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Bioautography, synergistic effect and HPTLC-MS and SEM analysis of antimicrobial and antioxidant compounds of inflorescence extract of *Sphaeranthus indicus*

Dhananjay Tandon[1,2*] and Ashwini Kumar Gupta[1]

Abstract

Background *Sphaeranthus indicus* L. is a well-known medicinal plant in folk medicine. A variety of biological activities and chemical substances in this plant have been reported. The phytochemical content and activity may vary according to geographic location. This study aims to determine the geographical significance, the analysis of the synergistic effect of phytochemicals, the identification of active compounds, and the determination of the action mechanism of *S. indicus* inflorescence methanolic extract against *Staphylococcus aureus* and *Klebsiella pneumonia*.

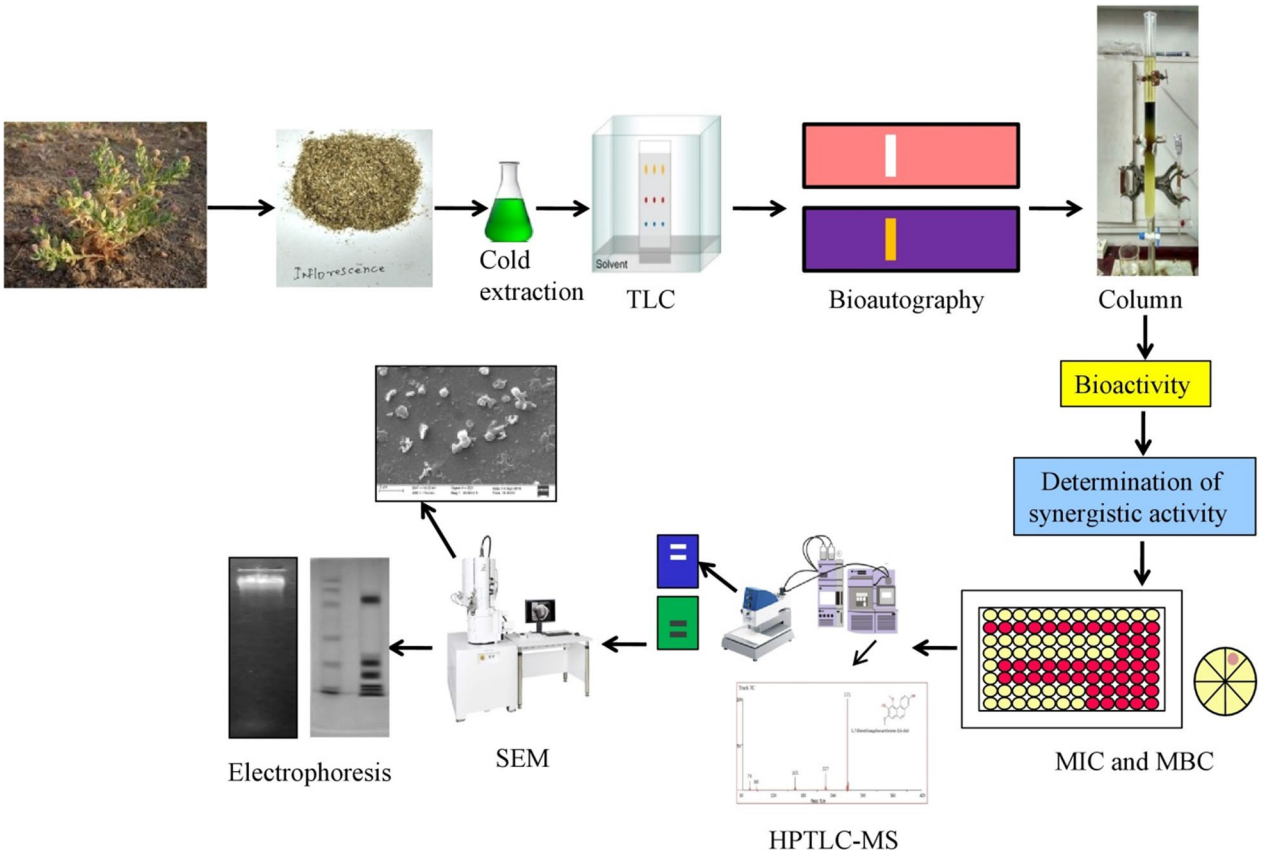
Results The bands with Rf values of 0.92 and 1.0 showed antimicrobial activity, while all bands showed antioxidant activity. The first fraction showed the highest antimicrobial activity, and the pool of the second fraction showed the highest antioxidant activity. The kinetics of the antioxidants differed among the fractions. Analysis of synergistic effects showed that several compounds were involved in the activities. The bands with Rf 0.45, 0.55, 0.68, 0.79, and 0.85 were active components of the extract. Leakage of cell contents was detected at 260 and 280 nm wavelengths. Six different proteins and one nucleic acid band were detected after electrophoresis. The SEM analysis showed that the phytochemicals caused severe membrane damage.

Conclusion The study revealed that the photochemical present in methanol extract of the inflorescence of *S. indicus* has a synergistic effect and acts on bacterial cell envelope. The five compounds were identified as active molecules belonging to the class of terpenoids. The result also signified the geographical area since thymol was identified for the first time in this plant at this location.

Keywords Bioautography, Antimicrobial, Antioxidant, HPTLC-MS, Synergistic effect, Detection of an active compound, Mode of action

*Correspondence:
Dhananjay Tandon
dhananjay.25t@gmail.com
Full list of author information is available at the end of the article

Graphical abstract



Background

Medicinal plants are an issue in the search for new medicines. At least 75% of the world's population uses medicinal plants in conventional home remedies to treat a variety of diseases and infections. Pharmaceutical companies around the world, including India, have discovered cutting-edge herbal medicines. Plants have a wide range of physiologically valuable compounds that can be used as a source of new molecules [1].

The evolution of antimicrobial resistance genes in bacteria is a very important topic that attracts most researchers in the field of phytochemistry. This is due to the fact that it has relatively few negative effects and it is difficult to generate resistant bacteria [2]. In 2020, the Indian Council of Medical Research published data on antibiotic resistance. According to the statistics, the most frequently isolated bacteria from 65,561 samples included *Escherichia coli*, *K. pneumonia*, *Pseudomonas aeruginosa*, *Acinetobactor baumannii*, and *S. aureus*. *Staphylococcus aureus* is one of the bacterial infections that have been found to have increased resistance to most antibiotics over time; however, no such trend was seen in MSSA isolates. When comparing MSSA and MRSA, MSSA was found to be more sensitive to erythromycin, clindamycin, ciprofloxacin, cotrimoxazole, and a high dose of mupirocin [3].

In the second aspect, free radicals are also a major cause for concern since they contribute to the development of many diseases, including hyperglycemia, rheumatoid arthritis, asthma, cataracts, neurological diseases, cancer, and aging [4–6]. When the antioxidant defense system is supported by antioxidants including ascorbic acid, alpha-tocopherol, glutathione (GSH), carotenoids, flavonoids, and antioxidant enzymes like SOD, catalase, and glutathione peroxidase, it is overrun by excessive ROS production, oxidative stress results [7, 8].

It has been observed that microbes are more likely to develop resistance to a single component than to a complex phytochemical mixture. The major phytochemicals

work together to fight the infections. Several components of the system works together to shield the active substance from biotransformation, drug resistance processes, transport across cell membranes, or breakdown by enzymes [9].

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It has been observed that microbes are more likely to develop resistance to a single component than to a complex phytochemical mixture. Most herbal medicines work together to fight infections. Several components of the system act together to protect the drug from biotransformation, drug resistance processes, transport across cell membranes, or degradation by enzymes [9].

Not every secondary metabolite must have a biological effect to be natural. Some of them are capable of doing so, but not all. These substances can be detected by bioautography, fractionation, activity comparisons, and phytochemical analyses using a variety of analytical tools.

A number of secondary metabolites reduce oxidative stress and microbial infection. Terpenoids, phenols, flavonoids, alkaloids, and coumarins are some of the classifications for these substances. The most widespread and structurally diverse group of natural products are the terpenoids, sometimes called isoprenoids. Turpentine hydrocarbons were originally given the general name "terpenes" with the suffix "ene" denoting olefinic boundaries. The isoprene rule categorizes this according to the number and structural arrangement of carbons formed by the linear arrangement of isoprene units, which is followed by the cyclization and rearrangement of the carbon skeleton [10]. Hemiterpenoids, monoterpenoids, iridoids, sesquiterpenoids, diterpenoids, triterpenoids, tetraterpenoids, polyterpenoids, and irregular terpenoids are only a few of the various terpenoids found in plants [11]. Terpenoids cause membrane damage and cellular content leakage [12].

Sphaeranthus indicus is an Asteraceae family member. This plant's applications are described in the text. The plant's entire body has medicinal applications. Folk medicine cures scabies, diarrhea, dysentery, piles, cough, gastric problems, dysuria, jaundice, mouth ulcers, stomachaches, swelling, and dysuria. In addition to these uses, the herb also has antiviral, antibacterial, antifungal, antiprotozoal, anthelmintic, macrofilaricidal, anxiolytic, and neuroprotective actions. From *S. indicus*, 81 chemicals have been isolated and identified [13, 14]. 7-Hydroxyfrullanolide was identified as an antimicrobial compound from *S. indicus* flowers [15, 16].

Geographical location, soil composition, and environmental stress all play a significant role in phytochemical synthesis. Depending on this, phytochemicals' composition and proportion may change [17–19]. Due to its extensive collection of aromatic and medicinal plants in both the plains and numerous tribal districts, Chhattisgarh is known as an "herbal state" [20]. The Chhattisgarh plains, which include numerous valuable medicinal plants, include Raipur. The investigation of bioactive metabolites and the mechanism of action of *S. indicus* inflorescence methanol extract against clinical samples of *S. aureus* and *K. pneumoniae* is the subject of the current study.

Methods

Plant material

Inflorescences were harvested from January to mid-February 2019 from Raipur, Chhattisgarh, India (Fig. 1). The sample was authorized by the professor at the National Center for Natural Resources, Raipur, Chhattisgarh, India. The specimen voucher number is 99,777. The plant material was washed with tap water, sterilized with 0.1% mercuric chloride for 5 min, rinsed with distilled water, and air dried under shade conditions at room temperature. It reached a constant weight and was grounded.

Preparation of an extract

Inflorescence powder (1 kg) was macerated in a 1:10 ratio with methanol at 40 °C for 72 h at room temperature. The extraction was repeated three times until a clear solvent was obtained. The extract was evaporated in a rotary vacuum evaporator at 40 °C. The methanol extract was further fractionated with chloroform and water in a separate funnel to isolate the brown fraction from the green fraction. The green fraction primarily contained chlorophyll and was not used for further analysis. The percentage yield of dried extract was 5.35%. The extract was kept at 4 °C in an airtight brown glass container.

High-performance thin-layer chromatography

HPTLC analysis was carried out following Gomathi et al. [21] with some modifications using the CAMAG system from Anchrom, India. The 10 mg of methanol extract of plant material was dissolved in 2 ml of methanol and filtered through 0.45 µm pore-size filter paper. A 4 µl aliquot of the above test solution was loaded as an 8 mm band width in a 5×10 cm silica gel 60F254 TLC plate (Merck, India) above 10 mm from the edge of the plate using a Hamilton syringe and LINOMAT 5 instrument

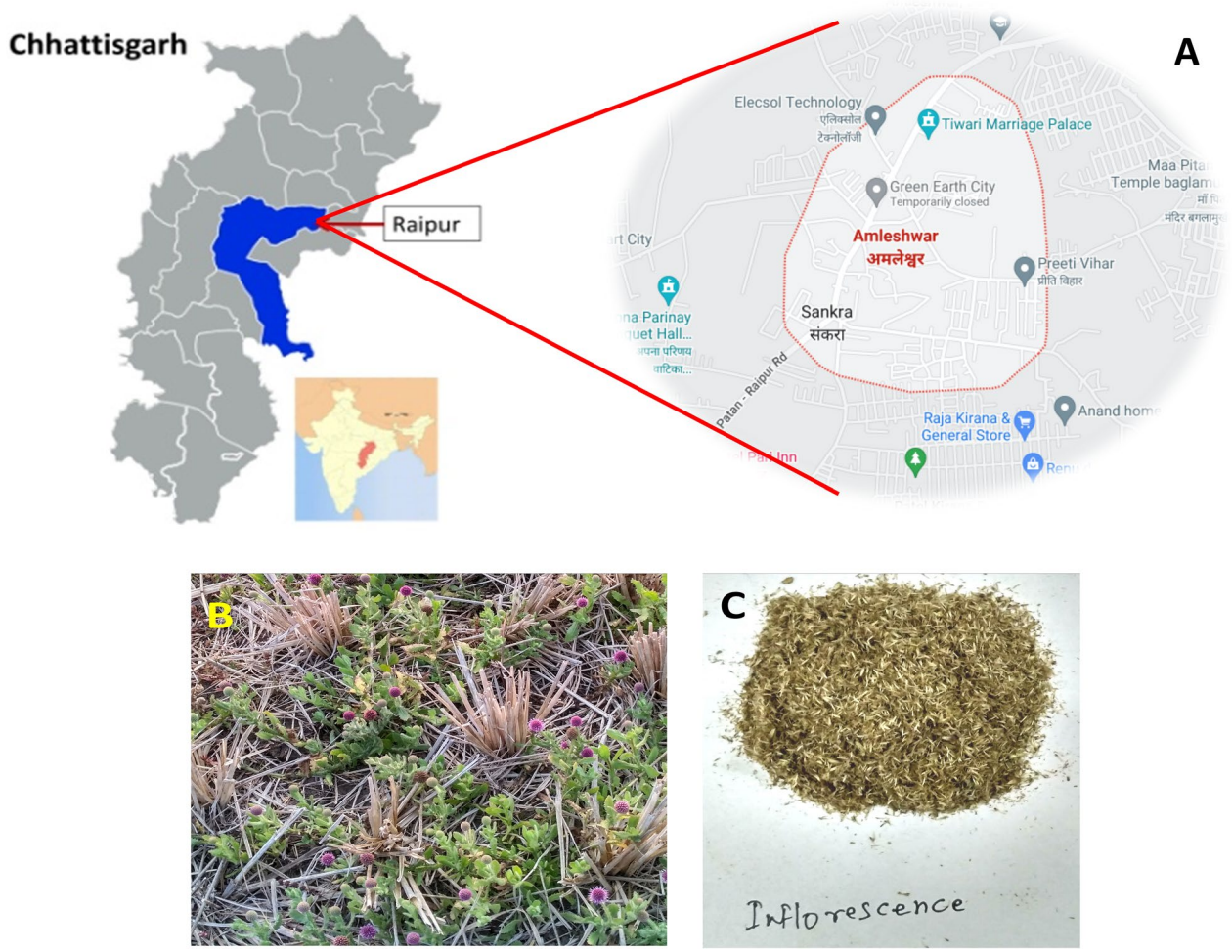

Fig. 1 **A** Location of harvesting site in Raipur, Chhattisgarh state, India (21.205366828509995, 81.58097462427807) The coordination was extracted from Google Maps. **B** The plant is grown in a paddy field, and processed in laboratory (**C**)

with a speed of 150 nl/s. The sample-loaded plate was kept in the TLC twin-trough developing chamber after saturation with the solvent vapor of the mobile phase [Ethyl acetate, Methanol, and Formic acid (100:18:9 v/v)] for 20 min, and the plate was developed up to 90 mm in height. The developed plate was dried with hot air to evaporate solvents from the plate. The plate was kept in the photo-documentation chamber (CAMAG Visualizer) and images were captured under white light and UV light at 254 nm and 366 nm. Densitometric scanning was done at 366 nm by the CAMAG TLC Scanner IV with a scanning speed of 20 mm/s, a data resolution of 25 µm/ step, and a 6×0.45 mm slit. The peak table, peak display, and peak densitogram were recorded.

Detection of the antimicrobial bioactive band(s) by bioautography test

The agar overlay assay described by Choma and Grzelak [22] was applied to bioautographic assays, with minor modifications. The clinical test bacterial samples, Gram-positive *S. aureus,* and Gram-negative *K. pneumonia,* were obtained from the Department of Microbiology, Pt. Jawaharlal Nehru Memorial Medical College, Raipur (Chhattisgarh), India, was used for assessment. *S. aureus* was used as a test strain based on susceptibility testing in previous studies [23, 40]. One milliliter of (1×108 CFU/ml) broth culture was transferred into 10 ml of Mueller–Hinton agar at 45 °C and one milliliter of the culture was poured over the chromatograms as a thin layer immediately. The plate was incubated at 37 °C for 18 h. After incubation, a filter-sterilized 2, 3, and 5-triphenyl tetrazolium chloride (2 mg/ml, HiMedia, India) solution was poured onto the bioautography strip. The strip was incubated at 37 °C for 1 h. The zone of inhibition of bacterial growth could be seen around the active chromatogram spot. The de-colored band was identified, and its Rf values were compared with those of the control chromatogram.

Detection of the antioxidant bioactive band(s) by bioautography test

The procedure was adopted by Cieśla et al. [24]. 50 µM DPPH (Sigma-Aldrich) solutions in methanol were poured on the developed chromatogram as mentioned above and incubated in the dark for 10 min. The bands were detected, and Rf was observed with a control chromatogram.

Extract fractionation by silica gel column

The fractionation of the extract was performed by Pandey and Gupta [25] with modifications. The 5 g extract was adsorbed with silica gel and subjected to a 500×350 mm glass column (Borosil) packed with silica gel (60–120 mesh, Merck). The constant flow rate of elution was 6 ml/min. The column was saturated with ethyl acetate, and elution was done with ethyl acetate-methanol and methanol-formic acid gradients (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100 v/v for each). The fractions were monitored by thin-layer chromatography using the same solvent system as in Section 2.3. The fractions were pooled according to their TLC profiles. A total of 10 fraction pools were obtained. The antimicrobial and antioxidant activities of each fraction pool were assessed.

Fractionation-guided antimicrobial activity

The antibacterial activity of the fractions was determined by the agar-well diffusion method [26]. Inoculums of *S. aureus* and *K. pneumonia* (1×10^8 CFU/ml) were streaked on sterile Mueller–Hinton Agar (Hi-media, India) plates using a sterile cotton swab. 6-mm-diameter wells were bored into the agar plate using a cork borer. The 0.05 ml of fractions dissolved in DMSO were introduced into the well at a concentration of 0.1 g/ml, and the plates were incubated at 37 °C for 18 h. Solvent control was also set up in parallel. The plates were observed for inhibition zones.

Determination of the minimum inhibitory concentration

The broth micro-dilution sensitivity test was performed following CLSI [27] with some modifications to determine the MIC of the fractions. The test was performed in a sterile 96-well microtiter plate (Tarson, India) using Muller-Hinton broth (Hi-Media, India). An inoculum stock (1×10^8 CFU/ml) was diluted in a 1:20 ratio with sterile saline and water to yield 5×106 CFU/ml. In each well, 0.1 ml of Mueller–Hinton broth was taken. The 0.05 ml (100 mg/ml concentration) of the first fraction was taken in the first well. Serial two-fold dilutions were prepared in successive wells and mixed thoroughly to give a final concentration ranging from 100 to 0.049 mg/ml. 0.01 ml of bacterial inoculum was inoculated to yield 5×105 CFU/ml. Appropriate solvents and streptomycin, as standard antibiotics, were used as negative and positive controls, respectively. The tests were repeated. The inoculated microplate was covered and incubated at 37 °C for 18 h. After incubation, 0.05 ml of TTC and a redox indicator were added and incubated at 37 °C for 30 min. Microbial growth was determined without shaking by observing the change in TTC color (pinkish-red formazan when growth occurs and clear solution when none occurs). The lowest concentration of the active fraction that inhibited bacteria growth was called the MIC. For each well showing no growth, 10 µl of broth was subcultured on a nutrient agar plate. This was done to verify the microorganism's growth after overnight incubation. No growth on the solid medium indicated that the specific concentration of the fraction inhibited bacteria.

Fractionation-guided antioxidant activity

Reducing power assay

Antioxidant activity was measured using the ferric-reducing power assay, as described by Oyaizu [28]. 1 ml of fractions (0.2–2.0 mg/ml) was mixed with 2.5 ml of phosphate buffer (0.2 M; pH 6.6) and 2.5 ml of 1% potassium ferricyanide, and the mixture was incubated at 50 °C for 30 min. After incubation, 2.5 ml of 10% TCA was added, and the reaction mixture was cooled to room temperature. 2.5 ml of the above mixture was mixed with 2.5 ml of deionized water (Milli Q, Merk) and 0.5 ml of 1% ferric chloride. The absorbance was recorded at 700 nm against a blank using a spectrophotometer. Ascorbic acid (30–70 µg ml-1) was used as the standard.

DMPD cation free radical scavenging assay

The radical scavenging assay was performed using the DMPD free radical scavenging assay, as described by Asghar et al. [29]. Briefly, the DMPD (Sigma-Aldrich) free oxidant was generated by adding 0.1 ml of DMPD aqueous solution (100 mM) to 0.05 ml of aqueous potassium persulfate (0.4 mM), and the final volume was made to 10 ml with sodium acetate buffer (pH 5.6). The solution was incubated in the dark for 5 h before use until it reached an optical density of 0.7–0.8 at 517.4 nm. The scavenging effect of fractions was measured by the addition of 0.2–2.0 mg/ml of extract into 3.5 ml of DMPD radical. This was incubated for different periods. The radical scavenging activity was expressed as a percentage of DMPD discoloration using the equation:

$$\text{DMPD Radical scavenging (\%)} = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100 \quad (1)$$

where A_{sample} denotes extract or reference compound absorption and A_{control} denotes DMPD solution

absorbance without extract addition. Ascorbic acid was used as the reference compound in the 40–80 µg concentration range. All assays were run in triplicate, and means were calculated. The IC_{50} was calculated using regression analysis. Results were expressed as µg ascorbic acid/ml plant extract. This assay is an end-point measurement assay, and radical absorbance increases after inhibition. Thus, the assay was carried out at a one-minute interval to check the reaction completion time.

DPPH scavenging assay

The DPPH assay was carried out according to Brand-Williams' method [30]. Briefly, a 50 µM DPPH solution was prepared in methanol. The scavenging effect of fractions was measured by the addition of 0.2–2.0 mg/ml of fractions to a 3 ml DPPH solution and incubated for different periods. The absorbance was measured at 517 nm. The radical scavenging activity was expressed as a percentage of DPPH discoloration using the equation:

$$\text{DPPH scavenging (\%)} = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100 \quad (2)$$

where A_{sample} denotes extract or reference compound absorption and A_{control} denotes DPPH solution absorbance without extract addition. Ascorbic acid was used as the reference compound in the 40–80 µg concentration range. All assays were run in triplicate, and means were calculated. The IC_{50} was calculated using regression analysis. Results were expressed as µg ascorbic acid/ml plant extract. The assay was carried out at a one-minute interval to check reaction completion time, and kinetics was done.

Analysis of synergistic effects between phytochemicals in the first fraction pool

The chromatogram of the first fraction pool was developed using the mobile phases chloroform, ethyl acetate, and methanol individually, as well as ethyl acetate and methanol (100:18 v/v) on a TLC plate of 2×10 cm dimension (Merck) to develop different band patterns. Bioautography was performed on each chromatogram for antibacterial activity against clinical *S. aureus*.

Detection of key compound(s) in the first fraction pool of inflorescence methanol extract

The key compound(s) were detected by comparing fraction band patterns and biological activities.

HPTLC-MS of biologically active fractions of S. indicus inflorescence and stem extracts

The experiment was performed following Kroslakova et al. [31]. The first fraction pool was dissolved in 2 ml of chloroform. A 2 µl and 4 µl sample was loaded onto a 100×100 mm silica gel $60F_{254}$ TLC plate in the form of an 8 mm wide band. The chromatography parameter was set to Section 2.3. The developed plate was derivatized in the derivatizing trough with an anisaldehyde sulfuric acid reagent. It was dried at 1000C in a CAMAG hot plate for 3 min, and visualized at 366 nm.

Assessment of membrane damage

The effect of the first fraction on membrane integrity was assessed against clinically isolated *S. aureus* and *K. pneumonia*.

Cell constituent leakage assay

The test was done following Diao et al. [32] with slight modifications. The overnight cultures were subcultured in nutrient broth and incubated at 37 °C with shaking at 150 rpm (Remi, India) for 10 h. Broth culture was harvested by centrifugation at 10,000 rpm for 10 min at 10 °C (Remi, India). The supernatant was discarded, and the precipitated cells were washed twice with sterile PBS (0.1 M, pH 7.4). The cells were resuspended in 2 ml PBS, and cell density was adjusted to an OD of 0.300 at 620 nm. The organisms were treated with different concentrations of the first fraction (1×MIC, 2×MIC, 4×MIC) and incubated at 37 °C for 12 h. The suspension was centrifuged at 13,400 g for 10 min at 10 °C. The control samples were prepared similarly, using untreated organisms and treated with DMSO. The aliquot absorbance was recorded at 260 nm and 280 nm. The aliquot was divided into two parts for protein and nucleic acid leakage determination.

Study of protein leakage by SDS-PAGE

Electrophoretic separation of protein samples was conducted using SDS-PAGE following Katoch [33]. Briefly, the aliquots were treated with chilled acetone at a 1:3 ratios for 24 h at 4 °C. Aliquots were centrifuged at 4 °C for 10 min at 10,000 rpm. The precipitates were dissolved in PBS. In a 1:1 v/v ratio, dissolved protein and sample buffer [0.5 M Tris-HCL, 10% SDS, 2% glycerol, 0.5% bromophenol blue, 10% β-mercaptoethanol] were added. The mixture was heated at 100 °C for 1 min and 20 µl samples were loaded onto 12% SDS-PAGE. Ladder protein was used as a standard. SDS-PAGE was carried out at 60 V for stacking gel and at 100 V for 180 min in resolving gel (Bio-Rad Laboratories, California). Staining was performed using the silver staining method. Briefly, the gel was soaked in methanol, acetic acid, and water (25:15:60 v/v) for 30 min. Then it was transferred to 50% methanol for 30 min. Washed with distilled water for 10 min and then dipped into 20% sodium thiosulfate for one minute. The gel was washed two times with distilled water for one minute. The gel was then transferred to

0.1% silver nitrate for 30 min with the addition of 0.2 ml formaldehyde. The gel was washed two times with distilled water for one minute. The gel was then transferred to a 3% sodium carbonate solution. After color development, the gel was stored in 7% acetic acid and analyzed to detect the release of proteins under the E-Gel Imager (Thermofisher).

Study of nucleic acid leakage by agarose gel electrophoresis

The test was done following Bickley [34]. The 1 ml aliquots were incubated with 0.5 mg/ml RNAase A at 37 °C for 1 h and then with 0.2 mg/ml proteinase K at 50 °C overnight. A phenol extraction of this mixture was performed. DNA in the aqueous phase was precipitated by 0.1 ml ammonium acetate (1/10 vol of 7.5 M) and 1 ml (1 vol) chilled isopropanol. The nucleic acids of both bacterial samples were re-suspended in TAE buffer (pH 8.0). The sample was mixed with a sample buffer (10 ml 1X TAE buffer, 1 mg bromophenol blue, and 1 ml glycerol) at a 1:1 ratio. DNA electrophoresis was performed in a 1% agarose gel containing 1 mg/ml ethidium bromide at 100 V, and DNA was analyzed with an E-Gel imager (Thermofisher).

Scanning electron microscopy (SEM) of a bacterial cell treated with the active fraction of a methanol extract of S. indicus inflorescence extracts.

To determine the efficacy of fractions on cell morphology changes of clinical *S. aureus* and *K. pneumonia*, SEM (Zeiss EVO 18) observation was performed on the tested bacteria using low vacuum following Wang et al. [35]. One milliliter of overnight culture was inoculated into sterile autoclaved 40 ml nutrient broth and incubated at 37 °C for 10 h. Cultures were harvested by centrifugation at 10,000 rpm for 10 min at 10 °C and washed twice with 0.1 M PBS (pH 7.4). Cultures were resuspended in 5 ml PBS. The first fraction was added at a concentration of 4×MIC to the suspension and incubated under agitation at 37 °C for 8 h. DMSO was used as a solvent control, and untreated bacterial cultures were used as a control. The cells were harvested by centrifugation at 10,000 rpm for 10 min at 10 °C and washed twice with PBS. A bacterial smear was prepared on a sterile slide measuring 1×1 cm. The smear was fixed with 2.5% glutaraldehyde in 0.1 M PBS overnight at 4 °C. After fixation, the cells were dehydrated by sequential exposure to 30%, 40%, 50%, 60%, 70%, 80%, and 90% ethanol for 10 min each. In addition, they were exposed to 100% ethanol for 1 h. The images were obtained at a voltage of 20 kV and a working distance of 7.5 mm. The surface of the bacterial cell was sputter coated with gold using the Quorum SC7620 sputter coater.

Statistical analysis

The results were analyzed by a one-way ANOVA. Tukey's test was used to identify significant differences among the mean (SPSS Statistics 20.0, IBM, Armonk, New York, USA). The difference among the means at the 5% level ($p < 0.5$) was considered statistically significant.

Results

HPTLC and bioautography of the polar portion of a methanol extract of inflorescence

Bioautography is an easy technique for the rapid detection of bioactive compounds from a mixture of components on a chromatography plate. A total of 6 bands were observed under UV light on the TLC plate (Fig. 2A). The bands were blue and pink. The bioautography test for antimicrobial activity revealed that bands near the solvent front with Rf values of 0.92 and 1.0 showed antimicrobial activity against clinical *S. aureus* (Fig. 2B). The inhibition was confirmed by a white zone on a pink background. Antioxidant activity was observed in all the bands that appeared, which was confirmed by the pink zone under the violet-colored area of the DPPH radical (Fig. 2C).

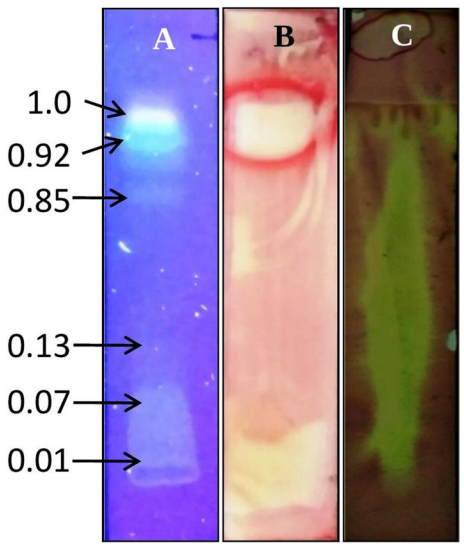

Fig. 2 Bioautography of inflorescence methanol extract. The figure shows a chromatogram under UV light (**A**); the Pink background expresses the bacterial growth while the white zone depicts antimicrobial activity, detected by 2,3,5-Triphenyltetrazolium chloride (**B**); The dark violet background exhibits the presence of DPPH radicals while yellow zone showing scavenged DPPH by phytochemicals present in extracts (**C**)

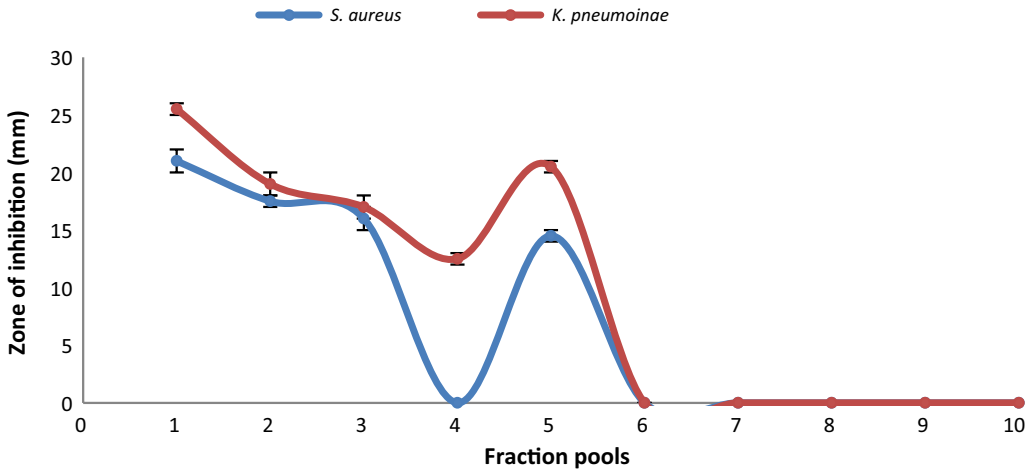

Fig. 3 Zone of inhibition (mm) of different fractions from active crude methanol inflorescence extract of *S. indicus* against *S. aureus* and *K. pneumonia* tested by agar well diffusion method at 350C for 18 h

Antimicrobial activity of fractions from a methanol polar extract of inflorescence

Antimicrobial activity was assessed against clinically isolated *S. aureus* and *K. pneumonia* due to their high susceptibility among other Gram-positive and Gram-negative bacteria, as recorded in previous work of this study [38]. The result is shown in Fig. 3. The zone of inhibition of the fraction pools ranged between 14.5 and 21.0 mm for *S. aureus* and 12.5–25.5 mm for *K. pneumonia*. The first fraction pool exhibited the highest activity against both bacteria, viz., *S. aureus* (21 mm) and *K. pneumonia* (25.5 mm).

Antioxidant activity of fractions from a methanol polar extract of inflorescence

The antioxidant activity was assessed by reducing power capacity and free radical scavenging ability using DMPD and DPPH radicals at a concentration range of 0.2–2.0 mg/ml.

The concentration-dependent analysis of reducing capacity revealed that all the fraction pools showed a linear pattern (Fig. 4A), which indicated that chemicals present in the fraction were not saturated at the tested concentration range; however, the reaction rate of the fifth, seventh, eighth, and ninth fraction pools was not consistent at certain concentrations. The highest absorbance at 700 nm was recorded in the second fraction pool (abs = 1.077) and the lowest was recorded in the 10th fraction pool (0.182) at 2 mg/ml (Fig. 4B).

Initially, the scavenging effect of fraction pools against DPPH and DMPD radicals was observed in a time-dependent manner to detect the complete inhibition time (data not shown). The inhibition kinetics of DMPD scavenging activity displayed a plateau formation in the second, third, sixth, seventh, and eighth fraction pools, while the fourth, fifth, ninth, and tenth fraction pools showed a sigmoid curve. The highest inhibition was detected at a concentration of 1.4 mg

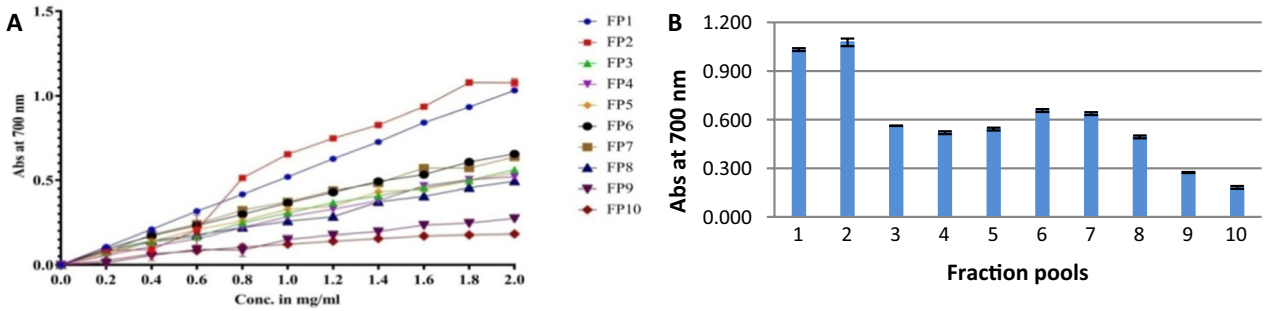

Fig. 4 Reducing power capacity of different fraction pools from inflorescence methanol extract of *S. indicus*. Figure **A** is showing Reducing power activity by fractions at different concentrations of fractions; Figure **B** is showing the comparison of activity at 2 mg/ml concentrations of fractions

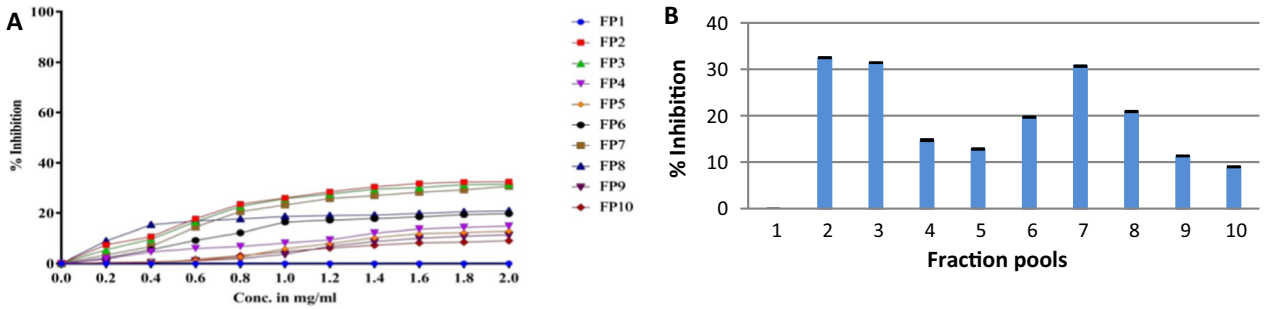

Fig. 5 DMPD scavenging activity by different fraction pools from inflorescence methanol extract of *S. indicus*. Figure **A** is showing reactions by fractions at different concentrations; Figure **B** is showing the comparison of scavenging activity at 2 mg/ml concentrations of fractions

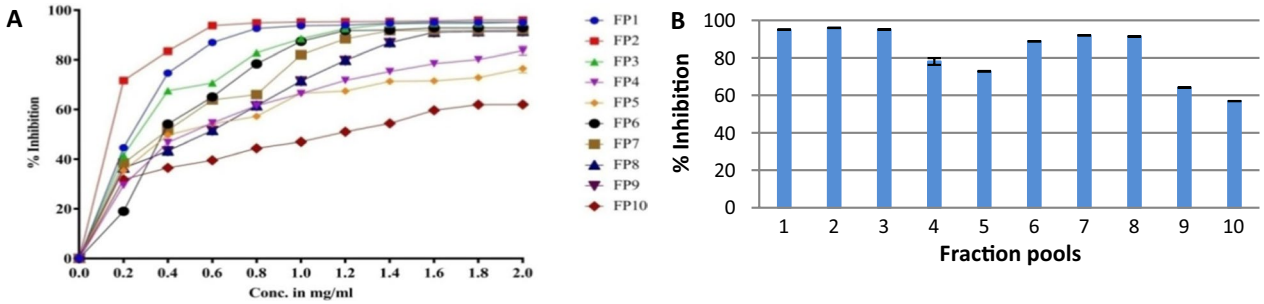

Fig. 6 DPPH scavenging activity by different fraction pools from inflorescence methanol extract of *S. indicus*. Figure **A** is showing reactions by fractions at different concentrations; Figure **B** is showing the comparison of scavenging activity at 2 mg/ml concentrations of fractions

from the second to fifth and ninth fraction pools, 1.0 mg in the sixth fraction pool, 1.6 mg in the seventh fraction pool, 0.4 mg in the eighth fraction pool, and 1.2 mg in the tenth fraction pool. The fraction pools displayed an inhibition range of 8.99–32.52% against DMPD cation radicals (Fig. 5A). Again, the second fraction pool exhibited higher inhibition capacity (32.52%), while the tenth fraction pool possessed lower inhibition (8.99%). The first fraction pool did not show any inhibition (Fig. 5B).

The plateau was observed during the scavenging activity of all the fraction pools against the DPPH radical in the graph (Fig. 6A). The steady-state started at 0.8 mg in the first fraction pool, 0.6 mg in the second fraction pool, 1.4 mg in the third, fifth, and seventh to ninth fraction pools, 1.6 mg in the fourth and tenth fraction pools, and 1.2 mg in the sixth fraction pool. In the case of DPPH free radical inhibition capacity, the fraction pools inhibited DPPH radicals in the range of 56.91–96.03%. The second fraction pool exhibited higher inhibition (96.03%), and the 10th fraction pool exhibited lower inhibition (56.91%), as shown in Fig. 6B.

The result revealed that the second fraction pool exhibited higher reducing power capacity and free radical scavenging ability than other fraction pools. The IC50 value of the potent fraction was calculated to determine the concentration that inhibited 50% of the radical and is presented in Table 1. The IC50 value

Table 1 IC_{50} of different fraction pools of methanol extract of the inflorescence of *S. indicus*

Fraction pools	DMPD inhibition (IC_{50} in mg/ml)	DPPH inhibition (IC_{50} in mg/ml)
1	0	0.22 ± 0.0004
2	0.75 ± 0.08	0.14 ± 0.0008
3	0.82 ± 0.06	0.29 ± 0.0011
4	3.31 ± 0.21	0.66 ± 0.0057
5	4.36 ± 0.15	0.74 ± 0.0056
6	1.95 ± 0.12	0.39 ± 0.0005
7	0.99 ± 0.13	0.41 ± 0.0011
8	1.43 ± 0.13	0.52 ± 0.0031
9	5.17 ± 0.08	1.30 ± 0.0072
10	6.03 ± 0.08	1.36 ± 0.0102

was 0.750.08 mg/ml against the DMPD radical and 0.14 ± 0.0008 mg/ml against the DPPH radical.

Synergistic effect of compounds present in the first fraction pool of the polar portion of the methanol extract of inflorescence

The results of bioautography, bands present in fractions, and fraction-guided biological activity revealed that no single compound could kill the tested microbes in this study. The growth of tested microbes was inhibited when multiple components containing fractions were used. The compounds present in these fractions may have combined effects. So, the synergistic effect was examined in this study.

This section of the study attempted to examine the synergistic effect of the potential first fraction pool, obtained from a polar portion of a methanol extract of inflorescence. The sample was separated using chloroform (100% v), ethyl acetate (100% v), methanol (100% v), and ethyl acetate: methanol (100:18 v/v). The Rf values and the results of bioautography are shown in Fig. 7. Nine clear bands were observed in the chloroform mobile phase, out of which three blue bands and one pink band were prominent, but in this condition, the bands did not inhibit the growth of *S. aureus*, when tested by redox indicator (TTC).

The bands obtained from ethyl acetate, methanol, and ethyl acetate: methanol (100:18 v/v) showed inhibition of bacterial growth. Nine bands were obtained in the ethyl acetate mobile phase, in which a blue band with an Rf value of 0.68 was closely attached to two other blue bands (Rf 0.64 and 0.70). The blue band (Rf 0.70) is attached to the pink band (Rf 0.73). These bands showed inhibition, while the prominent blue band (Rf 0.56), including other bands, did not show activity because these bands were separated; two bands were obtained from the methanol mobile phase, where bands with an Rf value of 0.91 showed antibacterial activity. Methanol clumped all the probable bands; when ethyl acetate: methanol (100:18) was used as the mobile phase, seven bands were obtained, in which bands with an Rf value of 0.78 and 0.81 exhibited activity. Two blue bands clumped together and gave an Rf value of 0.78; two blue bands and one pink band clumped together and gave an Rf value of 0.81. Both bands were close to each other and inhibited the growth of bacteria.

MIC and MBC of the active fraction

The minimum inhibitory concentration and minimum bactericidal concentration of the fraction pool were assessed against clinically isolated *S. aureus* and *K. pneumonia e* with regard to streptomycin (10 µg/ ml). The fraction showed a value of 0.31 mg/ml against *S. aureus* and 0.078 mg/ml against *K. pneumonia* (Table 2). The fraction also possessed bactericidal activity against both bacterial pathogens.

Detection of key compounds from fractions of the polar portion of methanol inflorescence extracts

The fractions in the polar portion of the methanol inflorescence extract contained multiple compounds. These fractions exhibited little to no activity. To resolve this mystery, an effort was made through the analysis of antimicrobial and antioxidant activities and the development of chromatograms of fractions. The chromatogram of fraction pools is depicted in Fig. 8a, b, and the chromatograms of each fraction pool are shown in Fig. 9 (FP1–10).

The fraction pools from 1 to 10 were subjected to HPTLC, and their bands were compared to the retention factor, which is shown in Table 3. The study was carried out to identify possible active bands and their potential activity. Since the first fraction pool possessed the highest activity of all, it was used as a reference. It contains more bands than other fractions, and many of them were unique to the first fraction, *i.e.*, bands with Rf values of 0.03 and 0.08 were unique in the first fraction pool, which might have enhanced the activity but were absent in other fractions, which resulted in reduced activity. Bands with Rf 0.16, 0.25, and 0.45 were absent in the second fraction pool, which might reduce activity.

The third fraction pool missed the Rf 0.70 bands, which can reduce activity more than the first and second fraction pools. The fourth fraction pool lacked most of the bands except Rf 0.54 and 0.60, which were not enough for better activity, hence showing less activity compared to the first, second, third, and fifth fraction pools. The fifth fraction pool also had bands with Rf 0.54 and 0.60, but it also had one extra band with Rf 0.13, which may have increased activity when compared to the fourth fraction pool. Bands with Rf 0.45 were also present in the first fraction pool, and bands with Rf 0.60 were present in fraction pools first through fifth. The absence of the band with Rf 0.54 might have caused no activity in the sixth fraction pool. Similarly, the seventh fraction pool missed the band with Rf 0.61, which nullified the activity; although it contained bands with Rf 0.35, 0.45, and 0.54, there was no importance to these without Rf 0.60. However, bands with Rf 0.79–0.81 were present in all the fractions; it may be possible that these compounds played a role in the activity. This study revealed that bands with Rf values of 0.13, 0.54, 0.60, 0.68, and 0.79 were active components, while others were associate components required for the activity.

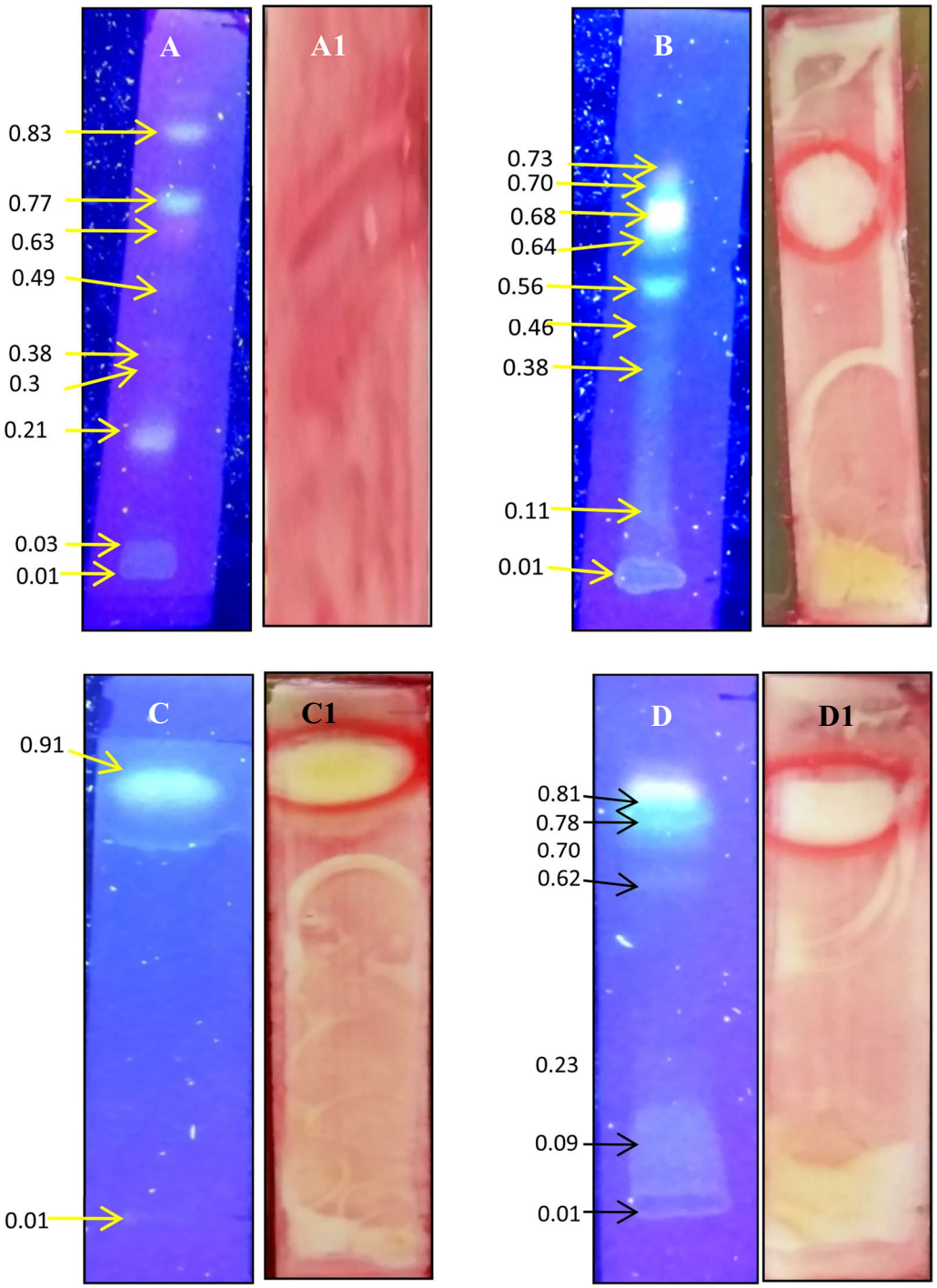

Fig. 7 The TLC chromatograms and bioautography test of the first fraction pool from inflorescence methanol extract of *S. indicus* against clinical *S. aureus*. The figures are showing TLC chromatogram and bioautography images developed by mobile phases of chloroform (**A** & **A1**), ethyl acetate (**B** & **B1**), methanol (**C** & **C1**), and ethyl acetate: methanol (100:18 v/v) (**D** & **D1**). White area is showing growth inhibition by bands while the pink background is showing growth of bacteria in the figures

Table 2 MIC of first fraction pool of inflorescence methanol extract of *S. indicus*

Bacterial cultures	Fraction (5 mg/ ml)	Positive control (1 mg/ ml)
Staphylococcus aureus	0.31 ± 0.20	0.00048 ± 0.03
Klebsiella pneumoniae	0.078 ± 0.15	0.125 ± 0.05

HPTLC-MS of active fractions of inflorescence

Selected compounds were analyzed for their molecular weight through a mass spectrophotometer after comparison and the detection of active bands in fraction pools. The terpenoids were detected in active fractions after derivatization. The chromatograms are shown in Fig. 10 I–IV. The mass spectral distribution of active bands was analyzed through the Orbitrap mass spectrophotometer. The chromatogram in Fig. 10 -V depicts the bands and their codes, which was used for detailed mass spectrophotometric analysis.

The mass spectrum of the bands in positive ion mode is shown in Figs. 11, 12, 13, 14 and 15. Band 7B (Rf 0.45) was identified as Thymol in the first fraction of the methanol extract of the inflorescence. Band 7C was identified as 5,7-Dimethoxyphenanthrene-2,6-diol (Rf 0.55), Sphaerindicin as band 7D (Rf 0.68), β-Sitosterol as band 7E (Rf 0.79), and Pheanthine as band 7F (Rf 0.85). The molecular weight, molecular formula, and MS fragment ions of compounds are given in Table 4.

Mode of action of compounds present in the first fraction pool: methanol inflorescence extract of *S. indicus*

The action of the first fraction pool of the polar fraction of the methanol inflorescence extract of *S. indices* on

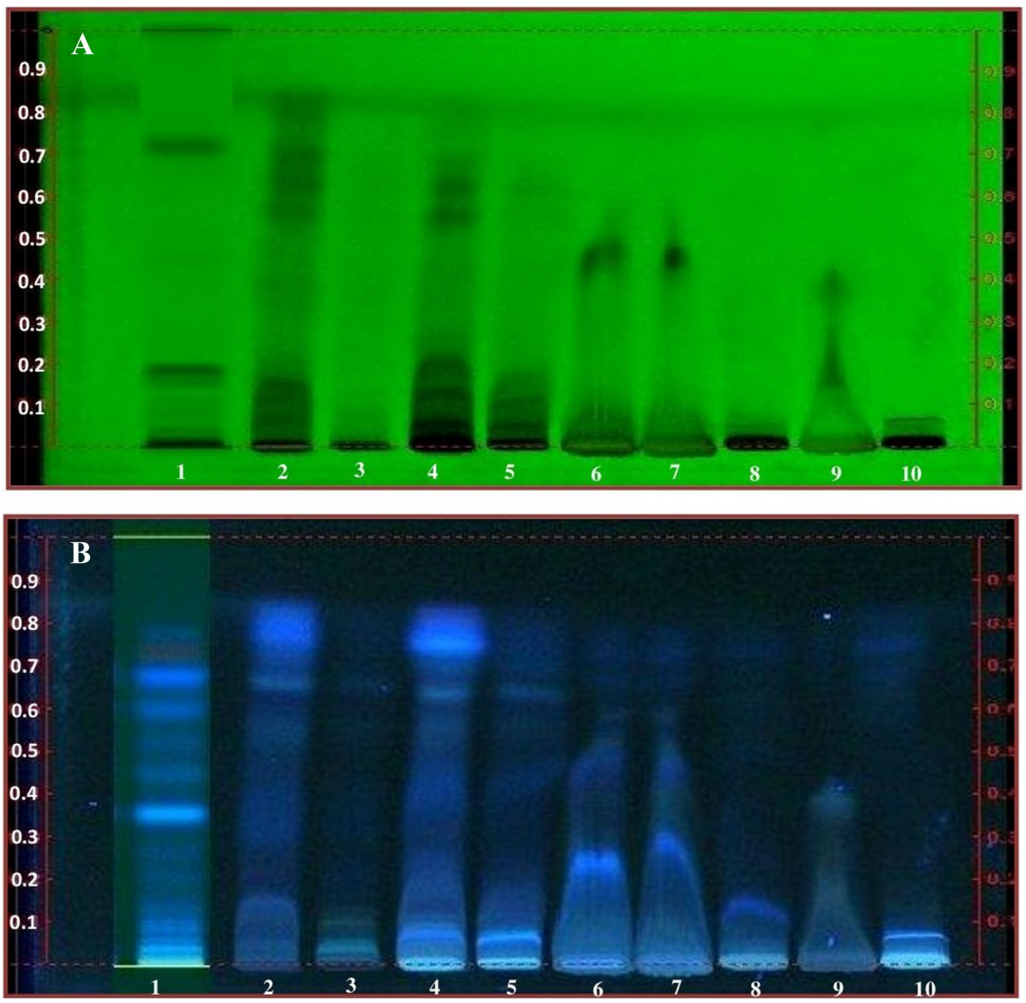

Fig. 8 The HPTLC chromatograms of fraction pools (1–10) from methanol inflorescence extract of *S. indicus* under 254 nm (**A**) and 366 nm (**B**) wavelengths

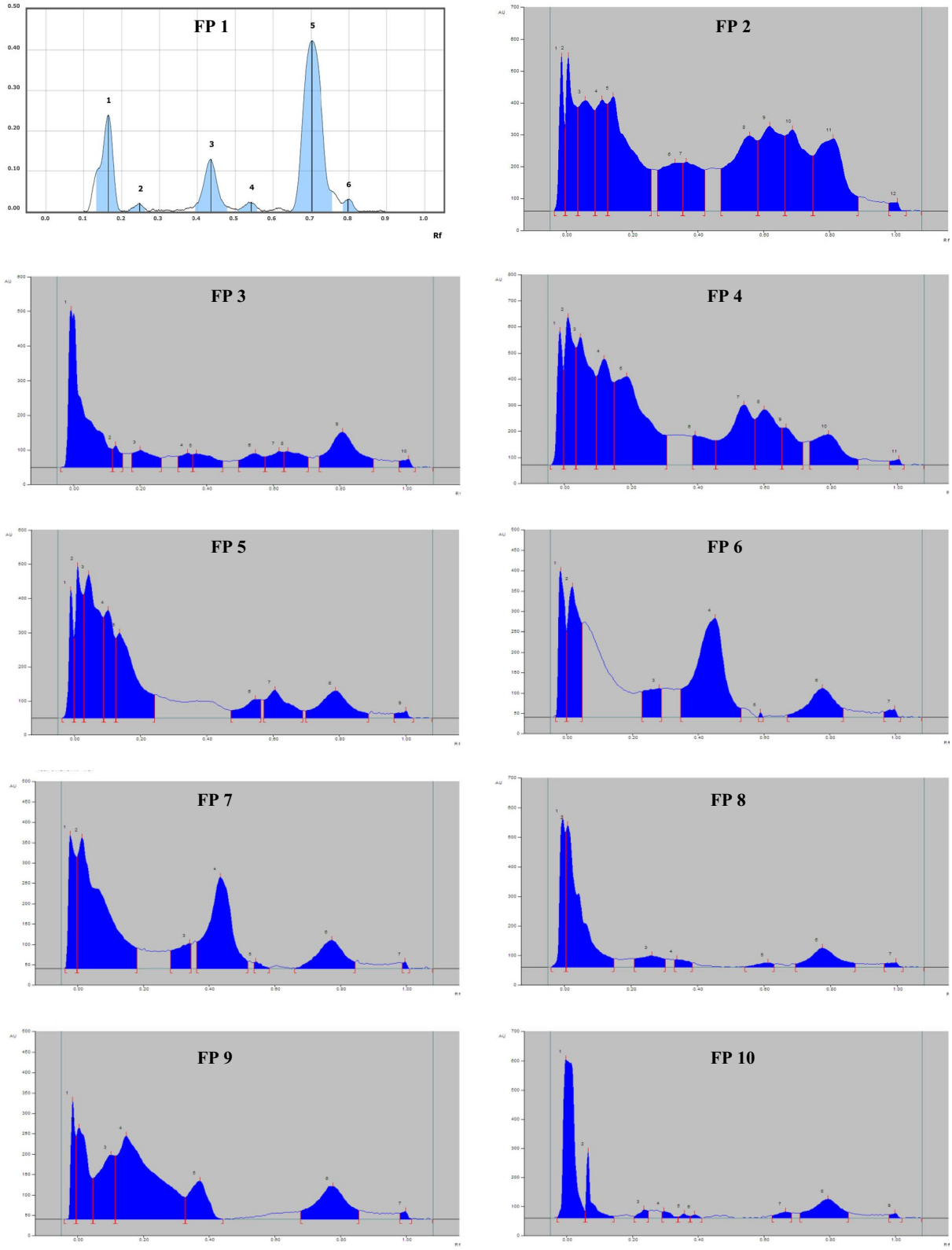

Fig. 9 HPTLC chromatograph of 1–10 fraction pools (FP 1–10) of inflorescence methanol extract of *S. indicus*

Table 3 Comparative Rf value of bands from fraction pools (FP) 1–10 of methanol inflorescence extract of *S. indicus* with the possible active compound(s) by HPTLC

FP1	FP2	FP3	FP4	FP5	FP6	FP7	FP8	FP9	FP10
0.00	−0.01	−0.01	−0.01	−0.01	−0.02	−0.02	−0.00	−0.01	−0.00
0.01	0.01		0.01	0.01	0.02	0.01	0.01	0.00	0.07
0.03									
0.05	0.06		0.05	0.04					
0.08									
0.09				0.10				0.10	
	0.11		0.12						
0.13	0.14	0.13		0.14					
0.16								0.15	
0.20		0.20	0.19						
									0.24
0.25							0.26		
					0.28				
									0.30
0.35	0.33	0.34				0.34	0.34		
	0.37	0.37						0.37	0.36
			0.39						0.39
0.45					0.45	0.44			
0.54	**0.56**	**0.55**	**0.54**	**0.55**		0.54			
0.60	**0.62**	**0.62**	**0.60**	**0.61**	0.59		0.61		
		0.65	0.67						0.67
0.68	**0.69**								
0.79	0.81	0.81	0.79	0.79	0.78	0.77	0.78	0.78	0.80
	1.01	1.01	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Bold number indicates Rf value of identified active molecule

FP Fraction pool

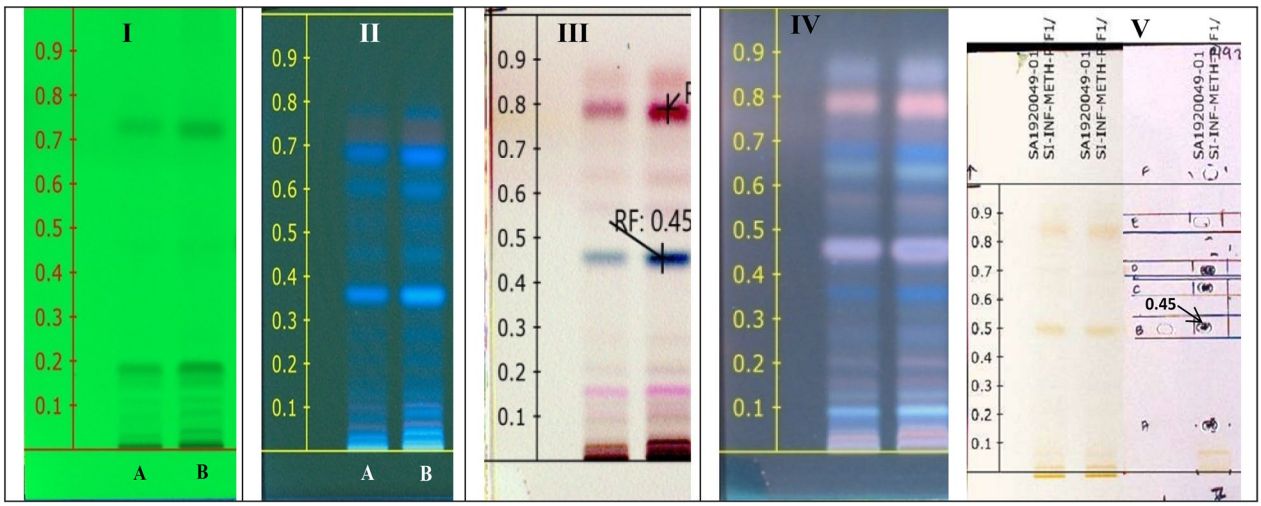

Fig. 10 The HPTLC chromatogram of first fraction pool of inflorescence methanol extract of *S. indicus* under 254 nm (**I**) and 366 nm (**II**); derivatized plate with anisaldehyde sulphuric acid reagent under visible light (**III**) and 366 nm (**IV**); marked area for Mass spectroscopy (**V**). Sample volumes were taken 2 µl and 4 µl for analysis

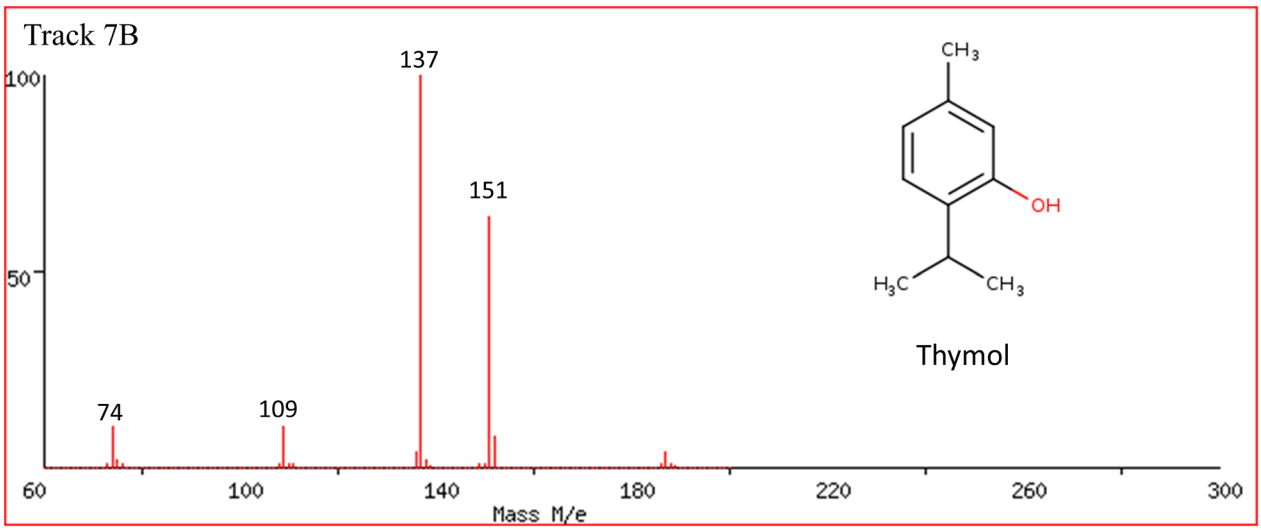

Fig. 11 Mass spectrum of thymol (Rf 0.45) present in first fraction pool from inflorescence methanol extract of *S. indicus* by using HPTLC-MS analysis

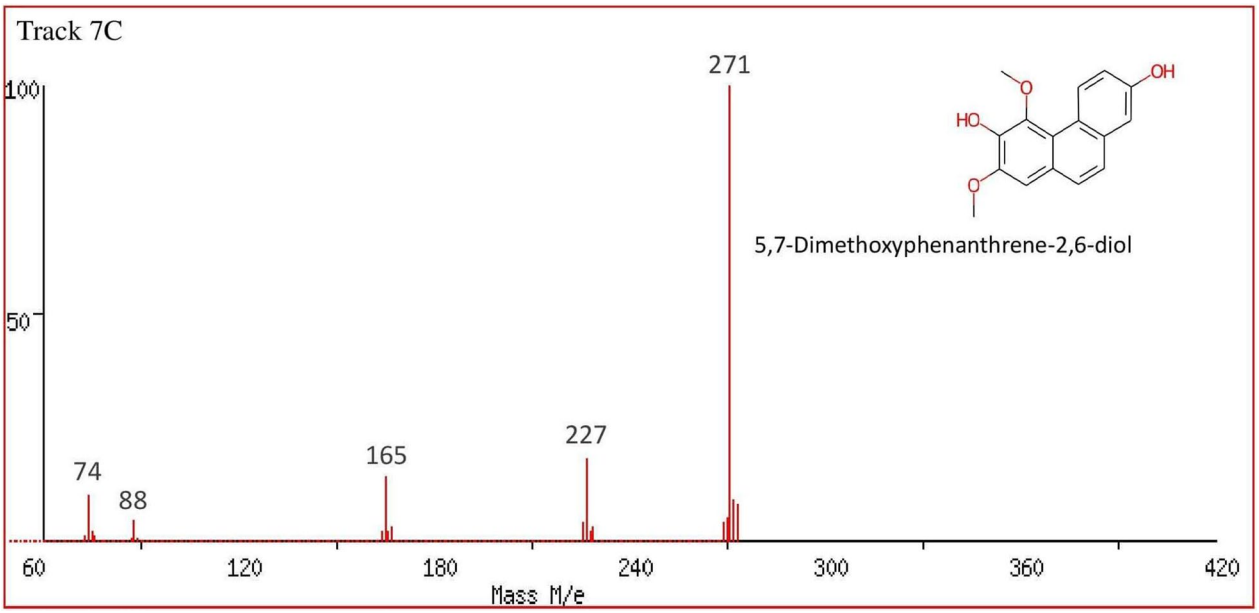

Fig. 12 Mass spectrum of 5,7-Dimethoxyphenanthrene-2,6-diol (Rf 0.55) present in first fraction pool from inflorescence methanol extract of *S. indicus* by using HPTLC-MS analysis

the bacterial membrane was assessed against clinical *S. aureus*. The bacterial cell was treated for 10 h with 1×MIC, 2×MIC, and 4×MIC concentrations of the sample. The protein and nucleic acid leakages were assessed in the UV range by a UV-VIS spectrophotometer at 260 nm and 280 nm, respectively (Fig. 16A–B).

After treatment with the first fraction pool at 4×MIC, SDS-PAGE, and agarose gel electrophoresis were used to detect leaked proteins and nucleic acids from control and drug-treated bacteria (Fig. 16C–D). Cells treated with fraction illustrated prominent bands, whereas untreated cells revealed no significant effect on the release of intracellular proteins and nucleic acids. Six different proteins were discovered, two of which had a mass of 25 kDa and one of which had a mass of 11 kDa.

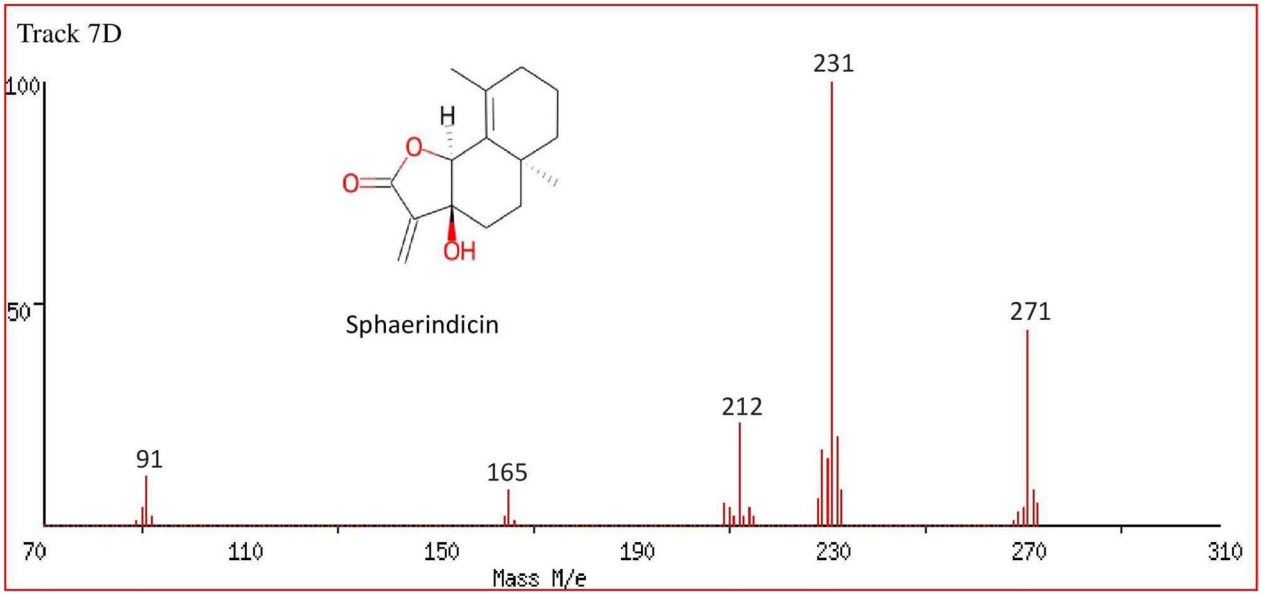

Fig. 13 Mass spectrum of Sphaerindicin (Rf 0.68) present in first fraction pool from inflorescence methanol extract of *S. indicus* by using HPTLC-MS analysis

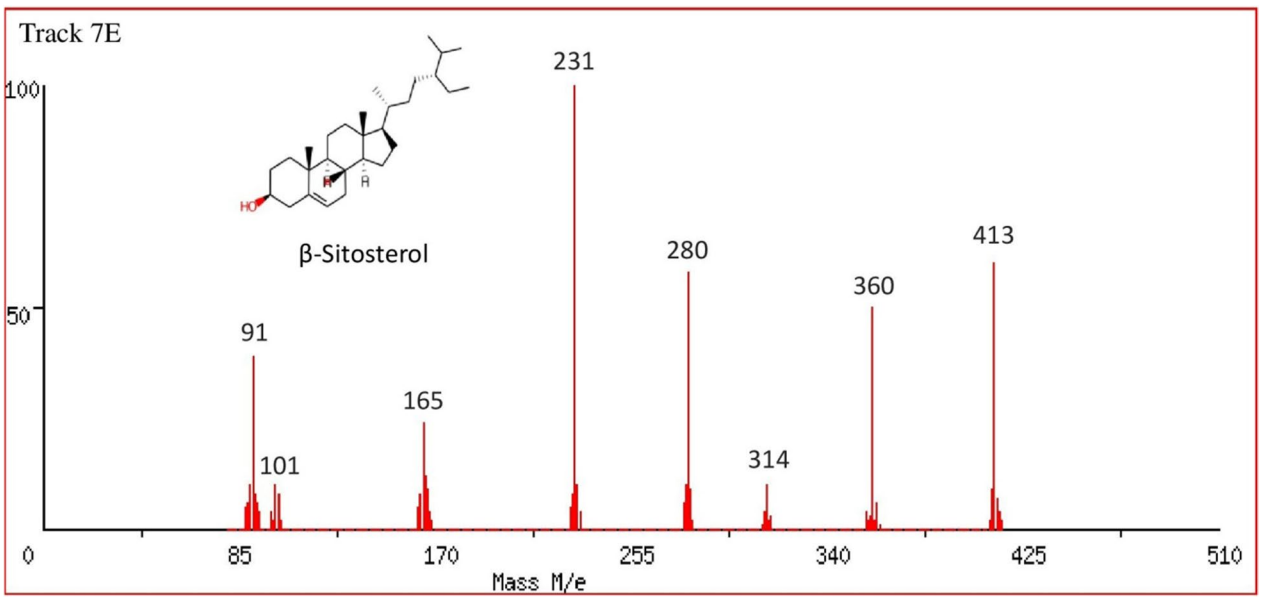

Fig. 14 Mass spectrum of β-Sitosterol (Rf 0.79) present in first fraction pool from inflorescence methanol extract of *S. indicus* by using HPTLC-MS analysis

The effect of the first fraction on the membrane integrity of clinical *S. aureus* was assessed under SEM. Bacterial morphology was disrupted at 4×the MIC concentration of the first fraction. As shown in Fig. 17A, B, the electron micrographs of bacteria demonstrated some alterations in membrane morphology. The comparison was made with the smooth cell surface of the untreated cells. Considerable structural changes were seen in treated *S. aureus*.

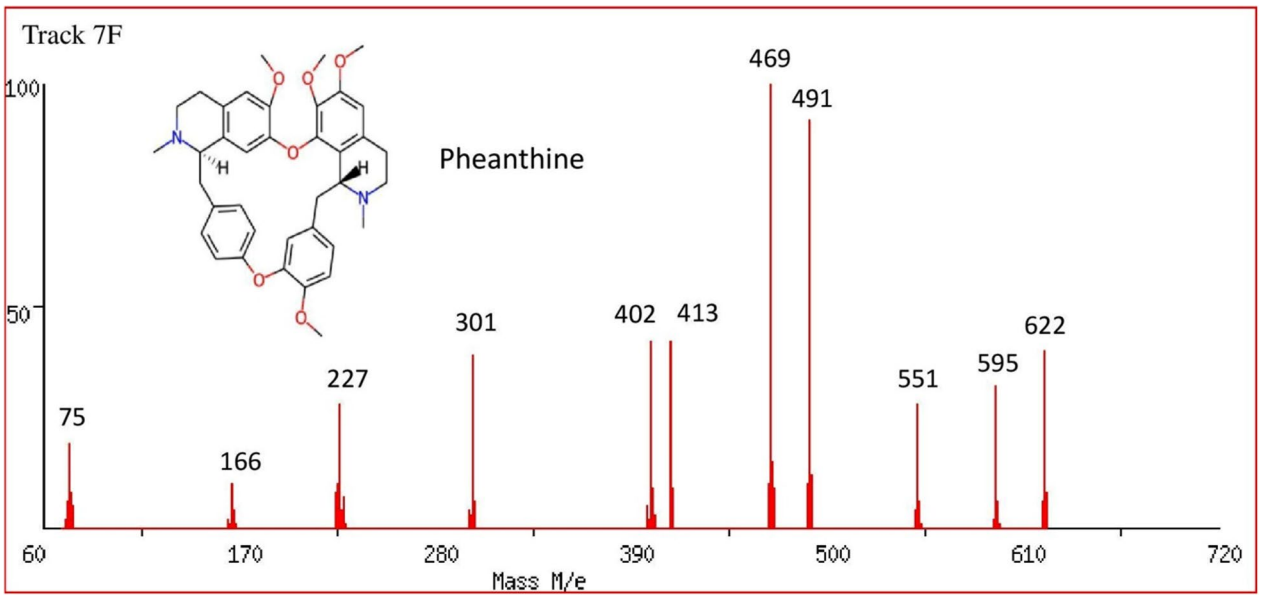

Fig. 15 Mass spectrum and of Pheanthine (Rf 0.85) present in first fraction pool from inflorescence methanol extract of *S. indicus* by using HPTLC-MS analysis

Table 4 ESI–MS spectrum data of molecules present in first fraction of inflorescence methanol extract of *S. indicus* L.

Band	Rf	m/z	Molecular weight	Compound	Chemical formula	Quantity (mg/ml)
7B	0.45	151, 137, 109 [M + H]	150.22	Thymol	$C_{10}H_{14}O$	0.675
7C	0.55	271, 227, 165, 74 [M + H]	270.28	5,7-Dimethoxy-phenanthrene-2,6-diol	$C_{16}H_{14}O_4$	0.097
7D	0.68	271.01, 230.09, 184.05, 74.10 [M + Na]	248.32	Sphaerindicin	$C_{15}H_{20}O_3$	3.01
7E	0.79	413.19, 360.27, 301.10, 231.09, 165.01, 91.25 [M + H]	414.72	β-Sitosterol	$C_{29}H_{50}O$	0.1
7F	0.85	75, 166, 227, 301, 402, 413, 469, 491, 551, 595, 622 [M + H]	622.76	Pheanthine	$C_{38}H_{42}N_2O_6$	0.102

Discussion

Plants are increasingly being used as medicine to treat microbial infections and to prevent the negative effects of free radicals. This is believed to be due to the safety conception attached to the plants, the high cost, numerous side effects, and the unavailability of orthodox drugs [36]. *S. indicus, an herb, had a variety of medicinal properties so it was used in this study* [37].

In this present study, authors attempted fractionation, antimicrobial activity, antioxidant activity, compound identification, and analysis of the mode of action of methanol extract of *S. indicus* inflorescence. At first, bioautography was performed to achieve these aims.

Bioautography is a rapid and useful tool to identify the active compound(s) in a mixture of chemicals [22]. The bioautography of the present extract accomplished two things: first that some common compounds exhibited antimicrobial as well as antioxidant activity, and second was that compounds with polar had no antimicrobial activity but exhibited antioxidant activity. Schmourlo et al.[38] and Suleiman et al.[39] examined the bioautography test of different plant species on several pathogenic microorganisms and found active bands. In another research study, DPPH-scavenging compounds were detected in *M. platytyrea* extracts [40].

The crude extract of the plant contains pigments, primary metabolites, and secondary metabolites. Here we focused only on secondary metabolites due to their variety of biological activities. The phytochemicals were separated via adsorption chromatography using Silica gel. The fractions were collected as per their chromatogram profile to achieve various targets. The activities of fractions were assessed, which revealed that the first fraction pool had high antimicrobial activity and the second fraction pool exhibited the highest antioxidant activity.

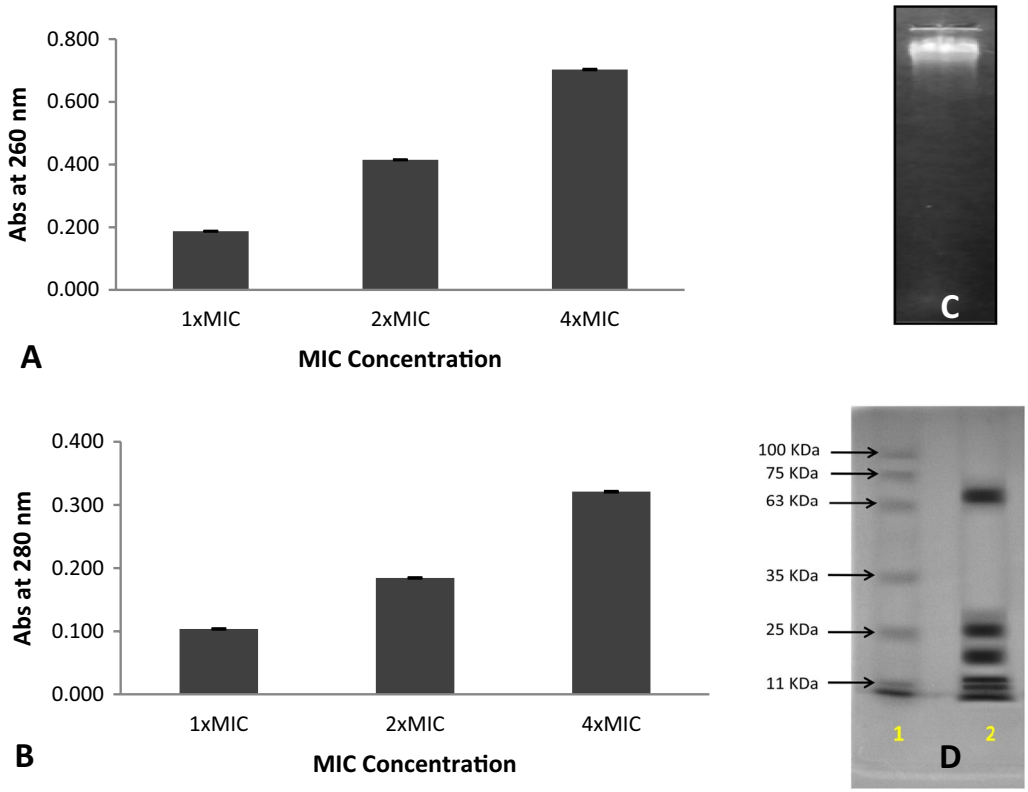

Fig. 16 Detection of release of cellular material from *S. aureus* at 260 nm (**A**) and 280 nm (**B**) treated with different MIC concentrations of first fraction pool of inflorescence methanol extract of *S. indicus*. The leaked content was cross checked by electrophoresis of nucleic acid (**C**) and protein (**D**) treated with 4xMIC concentration

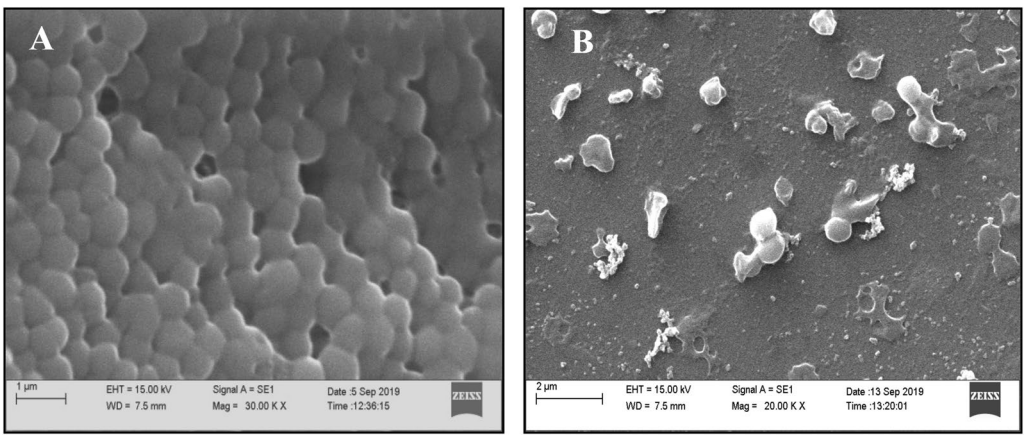

Fig. 17 Scanning electron micrographs of *S. aureus*. The images are showing untreated cell (**A**); treated cell with first fraction pool of inflorescence methanol extract (**B**) of *S. indicus*

The antimicrobial activity was highest against Gram-negative *K. pneumonia* as compared to Gram-positive *S. aureus*. It is believed that gram-positive bacteria are more susceptible than gram-negative bacteria due to the lack of an outer membrane [41]. But here we found that the phytochemicals of this plant are more toxic for gram-negative bacteria after fractionation, which may pave the way for the development of drugs against them.

The antioxidant activity of fractions was determined using DPPH inhibition, DMPD inhibition, and ferric-reducing capacity assays. Initially, the inhibition completion time and pattern of inhibition were assessed for the first two assays to achieve the accuracy of the result. The inhibition and reducing power were evaluated as a concentration gradient to determine which concentration is best for achieving the highest scavenging activity of phytochemicals present in the fractions.

The scavenging mechanism depends on the chemical structures, number of active groups, hydrophilicity, and lipophilicity of the components in fractions [42]. The reaction reached a stable state and produced a plateau in opposition to both radicals. The equation suggested by Mishra et al. [43] provides insight into this mechanism:

$$[R^{\cdot}] + [AH] \leftrightarrow R - H + \left[A^{\cdot}\right] \tag{3}$$

$$[R^{\cdot}]\left[A^{\cdot}\right] \rightarrow R - A \tag{4}$$

$$\left[A^{\cdot}\right] + \left[A^{\cdot}\right] \rightarrow A - A \tag{5}$$

The equation explains how antioxidants deliver hydrogen ions to radical scavengers at a quicker rate (Eq. 3), which causes antioxidants to transform into the radical form [A] (Eq. 4), which then reacts with free radicals to create radical-antioxidant complexes. There was a sluggish reaction at this moment, leading to a steady-state condition. The reaction was eventually terminated as the remaining [A] reacted among them (Eq. 5).

The antioxidants work in two modes: hydrogen atom transfer (HAT) or electron transfer (ET). DMPD and DPPH scavenging assays fall under HAT ability. These kinetic tests use a competitive reaction scheme in which the antioxidant and substrate vie for radicals, as stated in the previous equation. While methods based on electron transfer (ET), such as the Folin-Ciocalteu assay and reducing power, use color changes to gauge an antioxidant's ability to withstand oxidation [44], A chromogenic redox reagent is used in these tests. The results of the current study showed that every fraction pool had both mechanisms. The second fraction pool exhibited the highest antioxidant activity. Kinetic results on the fraction pools are in agreement with the kinetic data for both standard antioxidant compounds and other plant extracts [45, 46].

The result of bioautography and a plate assay for antimicrobial activity revealed that a single compound was unable to inhibit the bacterium's growth while organisms were inhibited in the crude extract. This indicates that phytochemicals may show synergistic effects. There are several methods to detect the synergistic effect between phytochemicals. If the sample volume and the number of compounds in the fraction are low, it can also be detected through the bioautography technique, which can be resolved by creating different band patterns in various mobile phases, which is a rapid technique instead of the separation of a large amount of a single compound by column chromatography. So the results revealed that when the compounds were present nearby, they inhibited the growth of bacteria, and when they were present far apart, they were unable to inhibit the growth. A similar result was obtained from the synergistic effect of different plant extracts. The combinations of plant extracts significantly inhibited aflatoxin production from *Aspergillus flavus* more than individual plant extracts [47]. Another research related to the antioxidant activity of flavonoids revealed that gallic acid and caffeic acid exhibited a synergistic effect to reduce the ferric ion [48]. The caffeic acid and α-chaconine from *Solanum tuberosum* L. possessed antifungal activity in the combined form [49]. As well as the extract of *Piper hispidum* with some antifungal drugs exhibited synergistic effects to inhibit the *Candida albicans* and *S. aureus* with FIC indices of 0.37 and 0.24 [50].

After the analysis of fraction-guided activity and synergistic effects between phytochemicals, one question arose: does every compound exert the same biological activity? To resolve this question, we made an effort to identify active compounds in the first fraction pool. We compared the results obtained from all the tests of antimicrobial activity and the chromatogram profile of each fraction pool. We interpreted that bands with Rf values of 0.13, 0.54, 0.60, 0.68, and 0.79 may exhibit antimicrobial activity. Based on their mass spectra and comparison with published data of *S. indicus* [51, 52], the bands were identified as thymol, 5,7-Dimethoxyphenanthrene-2,6-diol, sphaerindicin, -sitosterol, and pheanthine. Thymol is the first time it has been found in this plant. The antimicrobial and antioxidant compound thymol has been reported in some literature. Thymol inhibited the growth of *S. aureus*, *Listeria innocua, E. coli* O157:H7, *Saccharomyces cerevisiae*, and *Aspergillus niger* when released from microcapsule coated to plastic flexible films [53] and thymol also reduced ferric ion [54]. Purified compounds of *S. indicus* have been shown to have antimicrobial activity. A bicyclic sesquiterpene lactone isolated from the petroleum ether extract of the aerial part of *S. indicus* was reported to have antimicrobial activity against *S. aureus, E. coli, Fusarium* sp., and other microorganisms [55].

The mode of action of the fraction was also assessed. The phytochemicals caused leakage of cellular content, which was confirmed by spectrophotometer and electrophoresis techniques. Cells treated with fraction illustrated prominent bands, whereas untreated cells revealed

no significant effect on the release of intracellular contents. A similar result was seen in the report of Diao et al. [56] who examined the effect of *Foeniculum vulgare* Mill. seed essential oil on *Shigella dysenteriae*. They reported that essential oil caused leakage of electrolytes, the losses of proteins, reducing sugars, and 260 nm absorbing materials. In another report by Behbahani et al. [57], cumin essential oil increased membrane permeabilization of the cells and disrupted the membrane integrity of *E. coli* and *L. innocua*. The release of contents may be due to the damage and increment of bacterial cell walls and membrane permeability. It was cross-checked by SEM instrumentation. The first fraction pool severely damaged the cell membrane of *S. aureus*, resulting in cytoplasmic secretion and cell death. Also, the cell shape was seriously distorted. The observation was confirmed after comparison with the result of de Oliveira Galvão et al. [58] against *S. aureus*.

In the present study, the identified compounds are phenolic terpenoids. The antimicrobial effect of compounds depends on their molecular structure, water solubility, lipid solubility, bacterial cell wall/membrane structure, and net charge of the cell wall [59]. The hydroxyl group of the phenol moiety is essential for the antibacterial effect [60]. Hydrophobic aromatic terpenoids were much more efficient than the aliphatic family members, both in antimicrobial activity and increasing the permeability of the cell membrane. Thus, we conclude that a hydrophobic skeleton is advantageous for penetration through the hydrophobic layers of the membrane [61]. This hydrophobicity must be balanced, though, with a hydroxyl group. The hydroxyl group can act as a proton exchanger, retain terpenoid within the membrane layer, and affect various structural and dynamic properties of the membrane [62–64]. The structure of the bacterial cell wall also affects the activity of antimicrobial compounds. Since Gram-positive bacteria possess a permeable cell wall that usually does not restrict the penetration of antimicrobials, the major component of a Gram-positive cell wall is peptidoglycan, which is covalently linked to teichoic and teichuronic acids, which give the wall a net negative charge [65]. However, antimicrobial terpenoids target the bacterial cytoplasmic membrane. Gram-positive *S. aureus* contains the positively charged lipid lysylphosphatidylglycerol at levels between 15 and 40% [66]. The negative charge of the oxygen atom in the hydroxyl group in the phenol moiety may interact with this lipid and stay in the membrane with the hydrophobic benzene ring. Thus, they disrupt the membrane structure and function and cause the release of intracellular contents out of the cell, forming tube-like structures in the cell wall.

Furthermore, the outer layer of the Gram-negative outer membrane is composed primarily of lipopolysaccharide molecules and forms a hydrophilic permeability barrier, protecting against the effects of highly hydrophobic compounds [67]. This may explain the low sensibility of Gram-negative bacteria against antibiotics. But we found that K. pneumonia was also highly susceptible to the chemicals present in the first fraction pool. It may be due to the synergistic effect of active phytochemicals.

Cristani et al.[59] studied the inhibitory mechanism of some terpenes against the cell membrane of *S. aureus* and *E. coli*. They found that thymol was more effective against *S. aureus*. It possesses the highest capacity to increase the permeability of PC LUVs, together with the ability to migrate across an aqueous medium and to interact with phospholipidic membranes, and causes severe damage to the cell membrane when tested in model membranes. Ergüden [68] reported the antimicrobial effect of four monoterpenes against *S. aureus* and *E. coli*. Thymol was more effective against *S. aureus* and caused leakage of ions out of cells, which disturbs ion homeostasis.

This study is limited to in-vitro tests of photochemical against microbes and free radicals. Since the photochemical possess a synergistic effect and have lipophilic property, an ointment will be designed of identified bioactive molecules for the in-vivo test.

Conclusion

The herbs are good sources of antimicrobial and antioxidant compounds. This study revealed that the first fraction had the highest antimicrobial activity while the second fraction had the highest antioxidant activity. The bioautography and fraction-guided bioactivity of phytochemicals against bacterial pathogens and radicals were resolved. Another important finding in this study was that more than one compound showed antimicrobial as well as antioxidant activities, and some compounds exhibited both activities. Except for gram-positive *S. aureus*, the fraction had the potential to inhibit the growth of gram-negative *K. pneumonia*. The terpenoids were identified in a methanol extract of the inflorescence as well as in active fractions. Thymol, 5,7-dimethoxyphenathrene-2,6-diol, sphaerindicin, β-sitosterol, and pheanthine were identified as active compounds. The hydroxyl group of the phenol moiety of these compounds was responsible for damaging the membrane of bacteria and exhibited bactericidal activity. The presence of thymol is first reported in this plant, demonstrating the significance of geographical diversity. Thus, the methanol extract of *S. indicus* is a good source of antimicrobial and

antioxidant compounds. In the future, the emulsion and paste of this fraction will be prepared for external use.

Abbreviations

HPTLC-MS	High-performance thin-layer chromatography mass spectroscopy
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MRSA	Methicillin-resistant susceptible aureus
GSH	Glutathione
SOD	Super oxide dismutase
ROS	Reactive oxygen species
UV	Ultra-violet
DPPH	2,2-Diphenylpicrylhydrazyl
DMSO	Dimethyl sulfoxide
CFU	Colony forming unit
TTC	2, 3, 5,-Triphenyltetrazolium chloride
DMPD	N, N-dimethyl-*p*-phenylenediamine
IC_{50}	Concentration at 50% inhibition
F_{254}	Fluorescence at 254 nm
PBS	Phosphate buffer saline
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA
SEM	Scanning electron microscopy

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Status of plant
The plant *Sphaeranthus indicus* was harvested from local area i.e. paddy field of Raipur city, near Kharun river. The plant is not listed as endangered, endemic & rare flora and critical endangered flora as seen in Forest Medicine portal of Chhattisgarh Government, India (http://cgvanoushadhi.gov.in/). License is not applicable because there was no drug trial of phytomedicine in animal/plant.

Author contributions
DT performed methodology, investigation, formal analysis, and writing original draft. AKG performed supervision, conceptualization, funding acquisition, and review-editing the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials
The data sets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
The authors declare no conflict of interest.

Competing interests
The authors declare no competing interests.

Author details
[1]Microbiology Research Laboratory, School of Studies in Life Sciences, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh 492010, India. [2]Department of Applied Science, Shri Rawatpura Sarkar University, Dhaneli, Raipur, Chhattisgarh 492015, India.

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