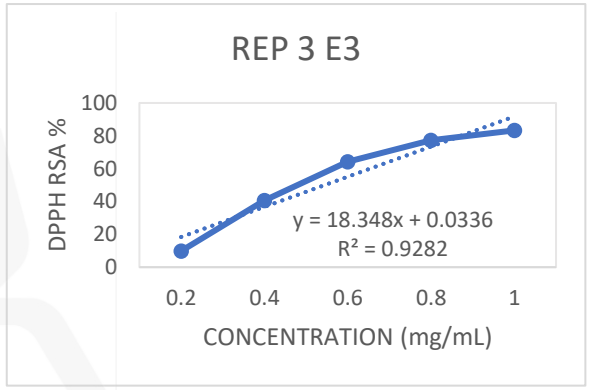


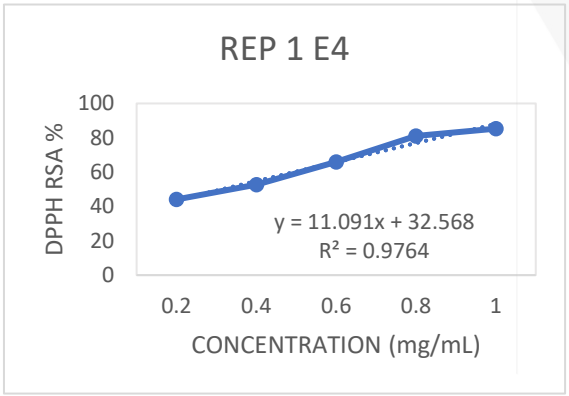


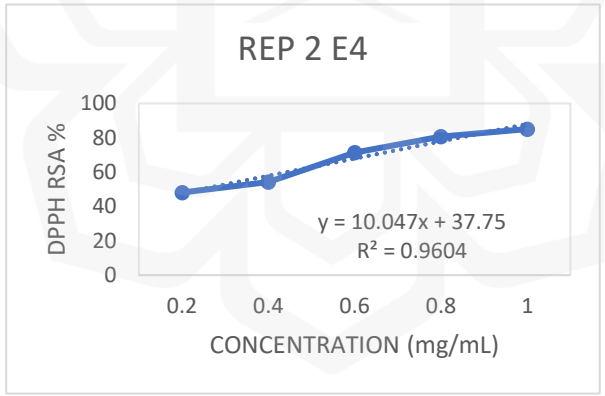
Appendix: Radical Scavenging Activity of E3 and E4 extracts

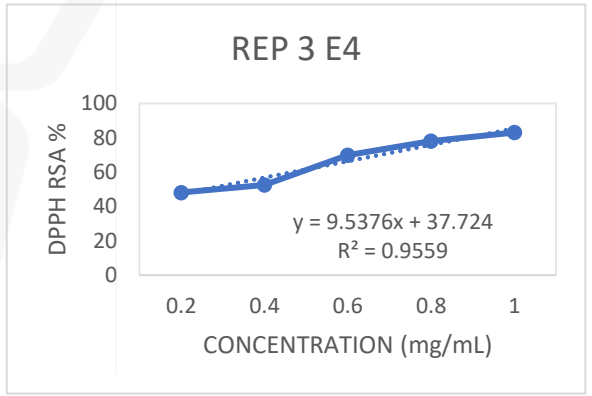




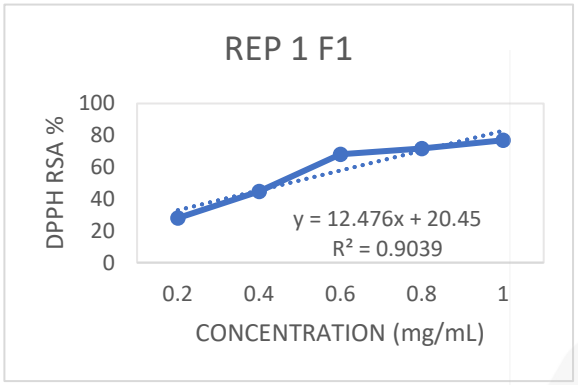


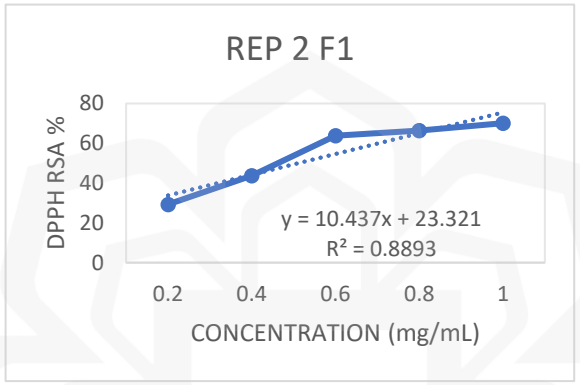


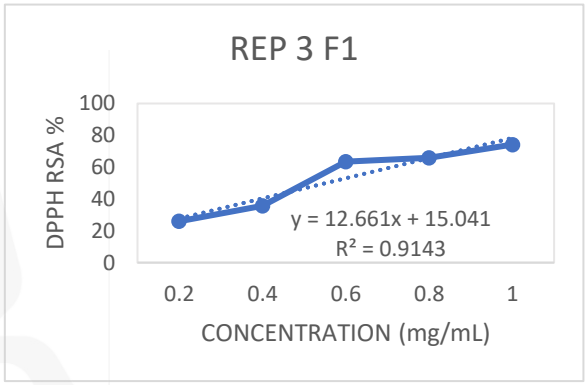


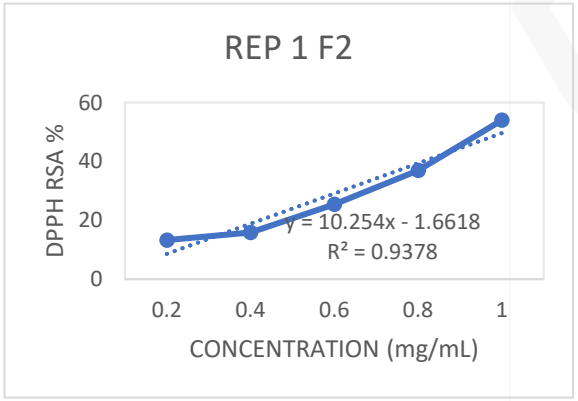


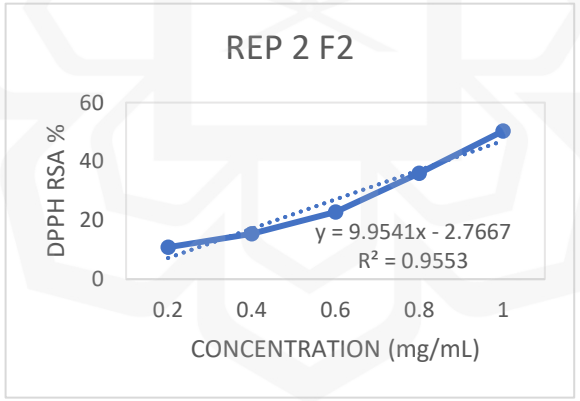
Appendix: Radical Scavenging Activity of F1 and F2 extracts

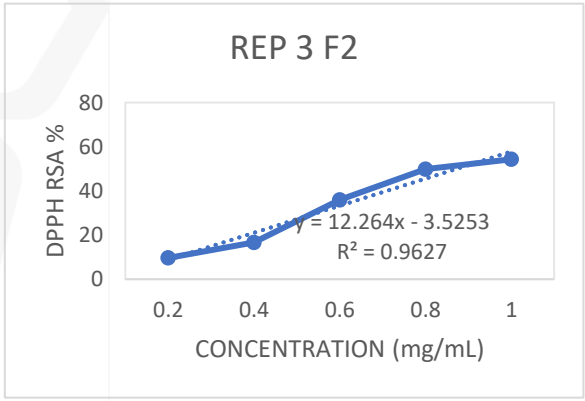




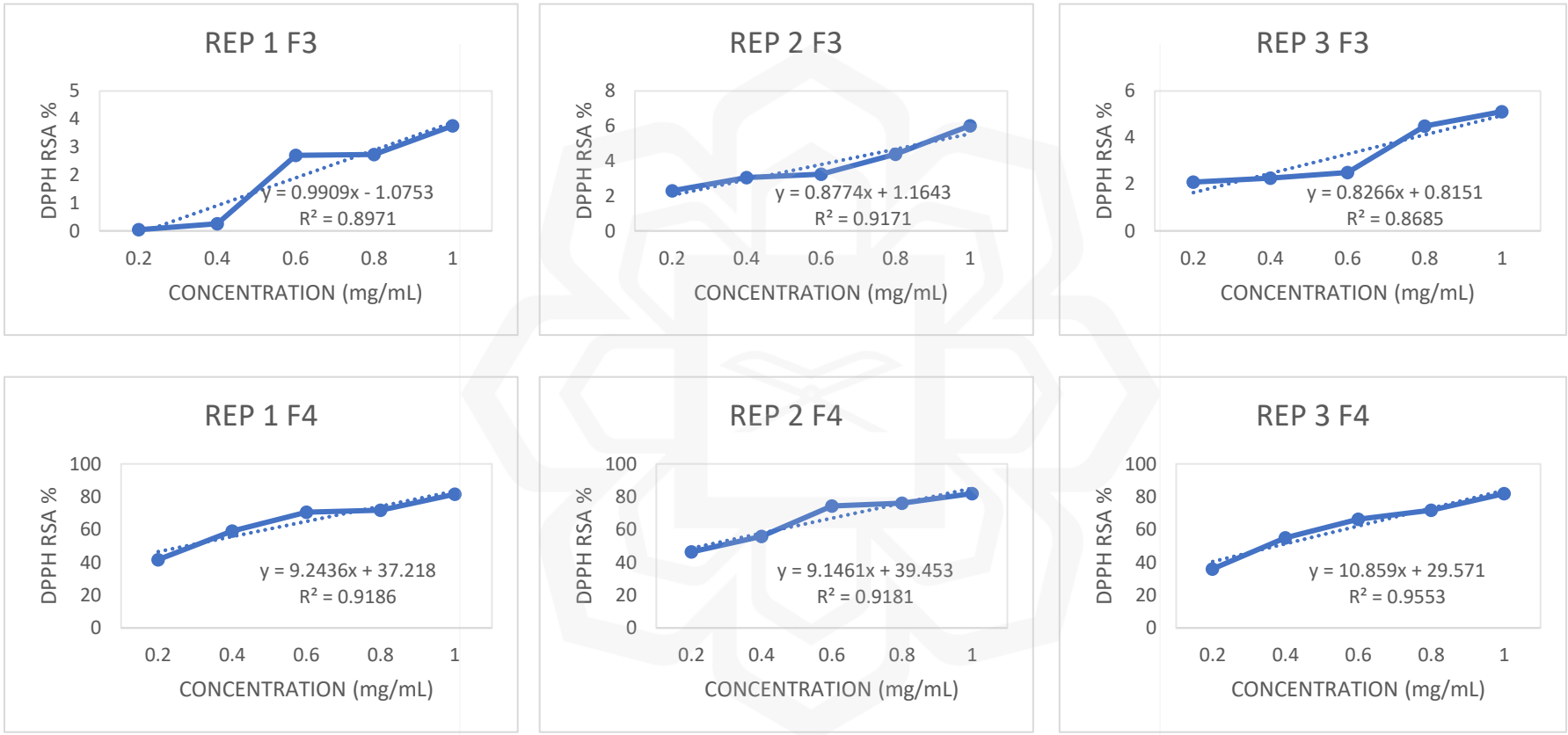




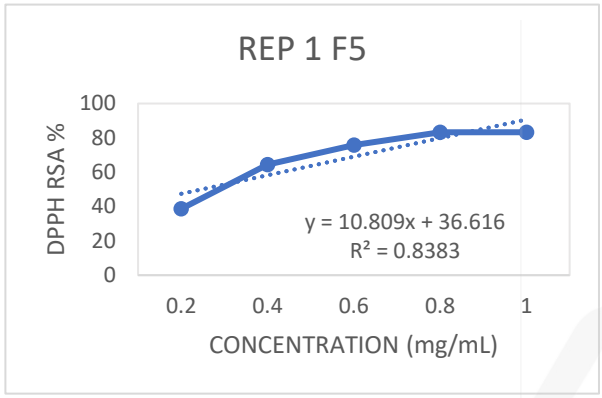


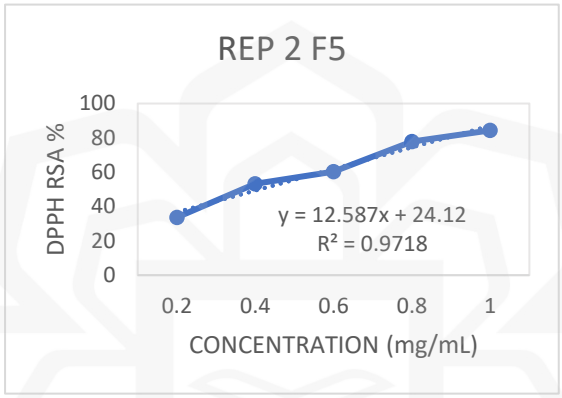


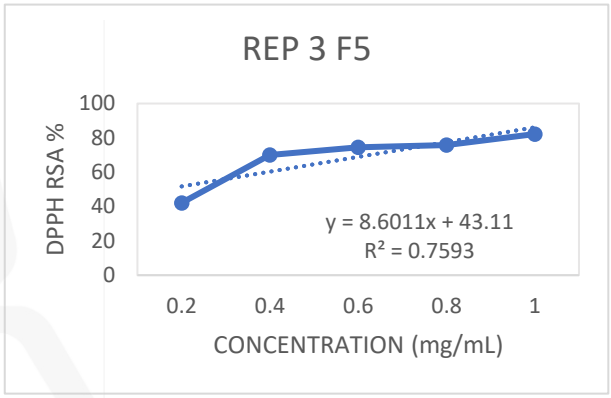
Appendix: Radical Scavenging Activity of F3 and F4 extracts

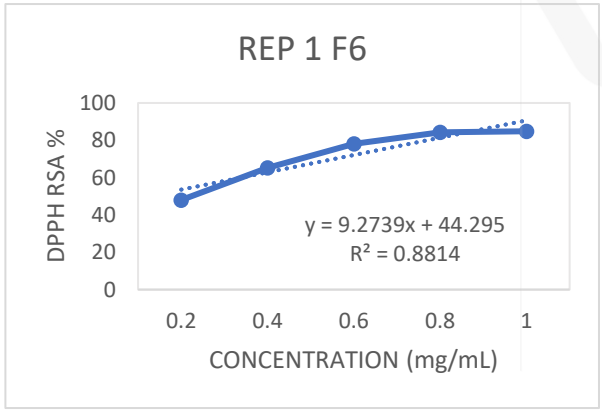


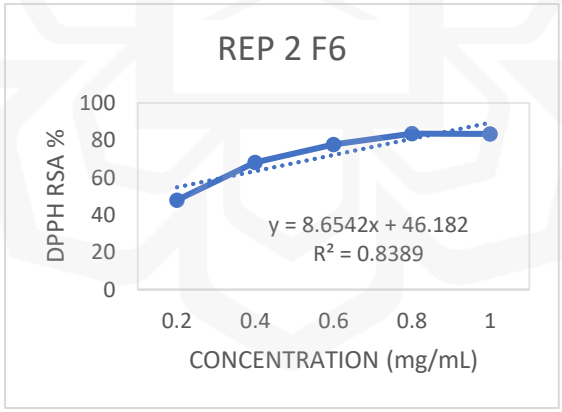
Appendix: Radical Scavenging Activity of F5 and F6 extracts

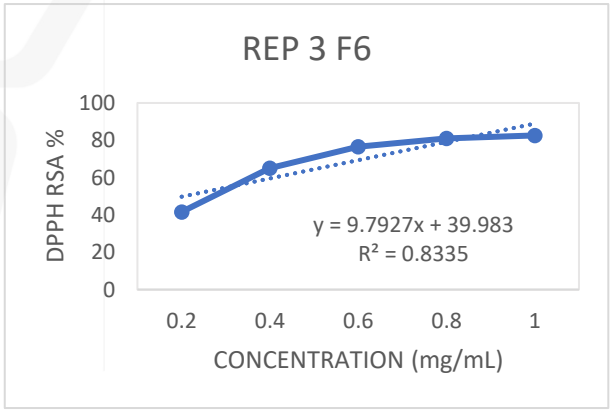




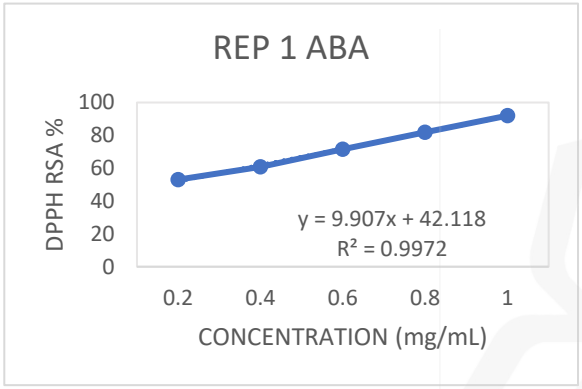


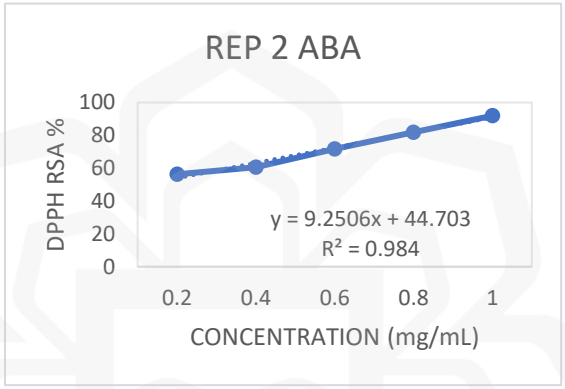


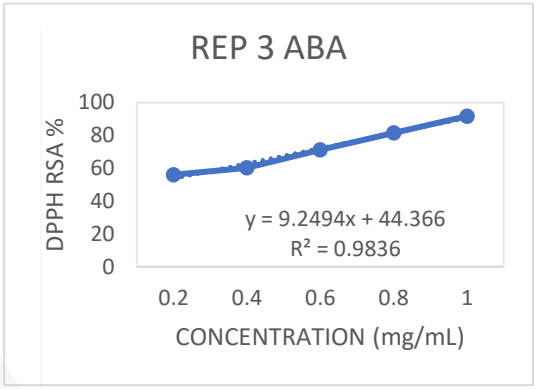




Appendix: Radical Scavenging Activity of ABA







APPENDIX H: STATISTICAL ANALYSIS FOR DPPH IC_{50}

Report DPPH IC_{50}

IC_{50}

SAMPLE	Mean	N	Std. Deviation	Std. Error of Mean
E1	2.411667	3	.0187705	.0108372
E2	3.415333	3	.3413800	.1970959
E3	2.765000	3	.0372424	.0215019
E4	1.359333	3	.1872868	.1081301
F1	2.562000	3	.1960689	.1132004
F2	4.901000	3	.4832898	.2790275
F3	55.569000	3	3.9802803	2.2980159
F4	1.472333	3	.3721308	.2148498
F5	1.365000	3	.6370659	.3678102
F6	.693000	3	.2987373	.1724761
ABA	.659333	3	.1197177	.0691191
Total	7.015727	33	15.6732343	2.7283599

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig.
IC_{50} * SAMPLE	Between Groups	(Combined)	7826.977	10	782.698	508.969	.000
	Within Groups		33.832	22	1.538		
	Total		7860.809	32			

Tests of Between-Subjects Effects

Dependent Variable: IC_{50}

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7826.977[a]	10	782.698	508.969	.000
Intercept	1624.274	1	1624.274	1056.226	.000
VAR00001	7826.977	10	782.698	508.969	.000
Error	33.832	22	1.538		
Total	9485.083	33			
Corrected Total	7860.809	32			

a. R Squared = .996 (Adjusted R Squared = .994)

DPPH IC_{50}

Duncan[a,b]

SAMPLE	N	Subset a	b	c	d
E1	3	2.411667	2.411667		
E2	3		3.415333	3.415333	
E3	3	2.765000	2.765000	2.765000	
E4	3	1.359333	1.359333		
F1	3	2.562000	2.562000		
F2	3			4.901000	
F3	3				55.569000
F4	3	1.472333	1.472333		
F5	3	1.365000	1.365000		
F6	3	.693000			
ABA	3	.659333			
Sig.		.083	.088	.057	1.000

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 1.538.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

RESEARCH OUTPUT

Rahim, N. A., Ferdosh, S., Zainuddin, N. A., & Sarker, M. Z. (2023). Extraction methodologies, phytochemical constituents, and biologicalactivities of Senna Alata Linn: A Review. *The Natural Products Journal*, *13*(2). https://doi.org/10.2174/2210315512666220427114719 (Review Article)

Nadirah Abd Rahim, Sahena Ferdosh & Nur Sabrina Ahmad Azmi (2022). Extraction Of Senna Alata (L.) Roxb Leaves Using Soxhlet, Maceration and Subcritical Fluid Method and antibacterial activities against *Staphylococcus aureus* and *Staphylococcus epidermidis*. In: *Malaysian Society for Microbiology Postgraduate Symposium (MSMPS2022)*, August 2022, Online. (Poster Presentation)

Ferdosh, Sahena and **Abd Rahim, Nadirah** (2021) Optimization of total phenolic content and antioxidant activity from Sukkari variety of date palm (*Phoenix dactylifera*) using supercritical fluid extraction method. In: 7th International Conference on Advancement in Science & Technology (iCAST2021), Online. (Oral Presentation)