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Research Article

PHYTOCHEMICAL SCREENING, ANTIOXIDANT, ANTIMICROBIAL, AND ANTIBIOFILM ACTIVITY OF *SAUROPUS ANDROGYNUS* LEAF EXTRACTS

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ABSTRACT

Objective: The objective of the study was to perform phytochemical screening, and evaluate the antioxidant, antimicrobial, and antibiofilm activities of *Sauropus androgynus* leaf extract against pathogenic organisms.

Methods: *Sauropus androgynus* leaves were shade dried, powdered, and extracted by Soxhlet hot extraction procedure using ethanol or methanol. The aqueous extract was prepared using a rotary shaker. The qualitative phytochemical screening was carried out by gas chromatography-mass spectroscopic (GC-MS) analysis and liquid chromatography-mass spectrometry (LC-MS). The antioxidant activity of plant extracts was estimated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH)-free radical scavenging assay. Antimicrobial and antibiofilm activity of extracts against various pathogens was performed using serial microdilution assay and microtiter plate method, respectively.

Results: Preliminary phytochemical analysis showed the presence of compounds such as tannins, flavonoids, alkaloids, and phenols. GC-MS studies also revealed the presence of biologically active compounds. Methanolic extract of *S. androgynus* showed higher DPPH Scavenging activity compared to other extracts. All the extracts were able to inhibit both bacterial growth and biofilm production; however, the methanolic extract showed the highest antibiofilm activity.

Conclusion: *S. androgynus* plant extracts possess significant antioxidant, antimicrobial, and antibiofilm activity.

Keywords: *Sauropus androgynus*, Extracts, Gas chromatography-mass spectroscopic, Minimum inhibitory concentration, Biofilm.

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INTRODUCTION

India is famous for its ancient heritage of using medicinal plants in traditional medicines. Many years of efforts in the search for good health, longevity, as well as for remedies to pain and discomfort, have led to the discovery of a variety of ethnomedicines. Plants are used as medicines in different systems of medicine including Ayurveda, Unani, Allopathy, and Homoeopathy [1,2]. From these medicinal plants, many new therapeutically effective drugs have been discovered and formulated as anticancer drugs or antimicrobials [3-6]. The use of plants for healing and as a source for antimicrobials has considerable advantages over current chemotherapeutic approaches [6,7]. Studies reveal that many medicinal plants are a good source of bioactive compounds such as peptides, glycosides, alkaloids, saponins, terpenoids, flavonoids, and phenols (most of which have antimicrobial activity) to treat microbial infections [8-11]. Antibiotic resistance of pathogens and the drastic side effects of antibiotic use have stimulated the search for new medicinal/bioactive compounds, especially from plants [12]. Resistance to antimicrobials can be caused by a variety of mechanisms, including biofilm formation. Various plant-based chemicals have been tested for their antibiofilm activity, and the alternative use of natural antibiofilm agents has gained importance [13,14]. Studies have shown the effect of plant extracts in preventing biofilm formation or inhibiting adherence of pathogenic microorganisms [15,16]. Plant-derived compounds have been found to have potential applications in pharmaceuticals, due to safety and less-toxicity properties [14-17].

Sauropus androgynus is known as Multivitamin Plant, and it is a good source of Vitamins A, B, C, and K, as well as carotenoids. Various parts of *S. androgynus* are commonly used to treat fever, diabetes, cancer, high cholesterol, allergies, urinary tract infections, and earaches [18-20]. Extracts of these medicinal plants are most often utilized for the preparation of raw drugs since their various medicinal properties could be due to additive or synergistic activity [12]. A survey of the literature reveals that so far, no studies have been reported about the antibiofilm activity of *S. androgynus* leaves extracts. Thus, the present research work was designed to evaluate the phytochemical constituents, antioxidant, antimicrobial, and antibiofilm activities of *S. androgynus* leaf extracts.

METHODS

Plant collection and extraction

The fresh leaves of *S. androgynus* were collected from Palakkad, South India. The plant with complete herbarium was identified (No.BSI/SRC/5/23/2016/Tech/1336) and authenticated at the Botanical Survey of India, Coimbatore, Tamil Nadu, India. Collected leaves were washed thoroughly under tap water, detergent water, and finally rinsed with distilled water until no foreign materials remained (Damaged leaves were removed). The leaves were then dried under shade for 10 days. The dried leaves were pulverized using a sterile electric grinder to obtain a powdered form. The powdered samples were stored in air-tight containers, protected from sunlight until required for further use.

The powdered leaves (25 g) were continuously extracted with different solvents, namely methanol, ethanol, or distilled water, for successive solvent extraction based on their polarity. The Soxhlet hot extraction procedure for methanol and ethanol solvents was performed for 12–16 h or until the color of the extracted solvent became clear [21]. The aqueous extract was prepared by soaking the plant powder in distilled water (plant powder to solvent ratio of 1:10 w/v) and extracted using Rotary shaker at 150 rpm for 24 h at room temperature [22]. The methanolic extracts of *S. androgynus* leaves (SALM), ethanolic extract

of *S. androgynus* leaves (SALE), and aqueous extracts of *S. androgynus* leaves (SALA) were concentrated under reduced pressure using a rotary vacuum evaporator. The extracts were then transferred into vials, dried in a desiccating chamber, and stored in an air-tight container at low temperature (@4°C). The extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions for further studies.

Preliminary phytochemical analysis

Qualitative phytochemical analysis of SALM, SALE, and SALA was performed for the identification of flavonoids, tannins, terpenoids, and alkaloids according to the standard methods [23]. The extracts were screened for the presence of various bioactive compounds including alkaloids, flavonoids, phenols, and tannins.

Gas chromatography-mass spectroscopic (GC-MS) analysis

Analytical techniques such as GC-MS are considered a powerful tool for the identification of secondary metabolites present in the plant extracts. Compounds such as essential oils, alcohols, acids, esters, steroids, amino, and nitro compounds can be identified using GC-MS. The phytochemical compounds present in the extracts of SALM and SALE were identified and confirmed using a Shimadzu GC-MS (Model Number: QP2010S equipped with Rxi-5Sil MS column) [24,25]. The plant extracts diluted in respective solvents were injected in splitless mode at an injector temperature of 260°C. The interface temperature was set at 280°C, and the oven temperature of the column was raised from 80°C to 260°C at 10°C/min. The phytochemical constituents were identified and compared based on the retention time, using the National Institute of Standards and Technology Mass Spectral Database (NIST 11), and the WILEY 8 library [26,27].

Characterization of SALA extract by liquid chromatography-mass spectrometry (LC-MS)

LC-MS analysis can be used to identify the fragmentation and structural information of both known and unknown compounds present in a sample [28]. The LC-MS analysis of SALA extract was carried out with the Waters Acquity H class ultra-performance LC BEH C18 column (50 mm×2.1 mm×1.7 µm; Waters India Pvt., Ltd., Bengaluru, India) in the Waters Xevo G2 Q-TOF mass spectrophotometer with Mass V 4.1 software. The compounds were identified by comparing with MS/MS Spectra using the ChemSpider database [29].

Antioxidant assay (2,2-diphenyl-1-picrylhydrazyl [DPPH]-free radical scavenging activity)

The antioxidant activity of all the extracts was determined based on hydrogen donation or radical scavenging ability using the DPPH assay, according to the standard protocol [30]. Samples (plant extracts) and standard (ascorbic acid) were taken at various concentrations, and the final volume was adjusted to 100 µL using methanol. The aliquots of samples were added to 3 ml of a 0.01 mM methanolic solution and mixed well. The negative control was also prepared by adding 100 µL of methanol in 3 ml of 0.1 mM methanolic solution of DPPH. The tubes were allowed to stand in dark for 30 min at room temperature. After 30 min, the absorbance of the sample was measured at 517 nm against the blank. The DPPH-free radical scavenging activity percentage was calculated using the following equation:

DPPH scavenging activity % = [(Control-Test sample)/Control]*100

Bacterial Isolates and culture media

A total of four American Type Culture Collection (ATCC) (originally from ATCC, Virginia, USA) strains were used for testing antimicrobial activity. An additional five clinical strains and three ATCC reference strains were also used for the antibiofilm assay. All strains were maintained in glycerol and stored at low temperature. Strains were subcultured onto suitable medium and grown at 37°C for 24 h before any assay. In order to determine the minimum inhibitory concentration (MIC) against bacterial strains, Tryptic Soy Broth (TSB) was used, whereas brain heart infusion (BHI) broth supplemented with 2% sucrose was used for biofilm formation and antibiofilm activity assays.

MIC

The MIC values of SALM, SALE, and SALA extracts were determined using the serial microdilution method [31]. Briefly, 100 µL of extracts from the stock solution (10 mg/ml) was added to the first row of a sterile 96-well polystyrene flat-bottom microtiter plate. Two-fold serial dilution was performed using the first well-containing stock solution after the addition of 50 µL of TSB to the remaining wells. Overnight bacterial culture developed from storage were re-suspended in TSB and incubated at 37°C for 3 h. 50 µL of bacterial cells (1.0–1.5×10^6 CFU/ml) were added to serially diluted wells of a microtiter plate, thereby making a final volume of 100 µL each. Two-fold dilutions of gentamycin and chloramphenicol were also included as positive controls. Bacterial cultures in TSB devoid of extracts were used as negative control. Wells filled with TSB alone were the blank reference. After incubation at 37°C for 24 h [32], to each well was added 40 µL of 0.2 mg/ml of p-iodonitrotetrazolium violet salts solution (INT). The microtiter plates were allowed to stand at 37°C for 30 min, and results were recorded. The formation of blue color on the wells indicates the presence of viable cells [33]. The MIC of extracts was determined visually as the lowest concentration which inhibited the bacterial growth. Each bioassay was carried out in triplicate.

Biofilm phenotypic assay

Biofilm production was determined using the standard Crystal Violet (CV) assay [34]. A total of 20 bacterial strains including 3 ATCC reference strains from fresh agar plates were inoculated into BHI broth (supplemented with 2% sucrose) and incubated for 18 h at 37°C in a stationary condition and diluted 1 in 100 with fresh medium. Individual wells of sterile, polystyrene, 96-well; flat-bottom microtiter plates were filled with 0.2 ml aliquots of the diluted cultures (broth only served as the control). The microtiter plates were incubated for 18–24 h at 37°C. After incubation, the plates were tapped gently to remove the content of each well. In order to remove the free-floating "planktonic" bacteria, the wells were washed 4 times with 0.2 ml of phosphate-buffered saline. Sodium acetate (2%) was used for fixing the biofilm formed by adherent "sessile" organisms in the plates. The plates were then stained with CV (0.1% w/v), and the excess stain was washed off thoroughly using deionized water. The plates were then allowed to dry. The quantification of biofilm production was carried out by the addition of 150 µL of 95% ethanol to each of the wells. After keeping the plates at room temperature for 30 min, the Optical Density (OD) of the stained adherent bacterial biofilms was read using a plate reader at 570 nm. The experiment was performed in triplicate. The average OD value of test organisms and controls was calculated, and the standard deviation above negative control (OD at 570 nm) was determined as cutoff value (ODc). The isolates were further divided in to non-biofilm producer = OD ≤ ODc (0), weak biofilm producer = ODc < OD ≤2 × ODc (+ or 1), moderate biofilm producer = 2 × ODc < OD ≤ 4 × ODc (++ or 2), and strong biofilm producer = 4 × ODc < OD (+++ or 3).

Effect of plant extract on biofilm formation

The activity of SALM, SALE, and SALA extracts on inhibition of bacterial biofilm was performed using microtiter plate method modified from Stepanović *et al.*, and O'Toole and Kolter [34,35]. Two-fold dilutions of SALM, SALE, and SALA extract stock solutions (10 mg/ml) were made in 96-well microtiter plates containing 50 µL of BHI broth (supplemented with 2% sucrose) in all wells except the first well of each row. Growth controls (cells + BHI), media control (BHI only), and blank control (plant extract + BHI) were also maintained. The plates were then incubated at 37°C for 48 h. After incubation, the plates were subjected to CV assay as described above. The assay was carried out in triplicate. The percentage of specific biofilm inhibition [36] was calculated using the following formula:

Biofilm inhibition percentage = [(OD Growth control – OD test sample)/OD Growth control]× 100

RESULTS

Qualitative phytochemical analysis

The qualitative phytochemical analysis of SALM, SALE, and SALA extracts showed the presence of various compounds, as listed in Table 1.

GC-MS analysis

Phytochemicals present in SALE, and SALM extracts was identified and matched using NIST 11 database and the Wiley 8 library. The names, peak area percentages, and biological activities of major phytochemicals present in SALM and SALE are listed in Fig. 1 and Table 2.

LC-MS

The total ion chromatogram of SALA extract is shown in Fig. 2. LC-MS analysis of SALA extract revealed the presence of several bioactive compounds. Further studies are needed to identify their structure and properties.

Table 1: Phytochemical analysis

Name of test	SALM	SALE	SALA
Carbohydrates	+	+	+
Proteins	+	+	+
Tannins	+	+	+
Saponins	+	-	+
Alkaloids	+	+	+
Flavonoids	+	+	+
Terpenoids	+	-	+
Glycosides	+	+	+
Phenols	+	+	+
Steroids	–	+	+

+: For the presence; –: For the absence

Antioxidant activity

From the results, it has been noted that the free radical-scavenging activity of SALM, SALE, and SALA is concentration dependent. The results also show that SALM, SALE, and SALA possess potent antioxidant activity (Fig. 3).

MIC of SALM, SALE, and SALA

The MIC values of SALM, SALE, and SALA were calculated to evaluate their effect on four ATCC strains (*Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Proteus vulgaris* NCIM 2027). SALM, SALE, and SALA were found to have a MIC value between 1.25 mg/ml and 5 mg/ml (Table 3). Plant extract at a concentration of 5 mg/ml was defined as the lowest concentration on inhibiting the growth of strains after incubation when compared to control. Chloramphenicol and gentamicin were used as reference antibiotics for Gram positive and Gram negative bacteria, respectively.

Biofilm production by microtiter plate method

All the 20 bacterial isolates were subjected to biofilm production on microtiter plates. After incubation, ODc values were determined. Of 17 clinical isolates, 2 strong biofilm producers (*Acinetobacter baumannii* and *Pseudomonas stutzeri*) and 3 moderate biofilm producers (methicillin-resistant *S. aureus*, Coagulase-negative *Staphylococcus*, and *Bacillus* species) were identified. Rest of the isolates were nonbiofilm producers. Among the 3 ATCC strains, 2 were strong (*E. coli* ATCC 25922, *S. aureus* ATCC 25923) and remaining one was a weak biofilm producer (*P. aeruginosa* ATCC 27853). Of 23 isolates subjected to biofilm production, 8 were selected for further studies (Table 4).

Antibiofilm activity of SALM, SALE, and SALA

The effect of plant extracts on biofilm inhibition was carried out in a microtiter plate. All three extracts were able to decrease biofilm

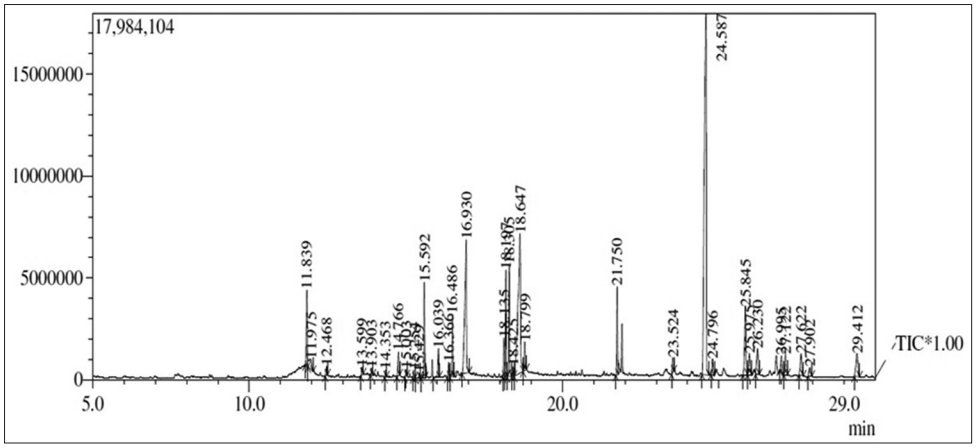

Fig. 1a: Gas chromatography-mass spectroscopic analysis of methanolic extracts of *Sauropus androgynus* leaves

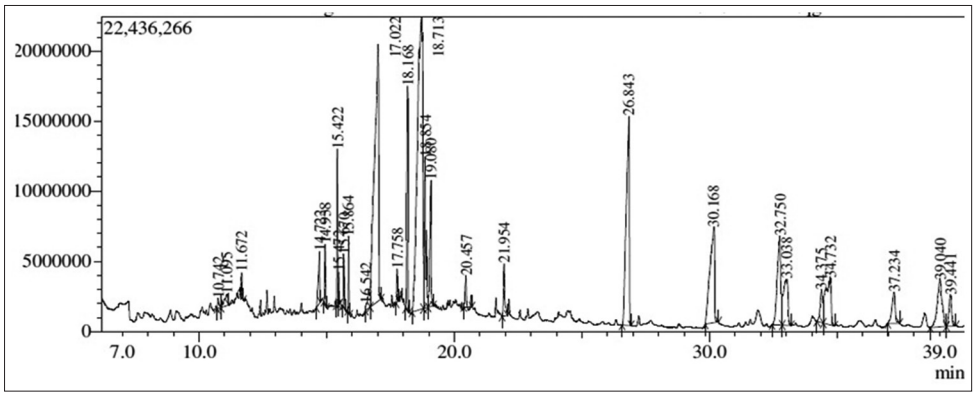

Fig. 1b: Gas chromatography-mass spectroscopic analysis of ethanolic extract of *Sauropus androgynus* leaves

Table 2: Gas chromatography-mass spectroscopic analysis of SALM and SALE

SALM				
S. No	**Retention time (min)**	**Compound name**	**Area%**	**Biological activity [37-57]**
1	24.587	Squalene	33.99	Antibacterial, antioxidant, anticancer, chemopreventive, immunostimulant, lipoxygenase inhibitor
2	18.647	Cis, cis, cis-7,10,13-Hexadecatrienal	14.95	Antioxidant activity
3	16.93	Hexadecanoic Acid	10.06	Antibacterial, nematicide, pesticide, lubricant, antiandrogenic, hemolytic 5-alpha reductase inhibitor, anti-inflammatory, phospholipase inhibitor
4	25.845	Solanesol	4.09	Antimicrobial, antihypersensitive, cardiac stimulant and lipid antioxidant, antitumor, anti-inflammatory, antiulcer
5	18.305	Phytol	3.97	Antimicrobial, anti-inflammatory, anticancer, antidiuretic, immunostimulatory, antidiabetic
6	15.592	Neophytadiene	2.92	Bactericidal, antifungal, antipyretic, analgesic, antioxidant, antihelminthic
7	11.839	Phenol, 2,4-Bis (1,1-Dimethylethyl)-	2.74	Antibacterial, antioxidant
SALE				
1	18.713	9,12,15-Octadecatrienoic acid, (Z, Z, Z)	25.43	Anti-inflammatory, antifungal, antiacne, antihistaminic, antieczemic, pesticide, antiarthritic, hypocholesterolemic, nematicide
2	17.022	Hexadecanoic acid, ethyl ester	17.25	Antimicrobial, antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic, hemolytic, alpha reductase inhibitor.
3	26.843	Squalene	10.19	Antibacterial, antioxidant, antitumor, chemopreventive, immunostimulant, lipoxygenase inhibitor.
4	30.168	Delta.-tocopherol	7.08	Vitamin, antioxidant, anti-inflammatory
5	32.75	Gamma.-Tocopherol	5.15	Antioxidant, antitumor, anti-inflammatory, hypocholesterolemic, cardioprotective.
6	18.854	Octadecanoic acid	3.68	Antimicrobial

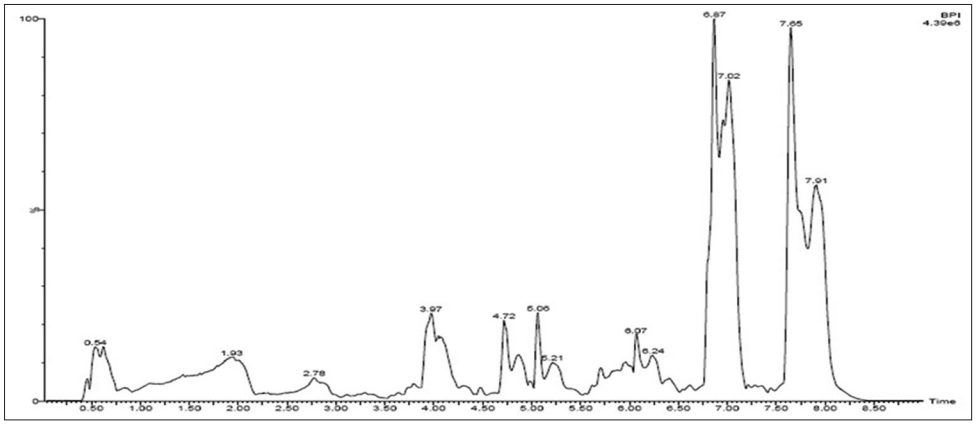

Fig. 2a: Liquid chromatography-mass spectrometry chromatographic profiles of SALA extract - Positive ion mode

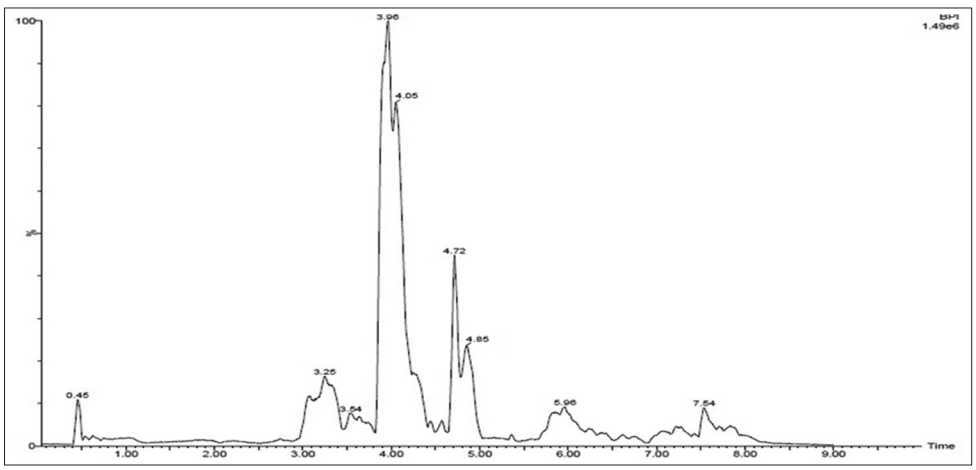

Fig. 2b: liquid chromatography-mass spectrometry chromatographic profiles of SALA extract - negative ion mode

production. Biofilm inhibition percentage of each extract against bacterial strains was calculated. It was found that all of the extracts were able to inhibit biofilm production. Among the extracts, SALM was found to possess the highest biofilm inhibition percentage against all biofilm producers (Fig. 4).

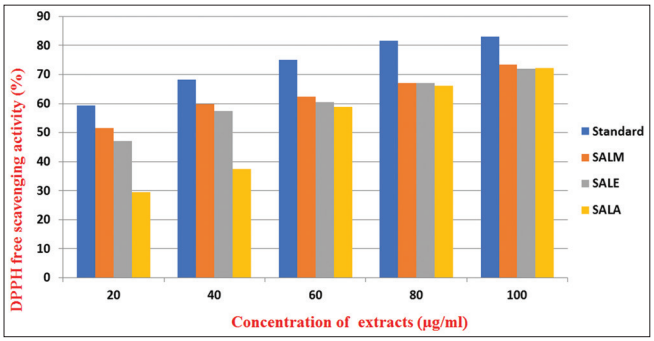

Fig. 3: 2,2-diphenyl-1-picrylhydrazyl activity of plant extracts at different concentrations

DISCUSSION

Plants are widely used to control common health complications as they possess several effective organic molecules called secondary metabolites. These bioactive compounds exhibit various pharmacological properties and are responsible for the medicinal activity of plants [58]. All the three extracts were found to have phytochemicals such as tannins, flavonoids, alkaloids, phenols, and steroids. The phytochemical tannins which are known for their astringent actions were identified in the plant extract. They are found to possess antimicrobial, antibacterial, antitumor, and antiviral activities [59-62]. GC-MS analysis showed that at least 15 compounds are present in both SALM and SALE. Squalene was the major compound identified in SALM extract whereas 9, 12, 15-Octadecatrienoic acid, (Z, Z, Z) is the highest compound identified on SALE. Various compounds identified on GC-MS analysis are known

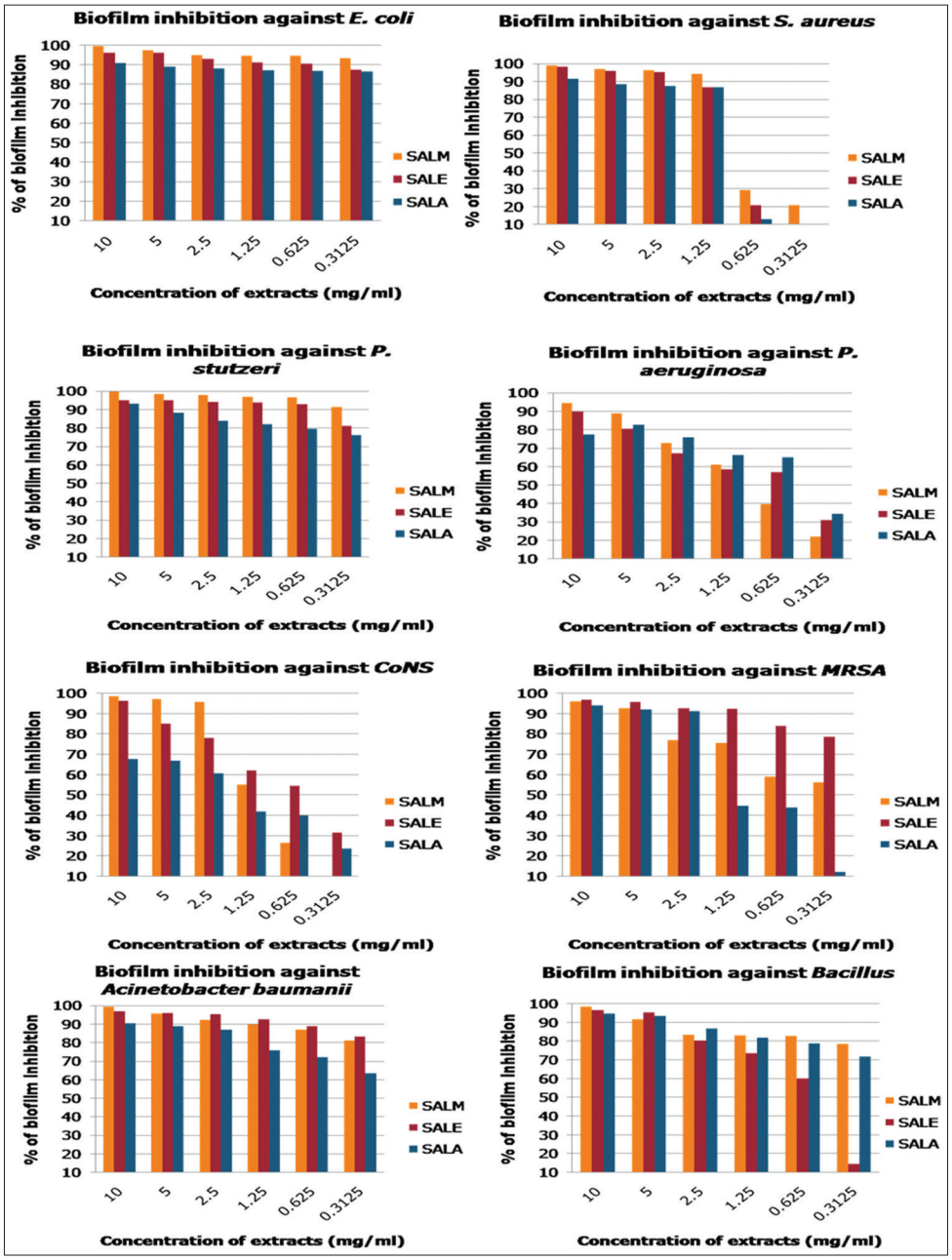

Fig. 4: Biofilm inhibition percentage of various extracts against bacterial strains

Table 3: MIC of plant extracts against bacterial strains

S.No.	Bacterial strains	MIC (mg/ml)				
		SALM	SALE	SALA	Chloramphenicol	Gentamicin
1	*S. aureus* ATCC 25923	5	5	5	0.625	-
2	*P. aeruginosa* ATCC 27853	1.25	1.25	1.25	-	ND*
3	*E. coli* ATCC 25922	1.25	1.25	1.25	-	ND*
4	*P. vulgaris* NCIM 2027	1.25	1.25	1.25	-	0.625

*ND: Not determined, MIC: Minimum inhibitory concentration, *S. aureus: Staphylococcus aureus, P. aeruginosa: Pseudomonas aeruginosa, E. coli: Escherichia coli, P. vulgaris: Proteus vulgaris*

Table 4: Biofilm production on a microtiter plate

Isolates	Nonbiofilm producer n (%)	Weak biofilm producer n (%)	Moderate biofilm producer n (%)	Strong biofilm producer n (%)
n=20	12 (60)	1 (5)	3 (15)	4 (20)

to possess antimicrobial, anticancer, anti-inflammatory, antifungal, antioxidant, and immunomodulatory properties [63,64]. In the current study, leaf extracts of *S. androgynus* exhibited both antimicrobial and antibiofilm activity. Bioactive compounds identified in SALM and SALE was found to be similar to those reported from previous studies [47]. Several studies reported the potential antioxidant activity of *S. androgynus* as well as its high flavonoid content [47,65,66]. All of the extracts were found to exhibit significant antioxidant properties compared to the standard. Among the extracts, SALM was found to have high antioxidant content which matches the findings of Badami and Channabasavaraj *et al.* [66]. Another experiment on DPPH scavenging activities of *S. androgynus* aqueous extract was found to have high polyphenol content and antioxidant activity [67]. The antibacterial activity of extracts could be due to the presence of multivitamins, peptides, glycosides, alkaloids, saponins, terpenoids, and flavonoids [68]. In the present study, antimicrobial activities of the extracts are in concordance with the findings of previous studies [47,69]. Furthermore, the MIC values of plant extract SALM, SALE, and SALA obtained against pathogenic strains at a concentration of 1.25–5 mg/ml were considered a promising result. Among the plant extracts screened, SALM was found to be a potential biofilm inhibitor compared to other extracts. SALM extracts were found to exhibit biofilm inhibition of 80–98% against pathogenic bacterial strains. Previously published studies have reported 80% biofilm inhibition at 100 µg/ml using marine actinomycetes (CAA-3) [70]. Antibiofilm activity of *S. androgynus* has not been reported using methanolic, ethanolic, and aqueous extracts. We report the first study on the antibiofilm activity of SALM, SALE, and SALA against pathogenic strains.

CONCLUSION

Plants are excellent sources of bioactive and phenolic compounds, and they can be exploited for beneficial uses. SALM, SALE, and SALA which contain essential compounds can be utilized for antimicrobial and antibiofilm activities. Antipathogenic therapy which targets only the biofilm and its associated virulence rather than harming the growth of pathogen is an alternative strategy for currently prevailing antimicrobial therapy. These findings justify the consideration of *S. androgynus* plant extracts as a potential natural source of products for not only preventing biofilm formation but also for use as an antiseptic (or topical) in the prophylaxis and treatment of skin/wound infections.

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AUTHORS CONTRIBUTION

Sujitha Kuttinath: All fieldwork, laboratory experiments, and preparation of documents. Haritha KH: Supporting laboratory experiments. Ram Rammohan: Guide, experimental designs, troubleshooting, preparation of the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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