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Thin Layer Chromatography - Direct Bioautography and Identification of Compounds from the Semi-purified Fraction of *Senna alata* (Linn.)

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ABSTRACT

The availability and affordability of medicinal plants, along with the fact that certain bacteria are resistant to synthetic drugs, have led to increased interest in natural products derived from plants for medical applications. *Senna alata* (Linn.), commonly known as "*gelenggang"* and "*daun kurap"*, has demonstrated a wide range of antibacterial properties against infections caused by bacterial pathogens. This study aimed to isolate and identify the bioactive compounds present in the leaf, stem, and root of *Cassia alata*, and to evaluate their antimicrobial activities. Thin Layer Chromatography-Direct Bioautography (TLC-DB) and Gas Chromatography-Mass Spectrometry (GC-MS) techniques were utilized for compound separation and identification. In the TLC separation, four, seven, and five spots were observed on the TLC plates for the leaf, stem, and root extracts, respectively. The bacterium *Mammaliicoccus* sp. exhibited a strong inhibition zone corresponding to one distinct spot on each TLC-DB plate: leaf (Rf = 0.47), stem (Rf = 0.40), and root (Rf = 0.42). In contrast, *Enterococcus* sp. showed a weaker inhibition at those same spots. GC-MS analysis of the active spots identified major bioactive compounds, including Phenol, 3,5-bis(1,1-dimethylethyl)-, Cholest-5-en-3-ol (3.beta.)-, carbonochloridate, Neophytadiene, Dodecane, 2,6,11-trimethyl-, 2-Pentadecanone, 6,10,14-trimethyl-, and Decane, 3,7-dimethyl-,. These findings suggest that *Senna alata* contains bioactive compounds with notable antimicrobial properties, supporting its potential application in developing alternative treatments for infections caused by *Mammaliicoccus* sp. and *Enterococcus* sp. This study highlights the importance of further investigating plant-derived compounds as promising candidates in the search for new antimicrobial agents.

Keywords: *Senna alata* (Linn.), bioactive compounds, Thin Layer Chromatography-Direct Bioautography, Gas Chromatography-Mass Spectrometry, antibacterial

Introduction

Traditional medicine has long relied on medicinal plants for their diverse biological activities, and plant-derived antibacterial compounds are found not only in medicinal species but also in common culinary ingredients and local vegetables, highlighting the broad potential of plant sources.[1,2] Due to the increasing prevalence of antibiotic-resistant microorganisms, the search for these compounds in medicinal plants is significant.[3,4] For example, the emergence of vancomycin-resistant enterococci infections has limited the number of antimicrobials used to treat these infections.[5] In addition, the fast-expanding antibiotic resistance repertoire of *Enterococcus faecium* makes it nearly resistant to all clinically used antimicrobials.[6] In other cases, researchers have become interested in the limited study of a novel genus, such as *Mammaliicoccus* species, which is associated with antibiotic resistance.[7] It has been observed that these species, the causative agents of mastitis in animals, may form biofilms and transmit virulence factors such as enterotoxins and hemolysins.[8] Given these challenges, plants with diverse bioactive profiles are considered promising alternatives for developing new antimicrobial agents. One of these is *Senna alata* (Linn.), a member of the family Fabaceae.[9]

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S. alata is a medicinal plant that has various ethnomedicinal uses, which include decoction and pounding of its leaves for the treatment of constipation, skin diseases, ringworm[10], haemorrhoids, and intestinal parasitosis.[9,11] This species is extensively distributed in tropical regions such as Southeast Asia, Northern Australia, Africa, and South America.[12] It has many local names, and in Malaysia is commonly known as "*gelenggang*" and "*daun kurap*".[13] The leaves, stems, and roots have been reported to possess various biological properties, including antibacterial, antifungal,[14] antioxidant,[15] anti-inflammatory,[16] and antihelmintic activities.[17] Although many studies have identified bioactive compounds in *S. alata*,[18] there is limited information on the specific compounds responsible for its antibacterial effects against emerging pathogens such as *Mammaliicoccus* sp. and *Enterococcus* sp. Information on the specific bioactive chemicals of medicinal plants and identifying the key molecules responsible for antibacterial action against pathogens are crucial in developing new treatments against bacterial infections.[19] Thus, the choice of bioassay method is crucial for identifying the bioactive compounds in plant extracts. Thin-layer chromatography coupled with direct bioautography (TLC-DB), offer simple, rapid, and cost-effective approaches for separating and screening plant extracts for antimicrobial activity.[20] Additionally, gas chromatography-mass spectrometry (GC-MS) allows precise identification of the chemical constituents present in active fractions, facilitating the characterization of potential active compounds.[21]

This study aimed to isolate and identify antibacterial components from the leaves, stems, and roots of *S. alata* extracts against *Mammaliicoccus* sp. and *Enterococcus* sp. by integrating thin layer chromatography (TLC) in combination with direct bioautography (DB) assay, and gas chromatography and mass spectrometry (GC-MS) analysis. The approach used here highlights the potential of *S. alata* as a promising source for developing plant-based antibacterial agents to address the growing problem of antibiotic resistance.

Materials and Methods

Plant Collection and Identification

S. alata was collected on 11th November 2020 from Kota Samarahan District in Sarawak, Malaysia (1˚27'40.968" N, 110˚24'50.5" E). Botanical identification of the plant was carried out by an experienced botanist Dr Meekiong B. Kalu of Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Malaysia. The specimen was deposited at the Herbarium of Institute of Biodiversity and Environmental Conservation (IBEC), Universiti Malaysia Sarawak, Malaysia under voucher no. CA-SL-02-2020.

The plant parts were divided into leaves, stems, and roots. The blooms, which grow from the plants in a vertical column and resemble a vivid yellow candle, were what allowed the plants to be recognized.[9] The samples were carefully washed and dried in an oven at 40 ˚C for 5 days or until they attained a constant weight. After drying, the samples were ground into a fine powder and stored at -4 ˚C until further use.[22]

Sample extraction

The sample extraction was performed using the Soxhlet extraction method with modifications.[23] Briefly, 15 g of powdered *S. alata* L. was weighed and mixed with the extraction solvent. Absolute chloroform (≥99.8%, Merck, Germany) was used as the extraction solvent. A total volume of 200 mL of solvent was put in a thimble of a Soxhlet apparatus, and the sample was extracted for 6 h. The extract was subsequently filtered using Whatman No. 1 filter paper (GE Healthcare, UK), followed by rotary evaporation at 40 ˚C and 474 mbar until only a small amount of solvent remained. The remaining crude extract was allowed to dry in the fume hood and kept at -4 ˚C for further analysis.

Compound separation

The bioactive compounds in *S. alata* extracts were analysed using Thin-Layer Chromatography (TLC) on silica gel G60 F_{254} aluminium plates (Merck, Germany). Briefly, 1 mg of crude extract was diluted in 1 mL of absolute dichloromethane (≥99.8%, Merck, Germany). The TLC plates (8 × 4 cm) were developed under saturated conditions using a solvent system of hexane (≥95%, Merck, Germany), ethyl acetate (≥99.5%, Merck, Germany), and chloroform (≥99.8%, Merck, Germany) mixed in a ratio of 8:2:1 (v/v/v). [24]

Standardisation of test bacteria

Two bacterial isolates, namely *Mammaliicoccus* sp. and *Enterococcus* sp., were obtained from the Department of Animal Science and Fisheries, Universiti Putra Malaysia Sarawak and Molecular Laboratory, Institute of Biodiversity and Environmental Conservation, Universiti Malaysia Sarawak, respectively. The bacteria were cultured on Mueller Hinton broth (MHB) (Oxoid, UK), incubated overnight at 37 ˚C for 18 h, and standardized to 0.5 McFarland at 600 nm using a spectrophotometer.[16,25]

Bioautography

The developed TLC plates were dipped in a standardised bacterial suspension for 10 seconds, air-dried at room temperature (26 ˚C) for 2 minutes, put in Petri dishes (150 × 15 mm) lined with moist tissue paper, and incubated at 37 ℃ for 17 h. After incubation, the TLC plates were sprayed with an aqueous solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 2.5 mg/ml) (Sigma Aldrich, USA) and further incubated for 2 h. Dehydrogenase activity was visualised using tetrazolium salt-based reagents. A drop of Triton X-100 (Merck, Germany) per 10 mL of aqueous MTT solution was added to increase colour intensity. The inhibition zones on the TLC plate were identified as a pale spot on a dark blue backdrop because metabolically active bacteria converted the MTT into formazan dye. The negative control was the developing solvent hexane: ethyl acetate: chloroform (8:2:1, v/v/v).[26]

Chromatographic purification

Semi-preparative TLC was used to extract the antibacterial compounds identified in TLC-DB. A 20 cm band was applied on TLC Silica gel 60G F_{254} 25 Glass plates (Merck, Germany). The plates were developed to a distance of 6 cm using the same solvent system as above. The separated fractions on TLC plates were dried and visualized under UV light (365 nm). Each identified fraction was scraped separately with silica, eluted with 20 mL of absolute methanol (≥99.8%, Merck, Germany) and filtered through Whatman No. 1 filter paper. The filtered fractions were dried in a fume hood and subjected to GC-MS analysis.[27]

GC-MS analysis

Gas Chromatography-Mass Spectrometry (GC-MS) was utilised to evaluate the chemical constituents of the active fractions.[28] The analysis was carried out using a Shimadzu GCMS-QP2010 Plus system (Japan), equipped with a quadrupole mass analyzer and a non-polar BPX-5 cross-linked column (5% phenyl polysilphenylene-siloxane), measuring 30 m in length, with an internal diameter of 0.25 mm and a film thickness of 0.25 µm. The diluted sample (1mg/mL) of 1 µL was injected. The mass spectrophotometer ionisation energy was set to 70 eV. The temperature was programmed from 50 °C to 260 °C at a rate of 10 °C/minute, and then held for 10 minutes. The temperatures of the injector and detector were both set at 280 °C. The detected compounds were identified by comparing their mass spectra with the National Institute of Standards and Technology (NIST) library.

Results and Discussion

Thin Layer Chromatography

To isolate and identify the bioactive compounds of *S. alata* extracts, TLC was performed as a qualitative method to document the extract constituents. The application of TLC is essential in analysing drugs and is frequently used for targeting medicinal plants in search of various physiological properties of botanical materials.[29] Figure 1 shows the TLC plates from the leaves, stems and roots of *S. alata* extracts.

In this study, four spots were observed in leaf extracts, seven in stem extracts, and five in root extracts. Observations under 365 nm revealed that TLC plates appeared with dark-coloured chemical compounds as a background emission, in which various colours were spotted, including yellow, red, light blue, and dark blue. The R_f values of each fraction were also calculated. The R_f value of L1 was similar to S1, with R_f values of 0.23. Other spots, such as S5, S6, and S7, were similar to R2, R3, and R4, with R_f values of 0.80, 0.87, and 0.92, respectively. Those with similar R_f were likely the same compound, though it was unnecessary.[30]

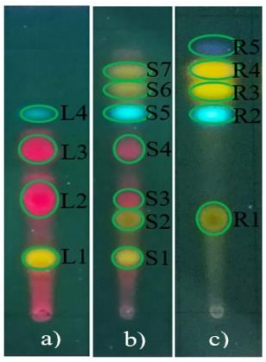

Figure 1: TLC plates of *S. alata* extracts under UV at 365 nm using a mobile phase, Hexane: Ethyl acetate: Chloroform (8:2:1). a) Leaves. b) Stems. c) Roots

Thin Layer Chromatography - Direct Bioautography

The direct bioautography assay was used to determine the antibacterial activity of the compounds separated on the TLC plate. The antibacterial activity was identified as a large clear zone of inhibition on a dark blue background sprayed with methylthiazol tetrazolium (MTT) solution. Figure 2(a) shows very weak activity and likely no inhibition was observed against *Enterococcus* sp. In contrast, in Figure 2(b), a strong inhibition was observed against *Mammaliicoccus* sp. Table 1 shows the colours and R_f values of the active spots. It was observed that the active spots were at similar R_f values for both bacteria. This explained that the potential compounds responsible for the previous antibacterial activity likely came from similar compounds.[31]

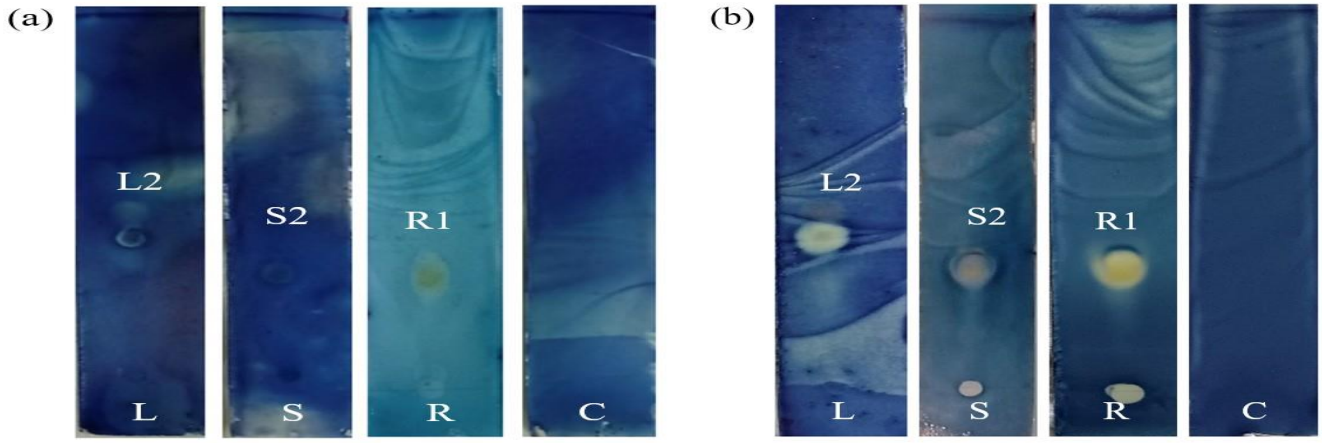

Figure 2: TLC-direct bioautography plates of *S. alata* extracts against (a) *Enterococcus* sp. and (b) *Mammaliicoccus* sp., where L: leaves, S: stems, R: roots, C: control (Hex: EtOAc: Chlo)

Among the identified spots, polar compounds were exhibited with inhibition. Significant antibacterial activity against *Mammaliicoccus* sp. was demonstrated by spots with R_f values of 0.47 (L2) from leaves, 0.40 (S2) from stems, and 0.42 (R1) from roots of *S. alata* extracts. However, the other spots without inhibition could be explained by either no activity, the evaporation of the active compounds, low content of active compounds[31] and disruption of synergism between the active compounds separated by TLC.[32]

This direct bioautography assay proved appropriate for identifying and evaluating compounds with antibacterial activity in *S. alata* extracts. This validates its significance in the exploration of novel antimicrobial agents. The assay also offers advantages such as cost-effectiveness, rapidity, ease of setup, and a minimal requirement for test extract.[20]

GC-MS Analysis

The spots with significant antibacterial activities from the TLC-DB assays were subjected to semi-preparative TLC for the isolation of the active compounds. Thus, L2, S2, and R1 spots were scraped and eluted with methanol, and the compounds present were identified using GC-MS analysis. In this study, a total of 16 compounds were identified from both L2 and S2, and 7 compounds were identified from R1. The identification of these compounds was confirmed based on their molecular formulas, retention times, and peak areas. The result of the chromatogram analysis is shown in Figure 3. Among these compounds, as shown in Table 2, 12 from L2, 10 from S2, and 5 from R1 had previously reported biological activities.

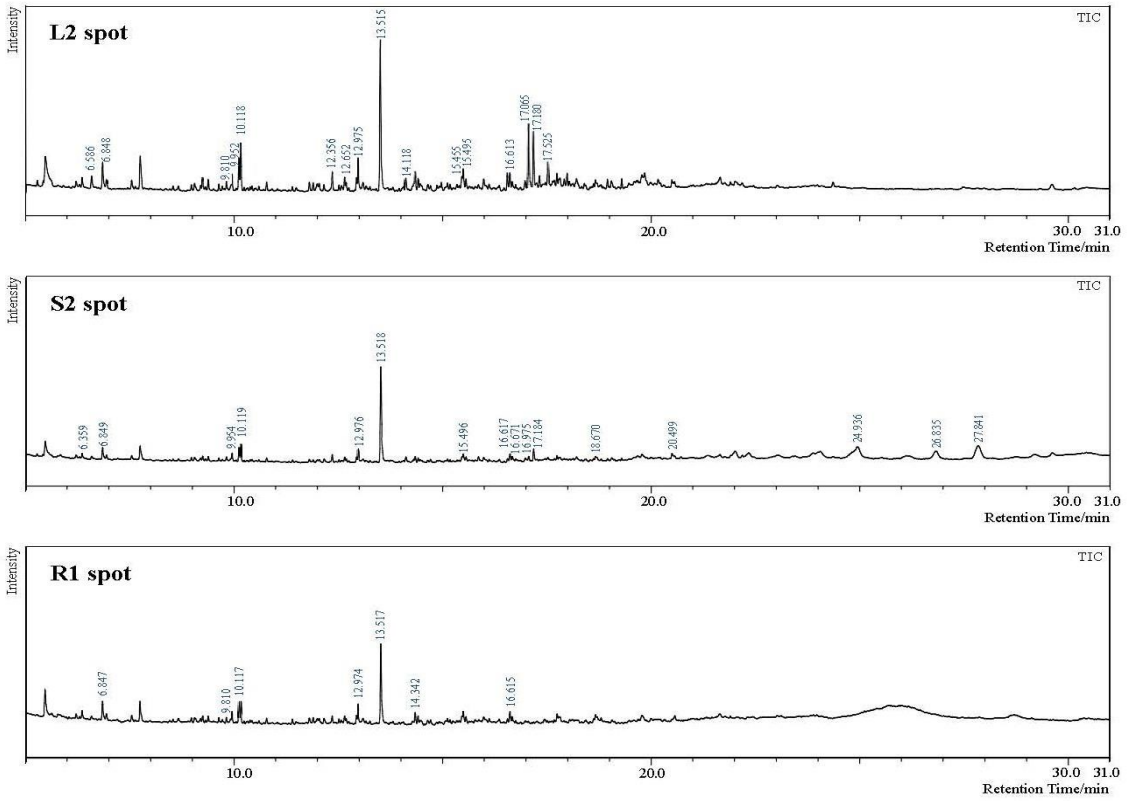

Figure 3: Chromatogram for L2, S2 and R1 spots

Table 1: Inhibition of growth on bioautographic TLC plates by *S. alata* extracts against *Enterococcus* sp. and *Mammaliicoccus* sp.

Parts	Spots	Colours	R_f values	Active spot	
				Enterococcus sp.	*Mammaliicoccus* sp.
Leaves	L2	Red	0.47	-	+
Stems	S2	Yellow	0.40	-	+
Roots	R1	Yellow	0.42	-	+

*(-) weak/no inhibition observed, (+) strong inhibition observed

According to the chromatogram, Phenol, 3,5-bis(1,1-dimethyl ethyl)- demonstrated the highest peak area, highlighting its predominance as a major compound, comprising for 32.07% in L2, 35.69% in S2, and reaching the highest proportion of 51.11% in R1. In a previous study of *S. alata*, a similar compound was found in crude extracts of stems with an area of 0.76 %.[16] This compound was also found in other plant extracts such as *Biden pilosa* leaves[33] and *Moringa oleifera*.[34] It is a phenolic compound that has various biological properties. This includes antioxidant,[33] antiseptic, disinfectant, flavouring, antibacterial,[35] and anthelminthic properties.[34] In this study, Phenol, 3,5-bis(1,1-dimethylethyl)- is a favourable compound because it might be the major contributor to the observed antibacterial activity against *Mammaliicoccus* sp.

Additionally, Cholest-5-en-3-ol (3.beta.)-, carbonochloridate showed a remarkably high area percentage in S2 (17.69%), but was not detected in L2 or R1. This compound has been reported in *Bacillus thuringiensis*, which has bio-insecticide and nematicidal effects.[36] It was also identified from mollusc *Purpura persica* extracts and exhibited antimicrobial activity against various bacterial pathogens and fungi.[37] Similarly, Cholesta-4,6-dien-3-ol (3β)- and Stigmasta-5,22-dien-3-ol, acetate (3β)- were found exclusively in S2 at 7.52% and 4.44%, respectively. The presence of these sterol derivatives supports the traditional use of *S. alata* for anti-inflammatory and hepatoprotective purposes.[38]

Table 2: Bioactive compounds identified from active spots L2, S2 and R1 of *S. alata* extracts

Compounds name	Spots	RT (min)	Area (%)	Molecular formula	Biological activities
Dodecane, 2,6,10-trimethyl-	L2	6.848	5.56	$C_{15}H_{32}$	Antimicrobial and cytotoxicity[42]
Dodecane, 4,6-dimethyl-	L2	9.810	2.88	$C_{14}H_{30}$	Antioxidant and antibacterial[39]
	R1	9.810	6.89		
Dodecane, 2,6,11-trimethyl-	L2	10.118	5.99	$C_{15}H_{32}$	Antioxidant and antibacterial[39]
	L2	12.975	5.75		
	S2	10.119	4.16		
	S2	12.976	3.60		
	S2	15.496	2.56		
	R1	10.117	10.46		
	R1	12.974	8.86		
3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	L2	12.356	2.92	$C_{18}H_{52}O_7Si_7$	Antimicrobial[43]
2,6,10-Trimethyltridecane	L2	12.652	2.51	$C_{16}H_{34}$	Drug development for shrimp culture system[44]
Phenol, 3,5-bis(1,1-dimethylethyl)-	L2	13.515	32.07	$C_{14}H_{22}O$	Antioxidant, antimicrobial,[33] antibacterial, antiseptic, disinfectant and flavouring[35]
	S2	13.518	35.69		
	R1	13.517	51.11		
2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	L2	14.118	2.45	$C_{11}H_{16}O_2$	Antimicrobial[45]
Eicosane	L2	15.495	3.37	$C_{20}H_{42}$	Antifungal[46]
1-Tricosene	L2	16.613	2.98	$C_{23}H_{46}$	Anticancer and anti-inflammatory[45]
Neophytadiene	L2	17.065	11.87	$C_{20}H_{38}$	Antioxidant and antimicrobial[47]
2-Pentadecanone, 6,10,14-trimethyl-	L2	17.180	9.73	$C_{18}H_{36}O$	Antibacterial[48]
	S2	17.184	3.97		
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	L2	17.525	4.05	$C_{20}H_{40}O$	Antimicrobial[49]
9-Eicosene, (E)-	S2	16.617	2.77	$C_{20}H_{40}$	Antibacterial[40]
	R1	14.342	5.39		
Heptadecane	S2	16.671	1.98	$C_{17}H_{36}$	Anti-inflammatory[50]
Tetrapentacontane, 1,54-dibromo-	S2	16.975	1.81	$C_{54}H_{108}Br_2$	Antioxidant[51]
1-Nonadecene	S2	18.670	1.93	$C_{19}H_{38}$	Antifungal, antibacterial,[52] anticancer[53]
Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)-	S2	24.936	4.44	$C_{31}H_{50}O_2$	Source of synthetic progesterone and exhibits antihepatotoxic, antiviral, antioxidant, cancer preventive, and hypocholesterolemic activity[53]
Cholesta-4,6-dien-3-ol, (3.beta.)-	S2	26.835	7.52	$C_{27}H_{44}O$	Antibacterial and antifungal[54]
Cholest-5-en-3-ol (3.beta.)-, carbonochloridate	S2	27.841	17.69	$C_{28}H_{45}ClO_2$	Antimicrobial activity,[37] nematicidal[36]
1-Hexadecanol	R1	16.615	5.56	$C_{16}H_{34}O$	Antibacterial[55]

Neophytadiene was also present at a considerable percentage (11.87%) in L2, while it was absent in both S2 and R1. This indicates a possible unique role of this compound in L2, potentially contributing to its specific antimicrobial properties. Similarly, 2-Pentadecanone, 6,10,14-trimethyl-, accounted for 9.73% in L2 and was lower in S2 (3.97%), but absent in R1. This suggests that L2 may have higher antibacterial potential compared to the other fractions. Both compounds have been reported in *S. alata* extracts that exhibited antibacterial activity against *Staphylococcus aureus*.[16]

Among the alkanes, Dodecane, 2,6,11-trimethyl- was present in multiple spots and higher percentages in R1 (10.46% and 8.86%) compared to L2 and S2, indicating its potential role in the strong antibacterial and antioxidant properties of R1. [39,40] Interestingly, 9-Eicosene, (E)- was relatively higher in R1 (5.39%) than in S2 (2.77%), and absent in L2, suggesting its specific enrichment in root extracts.

Overall, these findings demonstrate that each active spot exhibits a unique chemical profile, which may explain differences in bioactivity observed among L2, S2, and R1. The significantly higher proportions of phenolic and sterol compounds in R1 and S2 suggest stronger antioxidant and antimicrobial properties, respectively.[41] The quantitative dominance of certain compounds, such as Phenol, 3,5-bis(1,1-dimethylethyl)-, highlights them as potential biomarkers for further pharmacological investigations. It is also significant for these compounds to be further studied before formulating an effective natural antimicrobial drug against *Mammaliicoccus* sp.

Conclusion

The separation and detection of bioactive compounds were effectively achieved using simple and cost-effective TLC-DB, while GC-MS enabled the identification of chemical constituents present in *S. alata* extracts. This study confirmed that *S. alata* contains numerous bioactive compounds responsible for its antibacterial activity, with separated components providing more accurate and targeted information than crude extracts. Among the identified compounds, Phenol, 3,5-bis(1,1-dimethylethyl)- was the most dominant and consistently detected in the leaves, stems, and roots, indicating its major role in antibacterial activity. Other significant compounds included Cholest-5-en-3-ol (3.beta.)-, carbonochloridate, Neophytadiene and 2-Pentadecanone, 6,10,14-trimethyl-. These findings highlight the potential of developing purified bioactive compounds from *S. alata* as novel antibacterial agents to combat infections caused by *Mammaliicoccus* sp. and to enhance existing antibacterial therapies. Future research should focus on purifying and characterizing these active compounds, studying their mechanisms of action, and testing their effectiveness and safety in living systems. In addition, exploring their potential to work together with existing antibiotics and developing them into new natural antimicrobial products could help combat resistant bacteria and improve current treatments.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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