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Scanning electron microscopic evaluation with emphasis on *in vitro* antimicrobial activity of *Simarouba glauca* leaf extract against pathogenic bacterial strains

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Abstract

Herbal medicines have attracted much public attention due to its structurally diverse bioactive compounds which have promising antibacterial, antifungal, antitumor and cytotoxic activities and *Simarouba glauca* is a medicinal plant belonging to the family Simaroubaceae. The bark extract of *S. glauca* is eminent for pharmacological uses such as antidiarrhea, antidysenteric, antimalaria haemostatic, antihelminthic, antiparasitic and antipyretic. But their leaves also can be used as medicine. In this study we show that this plant is highly efficient against pathogenic strains of bacteria. Dried plant leaves of the plant were extracted with various non-polar and polar solvents including hexane, chloroform, ethyl acetate, 70% acetone, methanol and water. The plant extracts were subjected to preliminary phytochemical analysis. Then the extracts were used for the antibacterial studies using disc diffusion method and minimum inhibitory concentration (MIC). With the MIC concentration the scanning electron microscopy was done to see whether the cells destroying or reducing its number. Phenolic content of extracts was estimated and methanol extract showed the highest of 95.32 ± 1.6 mg/g. The antioxidant activity of the samples was evaluated by free radical scavenging assay and the IC₅₀ of methanol extract was found to be 10 µg. Zone of inhibition of plant extract were tested against pathogenic strains like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhimurium*, *Escherichia coli*, and *Proteus vulgaris*. The minimum inhibitory concentration of each extract was determined for each sample. Scanning electron microscopy of MIC concentration was performed with each bacteria mentioned above to confirm the destruction of the bacterial cell wall as well as the cytoplasm. *S. glauca* methanolic extract could replace the conventional drugs used as antibacterial drugs.

Keywords: Antibacterial activity, Scanning electron microscopy, *Simarouba glauca*,

1. Introduction

The major issues of scientists face against the fight with contagious diseases are the evolution of phenomena called resistance to the agents used to control them. There has been remarkable improvement in the prevention, control and even eradication of infectious diseases with better hygiene and development of antimicrobials and vaccines. However, infectious diseases still endure and are a leading cause of global disease burden with high morbidity and mortality, especially in the developing world.

Herbal medicines have attracted much public attention due to its structurally diverse bioactive compounds which have promising antibacterial, antifungal, antitumor and cytotoxic activities [1, 4]. These properties can be exploited for the development of more effective and safe antimicrobial agents which has stimulated multidisciplinary investigations on plant-derived compounds [5, 3]. One such plant is *Simarouba glauca* DC commonly known as Lakshmi Taru in India, which is a flowering tree, native to Florida. It is an evergreen, polygamodioecious, oil seed crop native of tropical rainforest regions of South and Central America and was first introduced to India in 1966 [6].

Simarouba glauca belongs to the family *Simaroubaceae* with more than 170 species. Quassinoids are one of taxonomic marker of this family. The bark extract is important and for pharmacological uses and is reported to control diarrhea, dysenteric, malaria with haemostatic, antihelminthic, antiparasitic, antipyretic and anticancerous properties [7, 8]. The phytochemicals present in leaf, fruit, pulp and seed of *S. glauca* are well known to possess medicinal properties such as antimicrobial, antianalgesic, antiviral, astringent and needed as a stomach tonic, emmenagogue and vermifuge [7, 9]. Quassinoids from *S. glauca* seed including glaucarubin, glaucarubinone, glaucarubol and glaucarubolone have exhibited *in vitro* cytotoxic activity against KB cells (human oral epidermoid carcinoma), [10].

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The esters of glaucarubolone, ailanthinone and glaucarubinone, show significant activity *in vivo* in the P388 lymphocytic leukemia model [11]. There are also a few reports of antimicrobial activity of *S. glauca* crude extracts [12, 13]. Petroleum ether and ethyl acetate extracts did not show any antibacterial properties in *S. glauca*, [9].

The objective of the present study is to establish the antibacterial property of methanol extract of *Simarouba glauca* leaves as potential new agent for controlling bacterial pathogens.

2. Materials and methods

Chemicals and Reagents

1,1-Diphenyl -2-picryl hydrazyl, gallic acid, ascorbic acid and methanol (HPLC grade) were purchased from Sigma-Aldrich. Sterile discs and other reagents were procured from Hi-media.

2.1. Preparation of plant material and extraction

Fresh and healthy plant leaves were collected from Kochi, Kerala. A specimen for the plant was submitted to Jawaharlal Nehru Tropical Botanical Garden Research Institute, Palode, Thiruvananthapuram, Kerala, identified and authenticated by Dr. E.S. Santhosh Kumar and submitted to Herbarium collection. (Herbarium Number TBGT 82861, submitted on 26.04.17).

Oven dried (40 °C) plant material (10 g) was crushed and powdered in an electric blender and extracted in hexane (100 ml), chloroform (100 ml), ethyl acetate (100 ml), methanol (100 ml), water (100 ml), and 70% acetone (100 ml) by agitation on rotary shaker (110 rpm) with the solvent for one week at 37°C. It is filtered and centrifuged (5000×g for 15 min), and dried to powder by lyophilization. The yield was calculated as% of yield = weight of the extract/dry weight x 100. The extracts were stored in the cold room (at 4 °C) for further use.

2.2 Test microorganisms

Bacterial cultures were procured from National Centre for Industrial Microorganisms (NCIM, Pune, India). Strains used in the study included *Staphylococcus aureus* NCIM 2127, *Pseudomonas aeruginosa* NCIM 2863, *Klebsiella pneumonia* NCIM 2957, *Salmonella typhimurium* NCIM 2501, *Escherichia coli* NCIM 2343 and *Proteus vulgaris* NCIM 2027. The strains were chosen based on their clinical and pharmacological importance. The bacterial cultures were grown in Muller Hinton agar and broth for 24 hours at 37°C and stored in glycerol in the cold room (at 4°C) for further use.

2.3 Antimicrobial activity by disc diffusion method

In vitro antimicrobial activity of the methanol extract was investigated by the disc diffusion method. The plant extract was diluted in 10% DMSO to get 1, 0.1, 0.01, 0.001 and 0.0001 mg/ml concentrations and serially diluted with MH broth. For the test, 6 mm sterile discs were impregnated with 20 µl of plant extract on the MH agar plates. Standard inoculum of 5×10^8 cfu/ml, of each selected bacterium was used for the study. 30 µg/ml Cefotaxime and 10% DMSO were used as positive and negative control respectively. The zone of inhibitions was measured after 24 hours of incubation at 37°C [14].

2.4 Minimum inhibitory concentration (MIC)

Dried plant extract of 1, 0.1, 0.01, 0.001 and 0.0001 mg/ml concentrations was serially diluted in 10% DMSO and MH

broth medium in 96 well plates and incubated with 10 µl microbial cultures. The minimum inhibitory concentration was determined by resazurin method [15]. Each plate had a set of cefotaxime (0.31- 1.5 µg/ml) and 10% DMSO as positive and negative controls. The tests were performed in triplicates.

2.5 Scanning Electron Microscope, SEM

Microbial culture was allowed to grow on a coverslip and subsequently fixed with 2.5% glutaraldehyde (Hi-media) for SEM analysis (JEOL Model JSM - 6390LV).

2.6 Qualitative analysis of phytochemicals

A preliminary phytochemical screening of the plant extracts were carried out for the presence of bioactive constituents of the plant by using standard methods as briefly described in [16].

2.7 Determination of Total Phenolic Content

Total phenolic content was estimated using Folin – Ciocalteu's reagent method. Total content of phenolic compounds of extracts was expressed in gallic acid equivalents (GAE) milligrams gallic acid per gram dry extract and was calculated by the following formula $C = c.V/m$, where,

- C= Total content of phenolic compounds, milligrams per gram plant extract, in GAE,
 c = Concentration of gallic acid established from the calibration curve, milligrams per ml
 V= volume of extract, milliliters
 M= weight of pure plant methanol extract (grams) [17].

2.8 DPPH (1, 1-Diphenyl -2-picryl hydrazyl) scavenging assay

The antioxidant activity was determined by DPPH method. Standard ascorbic acid was used as control. IC50 values were calculated from graph. Percentage of scavenging was calculated by $[AC - AT/ AC] \times 100$ where AC is the absorbance of control and AT is absorbance of test [18]. Assay was performed in triplicates.

2.9 Statistical Analysis

All the experiments were performed in triplicate and the results were statistically analysed. Analysis of variance was performed using two-way ANOVA and the significant differences ($p < 0.05$) between the means were performed.

3. Results and discussion:

3.1 Extract yield and total phenolic content

Simarouba glauca leaves were extracted with n-Hexane, Chloroform, Ethyl acetate, 70% Acetone, Methanol and Water. The highest yield was found in 70% acetone, 37.1% and lowest yield was found in water, 1.1% (Table 1), values have significant variation ($p < 0.05$). Total phenolics content of *S. glauca* extracts (expressed terms of gallic acid equivalence) were solvent dependent. Methanol extract had the highest content of 94.32 ± 1.6 GAE mg/g and lowest was in chloroform extract 06.12 ± 1.8 mg/g (Table 1). Eloff [19] reported that methanol was the most effective solvent for plant extraction compared to ethanol, n-hexane and water. Variations in the yield of different solvents used (Table 1) might be due to the differential solubility of the constituents of the leaves in these solvents.

Table 1: Extract Yield (%) and total phenolics with respect to Gallic acid equivalence

Sample	Solvent	Extract yield (%)	GAE mg/ g
<i>Simarouba glauca</i> leaves	Hexane	9.7 ± 1.7	8.49 ± 2.1
	Ethyl acetate	1.1 ± 2.2	51.12 ± 2.3
	Chloroform	8.9 ± 1.8	06.12 ± 1.8
	Methanol	12.3 ± 2.6	94.32 ± 1.6
	70% Acetone	25.8 ± 2.4	93.24 ± 2.1
	H ₂ O	17.1 ± 1.8	92.88 ± 2.0

3.2 Phytochemical Screening

The phytochemical screening of the plant extracts were done and it showed that leaves were rich in alkaloids, flavonoids, tannins and saponins as shown in Table 2, which is possibly

responsible for the medicinal property as well as the physiological activity [16]. The methanol extract exhibited higher amount of terpenoids and cardiac glycerides but it lacks saponins.

Table 2: Qualitative screening of phytochemicals in *Simarouba glauca* leaves extracted in various solvents, + indicates the intensity of the colour

S. No	Name of the constituent	Hexane	Ethyl acetate	Chloroform	Methanol	70% Acetone	Water
1	Proteins	+	+	-	+	-	+
2	Flavonoids	-	+	-	++	++	+
3	Tannins	-	+	-	++	++	++
4	Steroid	-	-	-	-	+	-
5	Terpenoids	-	+	-	+++	++	++
6	Saponins	-	-	+	-	+	+
7	Cardiac glycerides	++	-	+	+++	++	+
8	Alkaloids	++	++	++	++	+	+
9	Oil	+	-	+	-	-	-

Note: + shows the intensity of colour and – shows the absence of the colour

3.3 Free Radical Scavenging

In DPPH assay, IC₅₀ value of the extract was calculated by plotting graph of % scavenging v/s concentration of the extract and found to be 10 µg/ml as the IC₅₀ (Figure 1 (b)), as opposed to that of ascorbic acid (IC₅₀ 20 µg/ml) (Figure 1 a), which is much efficient antioxidant.

The result indicates that the plant extract may have an inherent compensatory mechanism against oxidative stress conferred tolerance to the plant causing damage and may be

associated with their ability to remove antioxidant species. The presence of phenolic compounds and radical scavenging activities are positively correlated which is in agreement with Zhang and Hamazu [20]. There are several studies which have reported a strong and significant correlation between the scavenging activity and total phenolic compound, as well as the flavonoid content and its significant contribution toward the total antioxidant activity [21].

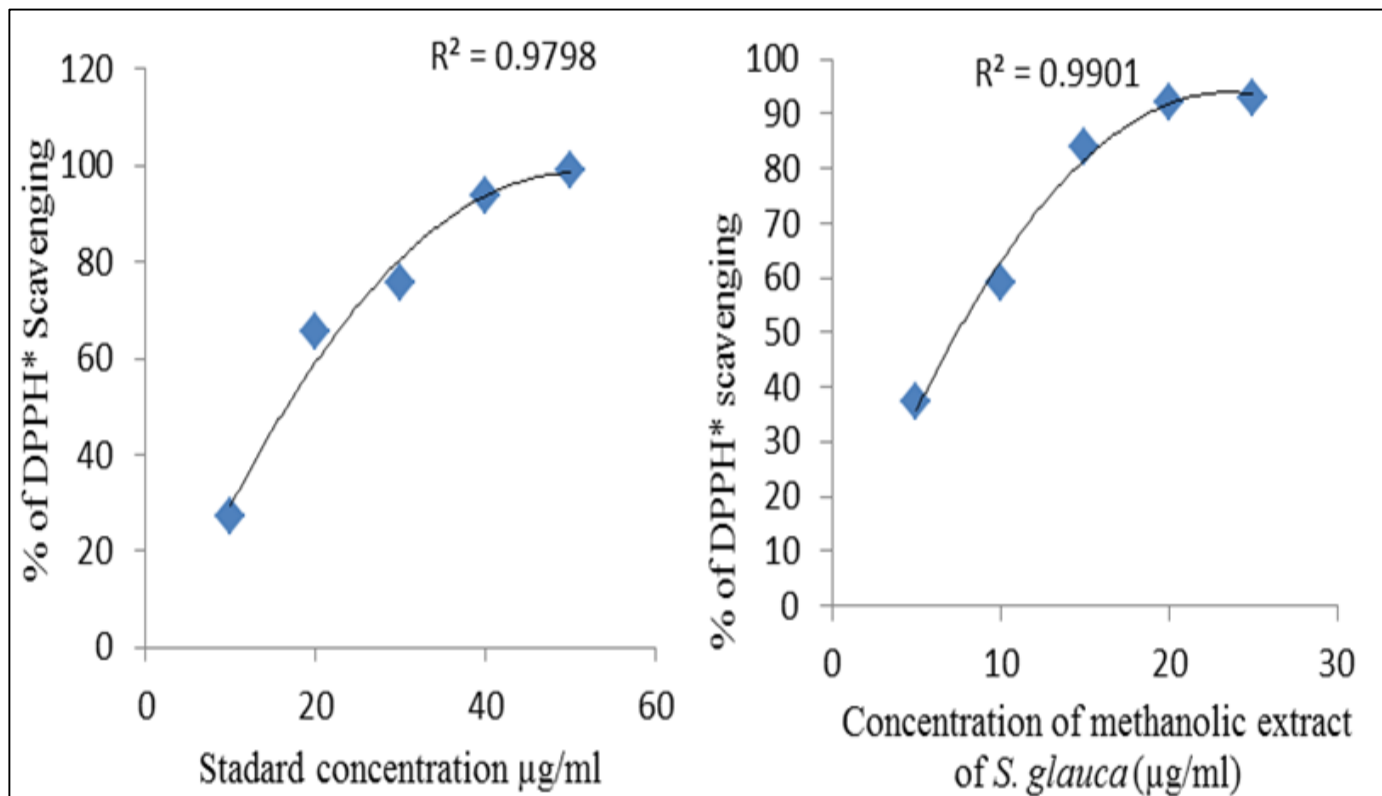


Fig 1: DPPH radical scavenging (a) Standard- standard ascorbic acid and (b) the plant extract, values were represented as mean, R² showing the correlation

3.4 Disc Diffusion method for Antibacterial activity

The *in vitro* antibacterial activity of the methanol extract of *S. glauca*, against the selected bacteria was qualitatively measured based on the presence or absence of inhibition zones. Results of antibacterial activity of *S. glauca* methanol extracts are presented in Table 3. Methanolic extract exhibited moderate potential for antibacterial activity against *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, *S. typhimurium* and *P. vulgaris* at all concentrations of 4 µg/ml, 20 µg/ml and 40 µg/ml where *E. coli* has showed the activity from 20 µg/ml (Table 3). The control did not inhibit the growth of any of the

tested bacteria. Cefotaxime 5mg/ml used as positive control [22] where 10% DMSO as the negative control. The results are in agreement with Jangale *et al* [12]. There are several reports shown that plant compounds show antioxidant and antibacterial effects against a broad spectrum of microorganisms [23, 26]. For correlation of concentration with the zone of inhibition, 4 µg/ml actively exhibit a zone of inhibition with a minimal concentration for all the test organisms except *E. coli*, which showed a correlation r^2 in percentage, 86% and it is 99% correlated with 40 µg concentration and the values are significant as $p \leq 0.05$.

Table 3: Zone of inhibition in centimeter on varying the bacterial strains and the concentration of the extract

NCIM No.	Bacterial strains	Extract 4 µg (cm)	Extract 20 µg (cm)	Extract 40 µg (cm)	10% DMSO	30 µg /ml Cefotaxime
2957	<i>K. pneumoniae</i>	1.2	1.3	2.0	0	2.2
2863	<i>P. aeruginosa</i>	0.9	1.3	1.4	0	1.9
2343	<i>E. coli</i>	0	1.1	1.3	0	1.7
2127	<i>S. Typhimurium</i>	1.35	1.4	1.67	0	1.9
2501	<i>S. aureus</i>	0.9	1.6	1.8	0	1.9
2027	<i>P. vulgaris</i>	0.8	1.4	1.8	0	2.2

3.5 Minimum Inhibitory Concentration

The Minimum inhibitor concentration (MIC) values varied from 0.16–1.25 µg/ml, respectively for the *S. glauca* methanolic extracts (Table 4). All the six microorganisms used were susceptible to methanolic extract but the MIC was different. MIC 0.16µg/ml value was obtained with *S. typhimurium* and *S. aureus* which was the lowest and most effective concentration among the methanolic extracts. Here, *S. typhimurium* is a gram negative and *S. aureus* is gram positive bacteria. As reported by Nikaido [27] the bacterial membrane allows molecules penetrate and disturb cellular function/ metabolism and loss of cellular constituents, leading their death. Cefotaxime showed MIC of a range 0.31- 1.5

µg/ml against these bacteria which served as a positive control. As per different reports 0.6 ~ 5000 µg/ml plant extracts completely inhibit the growth of the bacteria [28, 29]. Jangale *et al.* [12] reported that methanolic extract of *S. glauca* was effective against for food borne spoilage and pathogenic bacteria. Among the six bacteria tested, *S. aureus* and *S. typhimurium* were more sensitive to *S. glauca* methanolic extract but this is in discordance with Janglae [12] reported that *B. subtilis* was the most sensitive to *S. glauca* methanolic extract. At higher concentrations of methanolic extract with increasing concentrations with different bacteria showed significant results (p value ≤ 0.05).

Table 4: MIC of *S. glauca* for the test microorganisms and standard

S. No	NCIM No.	Bacterial strains used	MIC (µg/ml)	Cefotaxime (µg/ml)
1	2957	<i>K. pneumoniae</i>	1.25	0.62
2	2863	<i>P. aeruginosa</i>	1.25	0.31
3	2343	<i>E. coli</i>	1.25	1.25
4	2127	<i>S. typhimurium</i>	0.156	0.62
5	2501	<i>S. aureus</i>	0.156	1.25
6	2027	<i>P. vulgaris</i>	1.25	0.62

3.6 Scanning Electron Microscopy

Scanning electron microscopy revealed the structural changes when treated with the plant extract. In the control, cells displayed a smooth and intact surface texture (Fig. 1 a-f) where as in the test the texture was morphologically disturbed (Fig. 1 g- l). The treated cells showed deep craters with visible cell debris indicating the ultrastructural damages on both cell wall and cytoplasmic membrane of Gram-positive

and Gram-negative bacteria [30]. The underline mechanism could be as suggested by Miller [31]. When the cells are exposed to medium and plant extract, cells start to swell leading to the opening of mechanosensitive channels in the inner membrane which leads to release water, solutes, and ions into the periplasm and ultimate cell burst due to extreme turgor pressure, and death [31, 34].

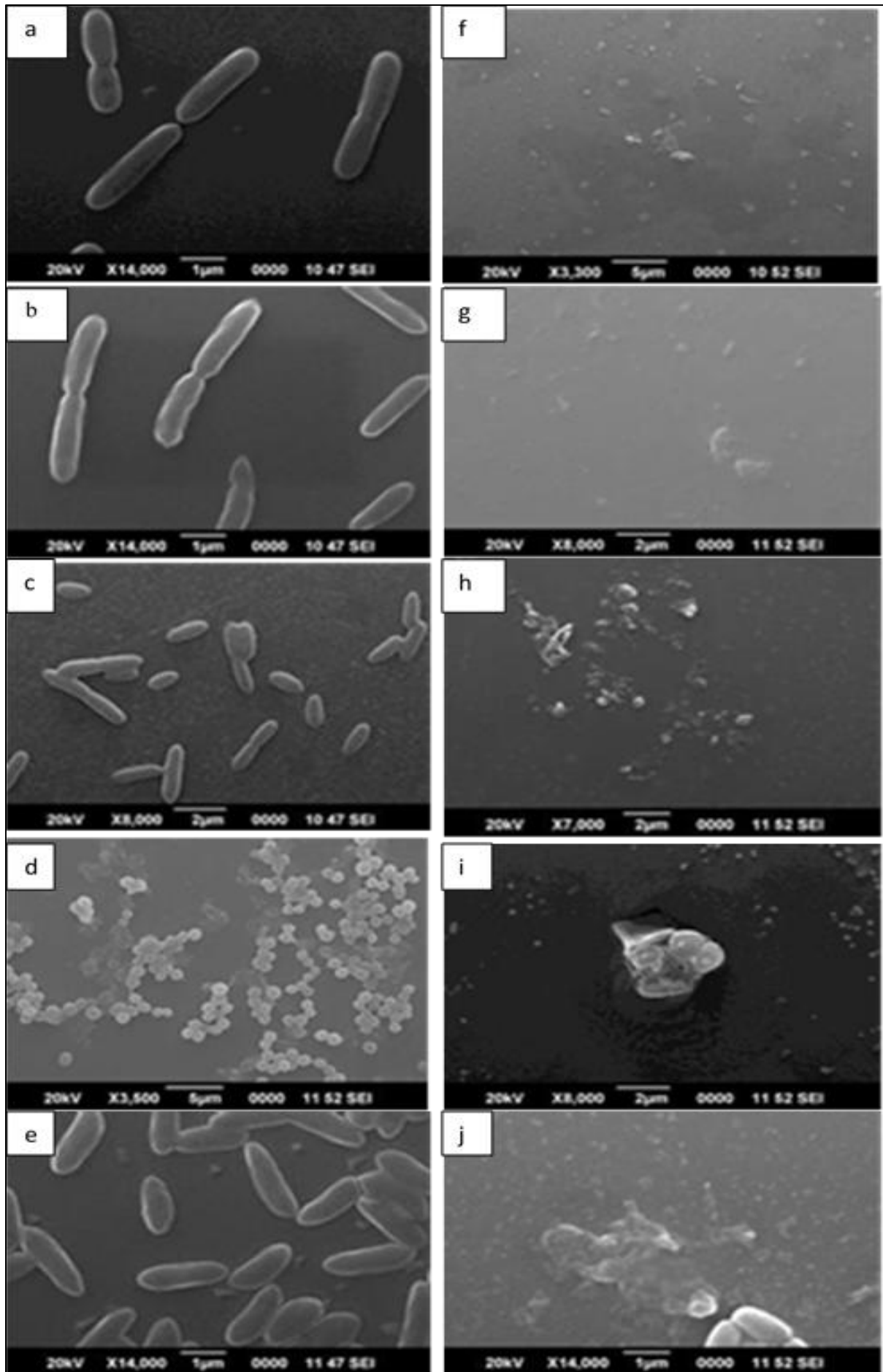


Fig 2 (a-f): SEM micrographs of untreated in (a) *E. coli*, (b) *K. pneumoniae*, (c) *P. aeruginosa*, (d) *P. vulgaris*, (e) *S. aureus*, (f) *S. typhimurium*, and treated in (g) *E. coli*, (h) *K. pneumoniae*, (i) *P. aeruginosa*, (j) *P. vulgaris*, (k) *S. aureus*, (l) *S. typhimurium* In LB medium the cells are long, intact, and evenly shaped (a-f), whereas in treated with plant extract MIC in medium they disrupted (g-l). After treatment, the cells appeared completely disrupted.

4. Conclusions

In the present investigation revealed the potentials for developing antimicrobial pharmaceutical substances from the leaf extract of *S. glauca*. Basic phytochemicals present are confirmed for triterpenoids, polyphenols, tannins and flavonoids. This study demonstrates that SEM images as well as the zone of inhibition are techniques to get insight into the antimicrobial effect of *S. glauca* plant extract. The lack of relevant literature has limited our ability to compare results. The results suggest that dried *S. glauca* leaves extracted with methanol can be used as a potential source of antibacterial drug which could be useful in controlling the growth of various pathogenic bacteria. Further studies are required to identify the compounds.

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7. Compliance with ethical standards

All the authors declare that there is no conflict of interest.

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