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ORIGINAL RESEARCH PAPER

Bioautography and liquid chromatography–mass spectrometry studies of *Meyna spinosa* Roxb. ex Link leaf methanolic extracts

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Abstract
Meyna spinosa Roxb. ex Link, locally known as "Haloo", was sampled from Surgana, Nasik, and is reported to have many ethnobotanical uses. This study aims at analyzing and isolating the bioactive components responsible for the antimicrobial activity of the plant's leaf methanolic extracts. Antimicrobial activity was studied using ditch-plate technique and bioautography. Ditch-plate method showed antimicrobial activity against *Staphylococcus aureus*. Bioautography in which thin-layer chromatography (TLC) is coupled to bioassay technique showed antimicrobial activity at the application spot against *Pseudomonas aeruginosa*. The compound/s were collected and concentrated by preparative TLC. The concentrated bioautography isolate (BI) and the leaf methanolic extract (ML) of *M. spinosa* Roxb. ex Link were subjected to liquid chromatography (LC) followed by electrospray ionization–quadrupole-time-of-flight–mass spectrometry (ESI–Q-TOF–MS), to compare the bioactive components. It was found that BI showed 10-deoxymethymycin, dihydrodeoxystreptomycin, 5E,8E,11E-hexadecatrienoic acid, deferoxime that have antibacterial properties. ML in addition shows quercetin, kaempferol, 3,4,5-trihydroxystilbene, swietenine, which have antioxidant and antimicrobial activities. We conclude that the antimicrobial activity exhibited by the ML was due to the synergistic action of all these compounds. The minimum inhibitory concentration (MIC) of ML by microdilution revealed to be 375 mg/mL for *S. aureus* and 125 mg/mL for *P. aeruginosa*.

Keywords Bioautography; Electrospray ionization · Quadrupole-time-of-flight · Mass spectrometry (ESI–Q–TOF–MS) · Fatty acids · Swietenine

1 Introduction

Meyna spinosa Roxb. ex Link is widely distributed across India and has various ethnobotanical uses. It is used as food, foliage, medicine for worms, dysentery, boils, swelling and as abortifacient [1–5]. It grows into large shrubs or small trees. Leaves are opposite and elliptic-oblong. Flowers are greenish yellow and fruits are fleshy, smooth and glabrous [6]. It has good antioxidant, antimicrobial and antidiabetic activity [7–9].

Antimicrobial activity has been reported in the flavonoid fractions of leaves against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [8]. Further, the fruit extract of *M. spinosa* and the aqueous extracts of *Vangueria spinosa* (synonym of *M. spinosa* Roxb. ex Link) leaf show antimicrobial activity against certain fungi [10, 11].

Certain compounds have been elucidated from leaf extracts of *M. spinosa* such as oleanolic acid, myricyl alcohol, β-sitosterol [12], different triterpenoid, and saponins [13, 14]. From the literature, the plant contains many fatty acids and esters. Flavonoids (−)-epicatechin-3-O-β-glucopyranoside [15] and (2-[3-cyclopropyl-4-hydroxyphenyl]-5,7-dihydroxy-4-oxo-4H-chromen-3-yl acetate) [16] were also elucidated from *M. spinosa* leaves.

Bioautography technique is a combination of thin-layer chromatography (TLC) and bioassay. In the current study, the overlay technique of bioassay was used. This method enables one to detect and further isolate the compounds responsible for antimicrobial activity by preparative TLC. Bioautography was performed with six plant extracts, the results of which indicated that the compound showing antimicrobial activity may be a flavonoid or terpene [17].

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Bioautography was also performed to study the antimicrobial activity of essential oil of peppermint, the results of which indicated that menthol was found to be responsible for antimicrobial activity in this oil [18]. The dichloromethane extract of the underground parts of *Eleutherine bulbosa* (Miller) Urban showed strong antibacterial activity with bioautography, which led to the fractionation and isolation of four quinonoid compounds from the extract, which further when isolated, all except one showed antibacterial activity [19]. When 40 Indian medicinal plants showed varied levels of antimicrobial activity, bioautography of certain extracts demonstrated the presence of common phytoconstituents in the extract such as phenols, tannins and flavonoids [20]. The cinnamon essential oil consists majorly of cinnamaldehyde and eugenol, which proved to have antibacterial effect against *Paenibacillus larvae*. This was determined using bioautography [21]. TLC−bioautography of *Eugenia jambolana* seed extracts revealed that the ethyl acetate fraction contained phenolics which were the major active phytoconstituents which inhibited the growth of multidrug-resistant human bacterial pathogens. The results justified for the use of these seed extracts in folk medicine to treat various infectious diseases [22]. The antimicrobial activity of plant extracts is derived from the plant's primary or secondary metabolites. Usually, one or more compounds act in synergy to provide antimicrobial effect to the plant. These metabolites can be studied by sophisticated instrumentation techniques such as nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), liquid chromatography−mass spectrometry (LC−MS), gas chromatography−mass spectrometry (GC−MS), among others, depending upon the level of structural elucidation required.

We targeted compounds responsible for the antimicrobial activity in the plant extracts using bioautography and high-performance liquid chromatography coupled with electrospray ionization−quadrupole-time of flight−mass spectrometry (LC−ESI−Q-TOF−MS) of the isolated compounds. LC−ESI−Q-TOF−MS of the whole leaf methanolic extract was also performed to determine the additional compounds that are contributing to the antimicrobial activity of the plant, apart from those present in the isolate.

2 Experimental

2.1 Sample preparation

M. spinosa Roxb. ex Link leaves were sampled from Surgana, Nashik, which was then dried, powdered and stored in airtight container, under dark conditions. For bioautography, an aliquot of 0.5 g of the dried leaf sample was mixed with 5-mL analytical reagent (AR) grade methanol and subjected to ultrasonication three times, 15 min each and was left overnight at room temperature (RT) for maximum extraction. For LC−ESI−Q-TOF−MS of whole leaf extract, an aliquot of 0.5 g of the dried leaf sample was mixed with 5 mL of AR grade methanol and was macerated on a shaker for 2 h at RT, after which the supernatant was collected for the study. Since the initial experiments involved ditch-plate technique followed by LC−ESI−Q-TOF−MS of the crude extract, the method was maintained the same for these two assays, while for bioautography, TLC had to be first performed. The ultrasonication method was finalized since it gave better profile on the TLC plate.

2.2 Thin-layer chromatography

TLC was performed to detect bioactive compounds. TLC plates silica gel $60F_{254}$ were used to run TLC. An aliquot of 5 µL of 100 mg/mL methanolic extract of leaf was applied on the plates using Linomat 5 applicator (CAMAG, Muttenz, Switzerland). The plates were then developed in ethyl acetate−water (10:1, *V/V*) mobile phase until the solvent reached 70 mm of the plate. The plates were dried using hair dryer, observed and scanned under white light and ultraviolet (UV) light (254 nm). The plate was derivatized using natural product reagent and scanned under 366 nm. It was then derivatized with anisaldehyde−sulfuric acid reagent. The plates were scanned again under 366 nm and white light. The compounds present were recorded in the form of peaks on the densitogram.

2.3 Bioautography

Two Gram-positive (*S. aureus, Corynebacterium diphtheriae*) and two Gram-negative (*E. coli, P. aeruignosa*) cultures were selected. Five microliters of leaf methanolic extracts (100 mg/mL) were applied on four TLC silica gel $60F_{254}$ plates and developed in ethyl acetate−water (10:1, *V/V*) mobile phase. For the positive control, 10 ppm cefixime antibiotic for *P. aeruginosa* and 10 ppm ciprofloxacin for *S. aureus, C. diphtheriae* and *E. coli* were applied at the application spot. The TLC plates were placed aseptically in sterile 6-inch Petri plates. A volume of 0.3 mL of 18−24-h-old culture adjusted to 0.1 optical density (OD) at 540 nm was added to 20 mL sterile Mueller−Hinton molten agar tubes and poured on the respective plates for the respective cultures. The plates were incubated at 37 °C for 24 h, after which they were observed for any zone of inhibition on the plate. A few drops of INT (iodonitrotetrazolium dye) indicator (0.2 mg/mL) were added to observe the actively growing culture. The zone on the plate, which is seen as a white patch against a background of pink (growth), is the region where the antibacterial compounds are present.

2.4 Ditch-plate technique

To determine the antimicrobial effect of the crude leaf extracts, 1 g of the dried powder was mixed with 10 mL methanol and macerated for 2 h at RT on a shaker, after which the supernatant was collected, evaporated and the extract was dissolved in 1 mL of 10% dimethylsulfoxide (DMSO). For ditch-plate technique, 6 cm by 1 cm ditch was made in the center of sterile Mueller–Hinton (MH) agar plate into which 1 mL of 1 g extract in 10% DMSO + 2 mL of sterile molten MH agar were poured and allowed to solidify. For culture preparation, 18–24-h cultures of *S. aureus, E. coli, C. diphtheriae* and *P. aeruginosa* were adjusted to 0.1 OD at 540 nm. Each of these was streaked across the ditch on MH agar plate and incubated for 24 h at 37 °C. Following incubation, the growth of the organisms was checked on and around the ditch [23]

2.5 Determination of minimum inhibitory concentration (MIC) by microdilution assay

For MIC, 1 g/mL of the extract was prepared in the same way in 10% DMSO as for the ditch-plate technique. The extract was diluted 1:1 in sterile MHB (Mueller–Hinton broth). For culture preparation, 18–24-h-old culture was adjusted to 0.1 OD at 540 nm, followed by dilution of 1:100 in sterile MHB. On an ELISA microplate, 50 µL of the diluted culture were added from well No. 2–8. 100 µL of the 1:1 MHB-diluted extract were then added to well No. 1 in all rows. From this well, 50 µL were pipetted into the well No. 2. This was mixed well and 50 µL were transferred from well No. 2 to 3 and so on until well No. 7. 50 µL liquid was then discarded from well 7. Following this, 50 µL of 1:100 diluted culture were added to all wells. The dilutions in the wells were as follows: well No. 1: 7.81 mg/mL; 2: 15.62 mg/mL; 3: 31.25 mg/mL; 4: 62.5 mg/mL; 5:125 mg/mL; 6: 250 mg/mL; 7: 500 mg/mL. Well No. 8 was the positive control. 1 mg/mL ampicillin and cefixime were used for antibiotic control, 10% DMSO was used as inhibitory control and the extract itself was used as negative control (no culture). The experiment was performed in triplicate. After the addition of culture, the plate was incubated at 37 °C for 24 h. After incubation, 30 µL of 0.015% resazurin indicator dye were added to each well. Wells remaining blue were assessed as 'No growth' and wells showing pink coloration were assessed as 'Growth'. The dilution at which the wells turned pink was determined as their MIC.

2.6 Preparative TLC

To collect the isolated compound band that shows antimicrobial activity, 20 TLC plates (0.25 mm thick) were applied with 400 µL of 100 mg/mL crude methanolic leaf extract (160 mm width) and developed in the mobile phase ethyl acetate–water (10:1, *V/V*). The band that showed antimicrobial activity was cut into thin small strips and soaked in methanol overnight along with ultrasonication. The isolate was concentrated by evaporating the methanol in nitrogen evaporator. Preparative TLC was repeated until no impurities and a single band was observed. The concentrated isolate was then subjected to LC–ESI–Q-TOF–MS and TLC with a different mobile phase.

2.7 LC–ESI–Q-TOF–MS analysis

LC–ESI–Q-TOF–MS analysis of methanolic leaf extract and bioautography isolate of *M. spinosa* Roxb. ex Link was performed on an Agilent Technologies 6550 iFunnel Q-TOF LC/MS, which included an Agilent 1290 Infinity ultra-high-performance liquid chromatography (UHPLC) having binary pump, thermostatted column compartment, autosampler, thermostat and jet stream. A two-solvent system was used which was as follows: Solvent A: water + 0.1% formic acid & Solvent B: 90% acetonitrile + 10% water + 0.1% formic acid; a gradient started with 95% Solvent A and ended with 100% Solvent B. The flow rate was maintained at 0.3 mL/min and the injection volume was 3 µL. The column used was C18 column (Hypersil Gold 3 micron 100 × 2.1 MM) and the column outlet was connected to a mass spectrometer via dual Agilent jet stream source (AJS) ESI. Both positive and negative modes of ESI were used to ionize the compounds which were channelized using Q-TOF. A photomultiplier plate was used to detect the *m/z* values of the ionized compounds. The MS spectra of the analyzed sample obtained were searched against the Metlin database (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage) to find the probable compounds in the sample by matching their mass values.

3 Results

3.1 Bioautography and TLC

Bioautography of the methanolic leaf extracts of *M. spinosa* Roxb. ex Link showed significant antimicrobial activity against *P. aeruginosa*. The zone of inhibition was observed at the application spot. However, as many other impurities are also present at the application spot, double development was performed. For this purpose, the band from that spot was lifted to 3 cm onto the TLC plate with methanol initially and then developed with the mobile phase ethyl acetate–water (10:1, *V/V*) up to 9 cm for the maximum separation. Figure 1 shows the comparison of the zone of inhibition spot on bioautography TLC plate with derivatized and underivatized TLC of the same extract at different wavelengths.

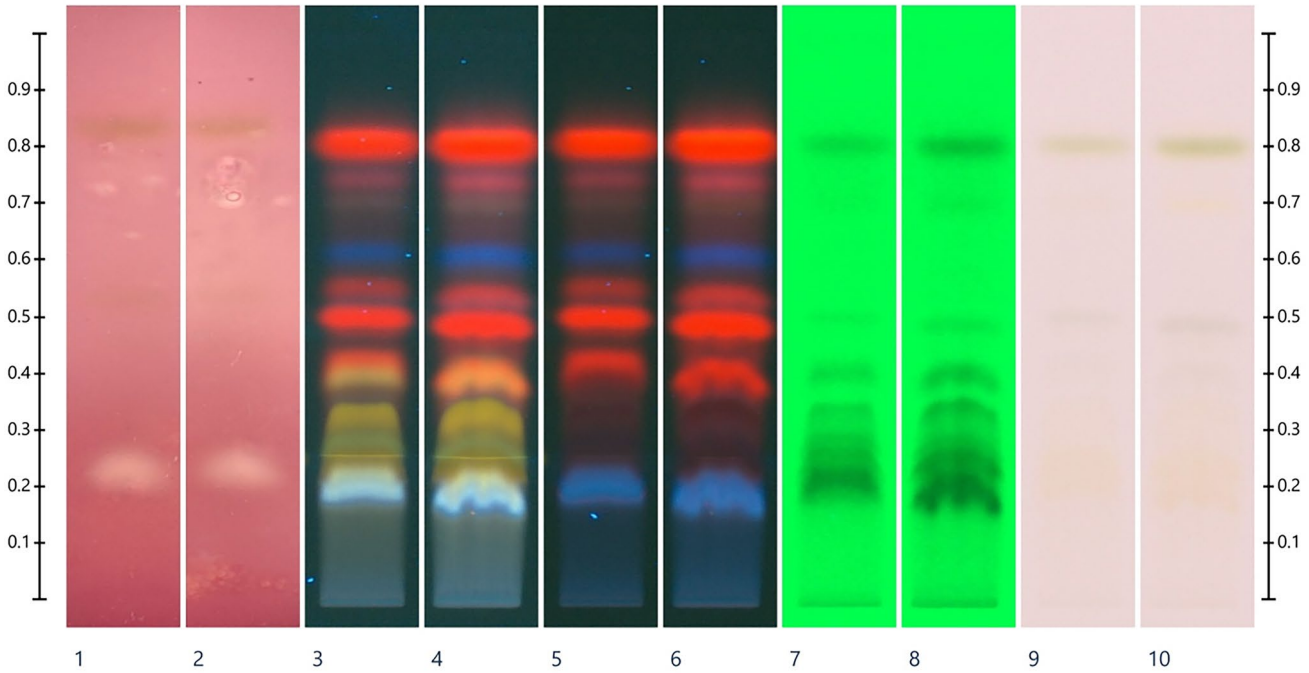

Fig. 1 Comparison of bioautography TLC plate (double development) with derivatized and underivatized TLC of the *Meyna spinosa* Roxb. ex Link leaf methanolic extract under different wavelengths. Lanes 1, 2: bioautography of extract with 5 and 10 µL, respectively; Lanes 3, 4: TLC of the extract with 5 and 10 µL, respectively, derivatized with natural product reagent scanned at 366 nm; Lanes 5, 6: TLC of the extract with 5 and 10 µL underivatized scan at 366 nm; Lanes 7, 8: TLC of the extract with 5 and 10 µL underivatized scan at 254 nm; Lanes 9, 10: TLC of the extract with 5 and 10 µL underivatized scan under white light. The markings on the sides represent the R_F values of the separated bands

From the image comparison, it can be seen that the zone of inhibition corresponds to the light blue band under 366 nm. From 254 nm scan, we also understand that these compound/s can absorb UV light as well. The positive control (antibiotic) was applied on the last lane and can be viewed in Supplementary Fig. 1.

3.2 Isolation of compound by preparative TLC

The compound isolated was subjected to TLC followed by derivatization and bioautography to confirm its bioactivity as shown in Fig. 2. The compound was lifted again to 3 cm and then developed in the mobile phase, *i.e.,* ethyl acetate–water (10:1, *V/V*), which was then derivatized with natural product reagent and anisaldehyde reagent. Another plate was developed by the same method which was subjected to bioautography.

The TLC plate showed a single band, which showed purple-colored band when treated with anisaldehyde reagent, which signifies carbohydrate or a sterol group.

The isolate was also subjected to TLC with a more polar mobile phase, *i.e.,* toluene–ethyl acetate–ethanol–water (2:4:3:1, *V/V*), which separated the isolate into different bands as shown in Fig. 3. All the bands from the isolate showed purple color with anisaldehyde reagent. Upon performing bioautography with the isolated bands of the bioautography isolate, no individual band revealed any antimicrobial activity (Fig. 3, lanes 11 and 12). Therefore, antimicrobial activity is hypothesized to be due to the synergistic action of more than one compound.

3.3 Ditch-plate technique and MIC

The crude methanolic extract tested against four organisms showed inhibition of only *S. aureus* by ditch-plate technique. The extract inhibits the growth on the ditch and not around the ditch. MIC by microdilution method of the extract against *P. aeruginosa* and *S. aureus* was performed. The MIC of methanolic leaf extract was found to be 375 mg/mL for *S. aureus* and 125 mg/mL for *P. aeruginosa*. Positive control and the negative control were maintained for MIC.

3.4 LC–ESI–Q-TOF–MS analysis

The crude methanolic leaf extract and the bioautography isolate of *M. spinosa* Roxb. ex Link, both were subjected to LC–ESI–Q-TOF–MS to identify compounds responsible for antimicrobial activity. Table 1 contains MS data regarding the compounds that are present in the bioautography isolate and has literature related to antimicrobial activity.

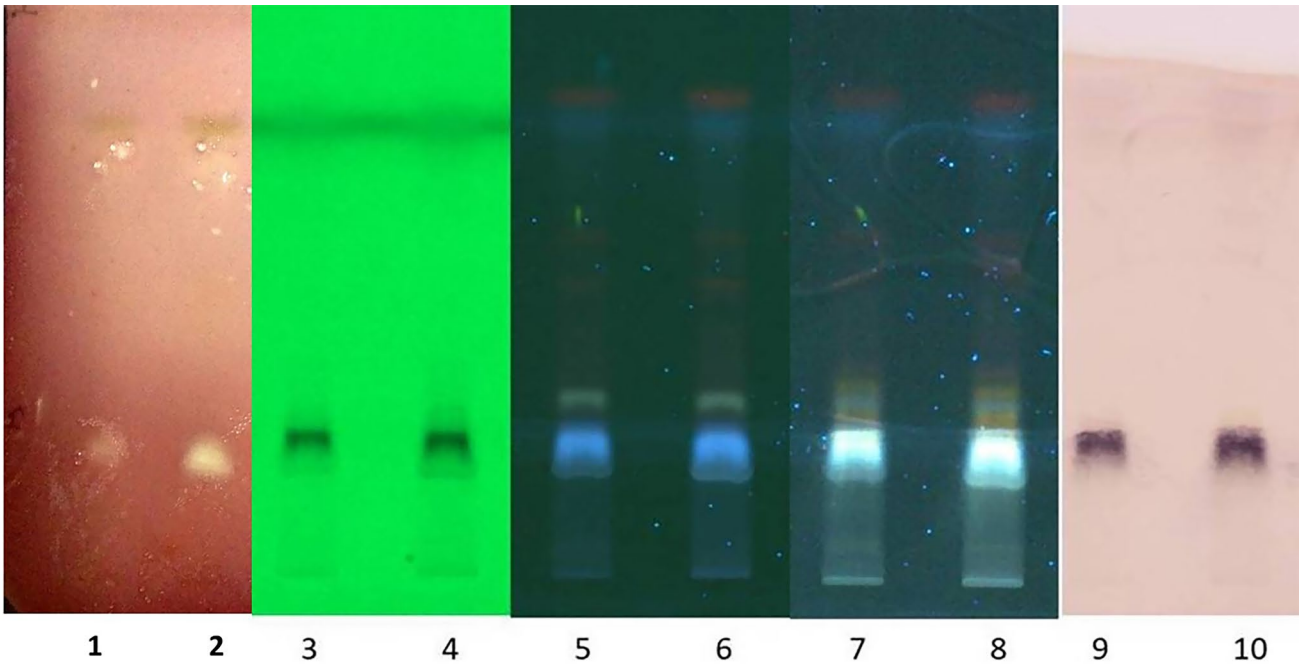

Fig. 2 Comparison of bioautography isolate with its TLC scan under different wavelengths. Lanes 1, 2: bioautography of isolate with 5 and 10 µL, respectively; Lanes 3, 4: TLC with 5, 10 µL isolate scanned under 254 nm; Lanes 5, 6: TLC with 5 and 10 µL isolate scanned under 366 nm; Lanes 7, 8: TLC with 5 and 10 µL isolate derivatized with natural product reagent scanned under 366 nm; Lanes 9, 10: TLC with 5 and 10 µL isolate derivatized with anisaldehyde reagent and scanned under white light

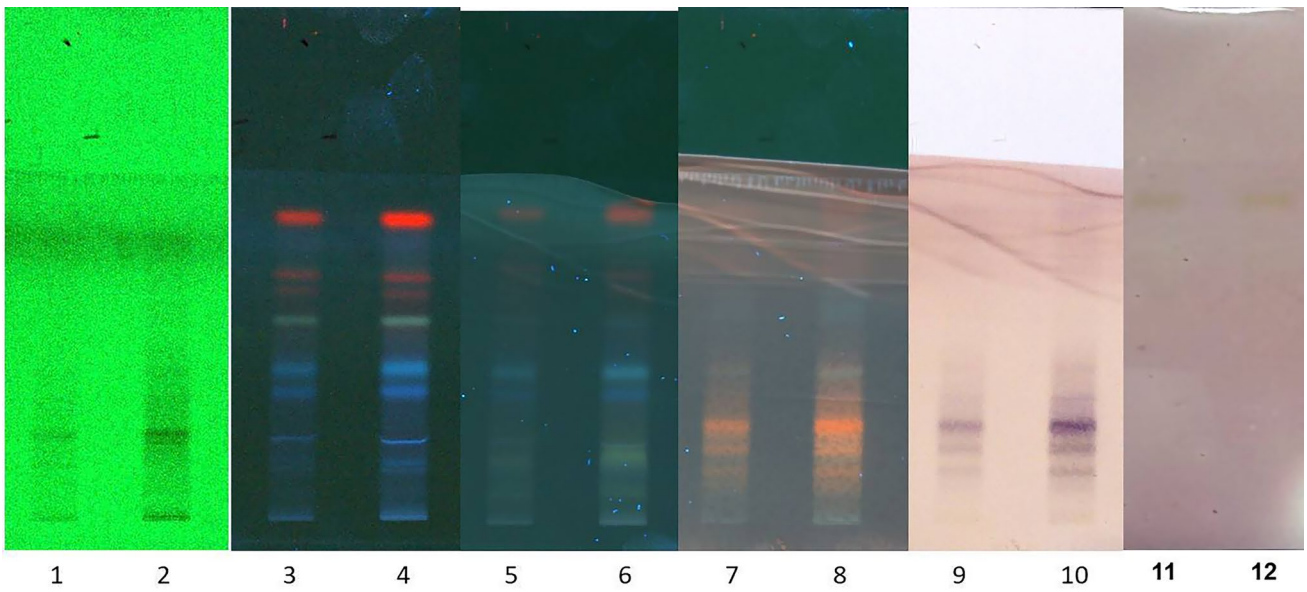

Fig. 3 Separation of bioautography isolate using the mobile phase toluene−ethyl acetate−ethanol−water (2:4:3:1, *V/V*). Lanes 1, 2: TLC of isolate with 5 and 10 µL, respectively, under 254 nm; Lanes 3, 4: TLC of isolate with 5 and 10 µL, respectively, under 366 nm; Lanes 5, 6: TLC of isolate with 5 and 10 µL, respectively, under 366 nm post-derivatization with natural product reagent; Lanes 7, 8: TLC of isolate with 5 and 10 µL, respectively, under 366 nm post-derivatization with anisaldehyde reagent; Lanes 9, 10: TLC of isolate with 5 and 10 µL, respectively, derivatized with anisaldehyde reagent and scanned under white light. Lanes 11, 12: bioautography with the separated isolated compounds

Table 1 MS data regarding the compounds that are present in the bioautography isolate and has literature related to antimicrobial activity

Compound name	Retention time	Mass	Formula	Mode of ionization
5E,8E,11E-Hexadecatrienoic acid	1.064	250.1901	C16 H26 O2	+ESI
10-Deoxymethymycin	5.952	453.3095	C25 H43 N O6	+ESI
Dihydrodeoxystreptomycin	9.825	567.2808	C21 H41 N7 O11	+ESI
Phthalic acid mono-2-ethylhexyl ester	15.304	278.1475	C16 H22 O4	+ESI
Eicosanedioic acid	11.553	342.283	C20 H38 O4	+ESI
Docosanedioic acid	13.259	370.3138	C22 H42 O4	+ESI
Deferoxamine	13.512	560.3641	C25 H48 N6 O8	+ESI
Adonixanthin	13.528	582.4078	C40 H54 O3	+ESI
Tetracosanedioic acid	15.112	398.3444	C24 H46 O4	+ESI
Cerebroside C	16.033	753.5719	C43 H79 N O9	+ESI
Oleamide	18.206	281.2674	C18 H35 N O	+ESI
Stearamide	19.323	283.2831	C18 H37 N O	+ESI

Table 2 contains MS data regarding the compounds present in the crude methanolic leaf extract of *M. spinosa* Roxb. ex Link. The extract also contains the compounds listed in Table 1 but has not been listed here to avoid repetition. The MS data chromatogram for both samples (BI and crude methanolic extracts) can be found in the attached Supplementary Material S1−S4.

4 Discussion

Previous studies on *M. spinosa* Roxb. ex Link extract indicate that it possesses antimicrobial activity. A few have also elucidated certain compounds in the extract. However, a correlation between the antimicrobial activity and the compounds responsible for it is reported for the first time by bioautography.

Table 2 MS data regarding the compounds present in the crude methanolic leaf extract of *Meyna spinosa* Roxb. ex Link

Compound name	Retention time	Mass	Formula	Mode of ionization
Tiletamine	0.814	223.1044	C12 H17 N O S	+ESI
Tranylcypromine glucuronide	1.992	309.1198	C15 H19 N O6	+ESI
Alprazolam	2.271	308.0886	C17 H13 Cl N4	+ESI
4-Hydroxylevamisole glucuronide	2.798	396.101	C17 H20 N2 O7 S	+ESI
Famciclovir	4.326	321.1432	C14 H19 N5 O4	+ESI
Acetylaminodantrolene	4.334	326.0986	C16 H14 N4 O4	+ESI
Hieracin	6.589	302.0407	C15 H10 O7	+ESI
Kaempferol	6.905	286.046	C15 H10 O6	+ESI
Quercetin	6.931	448.0977	C21 H20 O11	+ESI
9,12-Hexadecadienoic acid	9.49	252.2099	C16 H28 O2	+ESI
3,4,5-Trihydroxystilbene	13.33	228.077	C14 H12 O3	+ESI
13-oxoODE	15.045	294.2179	C18 H30 O3	+ESI
(Z)-2-hexacos-17-enamidoethanesulfonic acid	16.323	501.3872	C28 H55 N O4 S	+ESI
Tomatidinol	16.691	413.336	C27 H43 N O2	+ESI
Campesta-5,25-dien-3beta-ol	16.746	398.3549	C28 H46 O	+ESI
Swietenine	18.49	568.2684	C32 H40 O9	+ESI
Khivorin	19.097	586.2787	C32 H42 O10	+ESI
Khayanthone	19.099	570.2838	C32 H42 O9	+ESI
Trandolapril glucuronide	19.665	606.281	C30 H42 N2 O11	+ESI
O-desacetylcephalothin	7.095	354.0302	C14 H14 N2 O5 S2	−ESI

From the preparative TLC and bioautography data, we can deduce that more than one compound is responsible for the antimicrobial activity of the methanolic extracts of *M. spinosa* Roxb. ex Link against *P. aeruginosa*. TLC with a different mobile phase separated the bioautography isolate into different bands, which gave a purple colored reaction with anisaldehyde reagent. Further LC−ESI−Q-TOF−MS of this isolate showed two potential antibiotic derivatives, viz., 10-deoxymethymycin and dihydrodeoxystreptomycin fatty acids and amides were found to be present in the isolate. All the significant compounds deduced from LC−MS have reported to have certain antimicrobial activity, which is as follows:

The antibiotic derivative 10-deoxymethymycin is a 3,4,6-trideoxy-3-(dimethylamino)-β-D-xylo-hexoside of 10-deoxymethynolide, which is a carbohydrate derivative of macrolide [24] and dihydrodeoxystreptomycin has a glycoside entity [25] that explains the violet-colored band reaction with anisaldehyde reagent. Both, being antibiotic derivatives, should exhibit antimicrobial activity. Deferoxamine, iron chelator inhibits multiplication in certain microbes [26]. Phthalic acid was detected in the essential oil of *Leea indica* (Burm. f.) Merr. flowers which was reported to have moderated the antibacterial activity against Gram-positive and Gram-negative organisms [27]. There is also the presence of certain fatty acids such as 5E,8E,11E-hexadecatrienoic acid, eicosanedioic acid and tetracosanedioic acid, which were reported to be present in the crude extracts of *Spirulina platensis* [28]. Hexade-catrienoic acid was identified as one of the active constituents present in ethanol extracts of cyanobacterium *Nostoc verrucosum*, which inhibited Gram-positive bacteria [29]. Fatty acids have been previously reported to have antimicrobial activity, especially against Gram-positive bacteria [30]. Their mode of action is postulated to be due to one of the following: surfactant activity [31], inducing oxidative stress [32], uncoupling adenosine triphosphate (ATP) synthesis [33] and increased membrane fluidity [34, 35]. Fatty acid amide derivatives such as oleamide and stearamide were detected, which also have been previously reported to be present in the extracts of a few plants, algae and seed coats, which showed antibacterial activity [36–39]. Literature reports that amide derivative of fatty acids has antimicrobial activity [30, 40, 41]. All or a few of these compounds are responsible for the antimicrobial activity against *P. aeruginosa.*

LC−ESI−Q-TOF−MS of the crude methanolic leaf extracts of *M. spinosa* Roxb. exLinkshows compounds that include secondary metabolites. Of these, hieracin [42], swietenine [43] and 9,12-hexadecadienoic acid [44] have antimicrobial activity; Kaempferol [45], 3,4,5-trihydroxystilbene [46] and quercetin [47] have antioxidant activity; tranylcypromine glucuronide [48] and famciclovir [49] have antiviral activity. The synergistic activity of these compounds is postulated to be inhibiting the growth of *S. aureus*.

TLC separated the compounds in the crude methanolic leaf extract of *M. spinosa* Roxb. ex Link, thus ruling down the compounds that were responsible for the antimicrobial activity against *P. aeruginosa*. This paper reports the presence of tentative secondary metabolites and fatty acids and amides in *M. spinosa* Roxb. ex Link leaf extract and their correlation to its antimicrobial activity for the first time. For this reason, the future scope of this work lies in elucidating the structures of the compounds which can be done by NMR.

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Author contribution All authors contributed to the study conception and design. SK, SD and AP prepared the material, collected the data and its analysis. SK prepared the first draft of the manuscript and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Availability of data and material Data will be available on request.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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