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Original Article

Separation of Bio-active Compounds and *in vitro* Antibacterial Potency of *Callistemon linearis* Floral Extract with Mechanism of Action

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ABSTRACT

Introduction: Infection caused by bacteria is treated with antibiotics which caused several side effects in human and animals. This problem enforced scientist to work out plant originated compounds for treatment of several disease caused by morbific bacteria. **Objectives:** Present work revealed isolation of bioactive phytocompounds from different fractions of *Callistemon linearis* flower ethanol extract by chromatographic technique and determination of their antibacterial potency against selected bacteria with mode of action *in vitro*. **Materials and Methods:** Antibacterial potency of four different fractions of ethanol extract were done quantitatively via Agar well diffusion method along with determination of Minimum Inhibitory Concentration, and Minimum Bactericidal Concentrations. Further, phytochemical analysis, Fourier Transform Infrared spectroscopic analysis, thin Layer Chromatography separation and Gas Chromatography Mass Spectroscopy analysis of bioactive fractions were done simultaneously to assess the presence of bioactive phytocompounds responsible for the antibacterial potency of the fractions. Time-kill assay, Nucleic acid and protein leakage assay, and FESEM study were employed to determine the mode of activity of the isolated active phytocompounds on the tested bacteria. **Results:** Maximum antibacterial activity was observed in NHEX of ethanol extract against *Escherichia coli* (MTCC 739). Chromatographic separation of NHEX detected several bioactive compounds. Assessment of Time-kill assay in treated and untreated bacterial strains determined that bioactive compound of NHEX was bactericidal in nature. Nucleic acid and protein leakage assay of bacterial cells with isolated active compound showed cell damages and FESEM images strongly ascertained this data. **Conclusion:** The *C. linearis* floral floral extract possesses antibacterial activities against selected pathogens and causes cellular damages.

Keywords: *Callistemon linearis*, Antibacterial activity, FT-IR, GC-MS analysis, Bioactive compounds, Mechanism of action.

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INTRODUCTION

Deadly infectious diseases caused by pathogenic bacteria e.g., *Staphylococcus aureus* and *Escherichia coli* were documented as serious public threat in several countries.[1] In U.S. every year 2.8 million infections are caused due to infectious pathogens and about 35,900 deaths are also reported per annum so far.[2] At present, the main basis for dealing with these pathogenic bacteria is commercially available antibiotics. However, overuse, misuse, and repetitive exposure of antibiotics lead to increment of antibacterial resistance and development of bacterial multidrug resistant strains.[3,4] Synthetic antibiotics also possesses numerous undesirable materials which may cause side effects in human and other animals.[5] This rapid development of multidrug resistant bacteria leads the inventors to search new antibacterial drugs with lesser resistance affinity. World possesses a wide variety of medicinal plants and phytocompounds could be isolated from the vast array of medicinal plants.[6] Novel compounds isolation and determination of antimicrobial activity with these compounds could play a great role in drug development process.[7,8] Medicinal plant is a safest option in this scenario because compounds isolated from them is less harmful to human and other animals.[9]

Callistemon linearis, the narrow-leaved bottlebrush is a stiff shrub upto 3-meter height having linear leaves on woody stems. In the month of spring fiery red bottlebrush flowers are bloomed out. *C. linearis* belong to an evolutionary advanced family Myrtaceae which consists of several medicinally important members. The genus *Callistemon* comprises of 34 medicinally important species those exhibit several bioactivities like antifungal, antibacterial,

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cytotoxicity, cardioprotective, anthelminthic, neuroprotective, etc.[10-12] Several phytochemicals like flavonoids, terpenoids, etc. has detected from this species.[13] In previous mention literature leaves extract of *C. linearis* plant were reported for antibacterial and antioxidant activities.[14] It is important to understand the plant extracts capability on bacterial cell membrane i.e., the main basis of cellular structure damage in bacteria. Till date no detailed study on antibacterial activity and mechanism of action of active compounds of *C. linearis* floral extracts as antibacterial agents have been conducted. Therefore, the goal of this study is the isolation of bioactive compounds and evaluation of *in vitro* antibacterial activity of bioactive fraction of *C. linearis* floral ethanol extract on selected pathogenic bacteria along with explicating the possible mechanism of action.

MATERIALS AND METHODS

Plant Collection

Healthy and matured flowers were collected apparently during the flowering season of *C. linearis* from the surroundings of Burdwan, West Bengal, India and the plant specimen (Voucher specimen no. GCSD 01) was properly authenticated by plant taxonomist Dr. Ambarish Mukherjee, former professor of Department of Botany, Burdwan University.

Solvent Extraction

Collected flowers were rinsed thoroughly with tap water and distilled water for complete removal of dust and debris from the flowers. Then excess water was wiped out and left it for drying for around two weeks at room temperature. Shed dried, 200gm flowers were kept in thimble of Soxhlet apparatus and 2000 mL of absolute alcohol (99.9% ethanol) was taken in the round bottom of Soxhlet apparatus and placed in heating mantle.[15] The solvent was extracted at 40°C for seventy-two hours with maximum 8 hr per day. Extract that produced was filtrated through Whatman No. 1 filter paper and filtrate was dried and concentrated at room temperature. After complete evaporation of solvent, the yield of ethanol extract was 6.42% (w/w). It was then taken for fractionation serially with n-Hexane, Ethyl acetate, Dichloromethane and distilled Water.[16,17] For fractionation, 10 g of dry ethanol extract were mixed with 100 mL of distilled water and was taken in a separating funnel, then shaken it vigorously and rest it for settlement. Then 100 mL of n-hexane solvent was thoroughly mixed with this and allowed to settlement of solvent. After that, the aqueous layer was removed from the separating funnel and the upper portion of the separating funnel was poured into a beaker to get solvent fraction. Separation with n-hexane was continued until after adding n-hexane, clear solvent came out. Collected n-hexane fraction (NHEX) was left open in a safe place for evaporation of the solvent and after complete evaporation of solvent kept them in refrigerator. For each solvent extract same process was repeated. These dried fractions were used for further experiments.

Antibacterial Bioassay

Collection of Bacterial Pathogens

Pure cultures of four Gram-positive pathogenic bacterial stains specifically, *S. aureus* (MTCC 2940), *Bacillus subtilis* (MTCC 441), *B. licheniformis* (MTCC 530), and *B. mycoides* (MTCC 7343), and four Gram-negative pathogenic bacterial stains *E. coli* (MTCC 739), *Pseudomonas aeruginosa* (MTCC 2453), *P. fluorescens* (MTCC 103), and *P. putida* (MTCC 1654) were used. Bacterial strains were maintained on Nutrient Agar slants at 4°C temperature at our laboratory. Prior to antibacterial study, the pathogens were sub-cultured onto Muller-Hinton Agar (MHA) and bacterial inoculums were prepared in Muller-Hinton Broth (MHB). Concentrations of the bacterial inoculums were adjusted to 0.5 McFarland Standard before use.

Evaluation of Antibacterial Bioassay

For antibacterial assay standard procedures of Perez *et al.* (1990) and Burman and Chandra (2021) were used.[18,19] One milligram each of the n-hexane fraction (NHEX), ethyl acetate fraction (EA), dichloromethane fraction (DCM) and aqueous fraction (AQ) of ethanol extract was thoroughly mixed with 1 mL of 0.1% v/v Dimethylsulphoxide (DMSO) for antibacterial bio-assay. Autoclaved Nutrient Agar was solidified in the Petri-plates under laminar airflow. After that, four wells were made in each Petri-plate by using a 6 mm sterile metal cork borer. Bacterial culture was spread onto the Agar plates by sterile plastic spreader. In each hole, 100 µl of 1mg/mL extract was poured under laminar flow. A set of negative control (0.1% v/v DMSO) and a set of positive control (Tetracycline of 10 µg/mL concentration) were also used in this experiment. Then the plates were kept at room temperature for one hour to allow the extract to diffuse through the Agar medium under aseptic condition. Then the plates were incubated at 37°C for 24 hr. The antibacterial activity was indicated by diameter (in millimetres) of the clear zones around the wells. Three replicates for this experiment were completed and mean values were taken for each bacterium.

Antibacterial Activity of Bioactive Compounds

For determination of antibacterial potentiality of bioactive compounds, standard procedure of Perez *et al.* (1990) and Burman and Chandra (2021) were used.[18,19] One milligram of bioactive compounds (R_f value, 0.278) was thoroughly mixed with 10 mL of 0.1% v/v DMSO for antibacterial activity evaluation. Two wells were made in each Petri-plate by using a 6 mm cork borer and bacterial culture was spread onto the Agar plates by sterile spreader. In each hole, 100 µl of 100µg/mL extract was poured under laminar flow. 0.1% v/v DMSO was used as negative control and Standard Broad-Spectrum antibiotic Tetracycline (at 10 µg/mL concentration) was used as positive control. The plates were then incubated at 37°C for 24 hr. The antibacterial activity

was determined by measuring the clear zone of inhibition in millimetres. Three replicates were taken for each experiment.

Measurement of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of bioactive compounds

For determination of MIC and MBC values, procedure of Owuama *et al.* (2017) was followed with slight alterations.[20] Different concentrations of bioactive fraction were made like50, 45, 40, 35, 30, 25, 20, 15, 10, and 5 μg/mL with 0.1% v/v DMSO. 100 μl of each concentration of bioactive fractions was added to bacterial inoculums in 1 mL Nutrient Broth (0.5 McFarland Standard) for each bacterial strain. Test tube containing bacterial inoculum in Nutrient Broth and 0.1% v/v DMSO was used as control. Then these Test tubes were incubated at 37°C for 24 hr and MIC was determined. MBC was determined by serially dilution of concentrations that showed no noticeable growth after bacteria were inoculated in Nutrient Agar plate and incubated for 24 hr at 37°C. The assay was performed three times independently.

Determination of Mechanism of Antibiosis of Bioactive Compounds

Ratios of the MBC/MIC were calculated for determination of the mechanism of antibiosis of bioactive compounds. If the ratio of MBC/MIC is ≤ 4, then these compounds may act as Bactericidal and if the ratio of MBC/MIC is > 4, they act as Bacteriostatic.[21-23]

Identification of Bioactive Compounds

Phytochemical Analysis and FTIR Analysis

Preliminary phytochemical analysis was carried out for identification of basic phytochemicals in best effective extract, i.e., NHEX of ethanol extract by the standard procedure.[24,25] For identification of functional group in extract, FTIR analysis was done. NHEX was thoroughly mixed with KBr at room temperature and pellet was made. After that this pellet was scanned with a scanning range from 400 to 4500 cm^{-1} in FTIR spectrometer (Jasco, FT/IR-4700). KBr pellet without extract was used as control in respect of NHEX-KBr pellet. For validation of the result every experiment was repeatedly done.

Isolation of Bioactive compounds by Thin Layer Chromatography (TLC) and GC-MS analysis

Commercially available TLC plates were used (TLC Silica gel 60 F_{254} plates, 20×20 cm) for isolation of bioactive compound/s from NHEX. Sample was loaded at one end of the plate and developed it in solvent chamber containing n-heptane and ethyl acetate extract (in 8:2 ratio). After complete evaporation of solvents, bands were detected in developing plate by UV chamber. Bioautography of the active compounds in TLC plate was done by the procedure of Das *et al.* (2021).[26] After identification of bioactive bands in silica plate band (R_f value, 0.278), those bands were collected from the TLC plates after development of bands with the specific mobile phase. Bioactive was scraped and dissolved in n-hexane. Then, sample was filtered using Whatman no. 41 paper to remove silica. The isolated sample purification was assessed by GC-MS. For GC-MS analysis, Thermofisher Scientific India Pvt. Ltd., TRACE GC ultra with POLARIQ MS (30m×0.25mm×0.25μm) was used. 9 μL sample was injected for GC-MS analysis. Initial oven temperature was 60°C, which held for 3 min, the temperature increased gradually and attained a final temperature of 280°C. Helium was used as carrier gas and it was allowed to flow at the rate of 1 mL/min. Total run time was 60 min. The MS transfer line was maintained at a temperature of 280°C. Identification of the bioactive compound on the Mass spectrum was done by using the data on NIST (National Institute of Standard Technologies) library.

Antibacterial Mechanism of Action

Time Kill Assay Analysis of bioactive compounds

Antibacterial Time kill Analysis was carried out to evaluate bactericidal activity of bioactive compounds, according to the method of Sim *et al.* (2014) and Appiah *et al.* (2017) with little modifications.[27,28] Bioactive fractions were dissolved in two different concentrations (MIC and 2 × MIC) of 0.1% v/v DMSO. All the tested bacterial inoculums (at 1×10^8 CFU/mL concentration) and the bioactive fractions were added to 2 mL Nutrient broth in test tube. 0.1% v/v DMSO was taken as control and they were incubated at 37°C. Samples from each test tube were collected at selected time intervals (0, 1, 3, 6, 12 and 24 hr), serially diluted and plated into Nutrient Agar. After 24 hr of incubation at 37°C, CFU/mL on the Agar Plates were counted.

Bacterial Cell Membrane Integrity Loss by Bioactive Compounds

Leakage of cellular constituents and protein of each bacterium treated with bioactive compounds was determined by the process of Zhang *et al.* (2015) with slight alterations.[29] Each bacterial culture was grown at 37°C at Nutrient Broth. After overnight incubation, bacterial cells were washed with Phosphate buffer solution (pH, 7.4) and suspended in Phosphate buffer solution. Then bacterial cells were incubated with bioactive fractions of different concentrations (Control, MIC and 2 × MIC) at 37°C for 6 hr. Then samples were removed from incubator and centrifuged at 11000×g for 5 min. Collected supernatants were used for protein estimation by Bradford protein assay.[30] Cellular constituents mainly nucleic acids were determined by measuring the absorption of supernatants at 260 nm by UV-VIS-spectrophotometer (Shimadzu, UV-1800). Experiments were repeated thrice for obtaining accurate measurements.

Scanning Electron Microscopic observation of selected bacteria

Antibacterial potency of bioactive fraction on one Gram-positive strain namely, *B. subtilis* and one Gram-negative strain namely, *P. aeruginosa* were observed by Field Emission Scanning Electron Microscope (FESEM) observation. Bacterial cells (of 1×10^8 CFU/mL concentration) were treated with MIC concentration of bioactive fraction and kept it in incubator for 24 hr at 37°C. Bacterial cells with 0.1% v/v DMSO treatment were taken as control. After 24 hr, bacterial cells were suspended in 0.1 M PBS (pH 7.4) and centrifuge the sample at 1500 g for 10 min. Next the precipitation was washed with PBS solution and spread onto the cover-slip and allowed to air-dry. Then the samples were fixed in 2.5% glutaraldehyde and alcohol solution. Samples were coated with gold particles and observed it under FESEM (Carl Zeiss, UK. Model: Sigma).

Statistical Analysis

All the experiments were repeatedly done [triplicate] to confirm the data. The data were analysed by Microsoft Excel Software (MS Excel 2007; Microsoft Corporation) and stated as mean ± Standard Deviation (SD) of three replicates.

RESULTS AND DISCUSSION

Pathogens develop several mechanisms of resistance against available antibiotics, which enforce the search of novel antimicrobial compounds of good effectiveness against bacteria for the benefit of mankind. Most of the plants are rich source of secondary metabolites and from these natural sources isolation of pure compounds can be done for exploring their potential antimicrobial efficacies.[31,32]

Antibacterial Assay

Results of different extracts against the selective Gram-positive and Gram-negative bacteria were depicted in Table 1. Tetracycline (10 µg/mL) showed variability in efficacy against the bacteria under study whereas 0.1% v/v DMSO showed no Zone of inhibition. Current study established antibacterial potency of floral extracts of *C. linearis* against eight human and fish infective bacterial strain by Agar well diffusion method. All fractions of ethanol extracts and positive control Tetracycline (10 µg/mL) showed good range of inhibition against the tested pathogens. Among all the extracts tested NHEX showed highest inhibition zone in *E. coli* (21.5 ± 0.50 mm) followed by *P. aeruginosa* (20.33 ± 0.47 mm), *P. fluorescens* (19.50±0.40 mm), and *P. putida* (19.17 ± 0.84 mm). Among the Gram-positive bacteria tested best inhibition zone was found in *S. aureus* (20.67 ± 0.57 mm) followed by *B. licheniformis* (20.17 ± 0.23 mm), *B. mycoides* (19.67 ± 0.63 mm), and *B. subtilis* (19.33 ± 0.47 mm). DCM was the second-best effective extract and highest zone in this extract was found against *E. coli* (18.33 ± 0.57mm). DCM also had efficacies against the Gram-negative bacteria, *P. aeruginosa* (17.33 ± 0.47 mm), *P. putida* (16.33 ± 0.47 mm) and *P. fluorescens* (15.33 ± 0.47 mm). Gram-positive bacteria, *B. subtilis* (16.33 ± 0.47 mm) followed by *B. mycoides* (15.67 ± 0.47 mm), *S. aureus* (15.33 ± 0.57 mm) and *B. licheniformis* (14.83 ± 0.23 mm) were also susceptible to DCM. Other two fractions, EA and AQ of ethanol extract also effective against selected tested pathogens. Positive control Tetracycline (10 µg/mL) showed various degree of inhibition zone and highest zone of inhibition was resulted in *S. aureus* (14.33±0.57 mm). It had antibacterial potencies against the Gram-positive bacteria B. *subtilis* (12.50 ± 0.50 mm), *B. mycoides* (10.67 ± 0.57mm) and *B. licheniformis* (11.33 ± 0.57 mm). In Gram-negative bacterial strain *E. coli* (11.83 ± 0.76 mm) was most susceptible to Tetracycline followed by *P. putida* (10.67 ± 0.57 mm), *P. aeruginosa* (9.83 ± 0.76 mm) and *P. fluorescens* (9.67±0.57 mm). Negative control i.e., 0.1% v/v DMSO, showed no inhibition zone against any of the tested strains. Thus, all tested fractions of ethanol extract of *C. linearis* flower were found to be effective against the tested pathogens and in most cases gave better results than positive control. Figure 1 presents antibacterial efficacy of bioactive compounds on different bacterial strains. Best inhibition zone was found in *E. coli* (15.33±0.47 mm) followed by *P. aeruginosa* (15.17±0.23 mm). Rest of the bacteria are also susceptible to active fraction. Table 2 presents results of MIC, MBC and mechanism of antibiosis. Data of MIC and MBC values of bioactive compounds and MBC/MIC values clearly indicate strong broad-spectrum bactericidal activity of the active fraction of the respective plant part for the first time.

In an earlier report by Kavita and Satish (2013) explained that they had found antibacterial activity of *C. lanceolatus* leaf extracts against *E. coli, P. aeruginosa, S. aureus*, and *B. subtilis* at 100 mg/mL concentration.[33] But our study indicates a much better result at low concentration. In another experiment Cock (2012) reported antibacterial activity of floral extract of *C. citrinus* and *C. salignus* at 37mg/mL concentration against 14 bacteria, out of which only 9 bacteria showed inhibition zone.[34] So, we can accomplish that NHEX fraction of ethanol floral extract of *C. linearis* shows better antibacterial activity compared to other plant species of the genus *Callistemon*.

Identification of Bioactive Compounds

Peaks detected from FTIR analysis (Figure 2) are tabulated in Table 3. Phytochemical analysis of best active fraction showed the presence of phenolic, terpenoid and flavonoid compounds. They are very useful secondary metabolites of plants and can cause several damages to microbes.[36,37] FTIR-spectrum data (Figure 2) of NHEX extract shows a number of absorption bands implying that several active functional groups are present in NHEX of *C. linearis* flower. Existing literatures report that various functional groups such as alcohol, aromatics, esters, aldehydes, etc. have various degree of antibacterial activity.[38,39] From GC-MS analysis (Figure 3 and Table 4) of the bioactive fraction band (R_f value, 0.278), б-Phellandrene, a terpene compound has been detected,

Table 1: Antibacterial activity of n-hexane fraction (NHEX), Ethyl acetate fraction (EA), Dichloromethane fraction (DCM), and Aqueous fraction (AQU) of ethanol extract of *Callistemon linearis* flower.

Name of Bacterial isolates							
Gram-positive strains				Gram-negative strains			
Staphylococcus aureus (MTCC 2940)	*Bacillus subtilis* (MTCC 441)	*Bacillus mycoides* (MTCC7343)	*Bacillus licheniformis* (MTCC530)	*Escherichia coli* (MTCC 739)	*Pseudomonas aeruginosa* (MTCC 2453)	*Pseudomonas fluorescens* (MTCC103)	*Pseudomonas putida* (MTCC 1654)
Zone of inhibition of NHEX (in mm)							
20.67±0.57	19.33±0.47	19.67±0.63	20.17±0.23	21.5±0.5	20.33±0.47	19.50±0.40	19.17±0.84
Zone of inhibition of EA (in mm)							
12.33±0.57	14.33±0.47	14.33±0.47	13.33±0.23	14.67±0.57	15.00±0.81	12.67±0.47	13.5±0.40
Zone of inhibition of DCM extract (in mm)							
15.33±0.57	16.33±0.47	15.67±0.47	14.83±0.23	18.33±0.57	17.33±0.47	15.33±0.47	16.33±0.47
Zone of inhibition of AQ (in mm)							
11.33±0.57	11.33±0.47	12.33±0.47	11.50±0.40	12.17±0.76	11.67±0.47	12.33±0.47	11.17±0.84
Zone of inhibition of positive control (in mm)							
14.33±0.57	12.50±0.50	10.67±0.57	11.33±0.57	11.83±0.76	9.83±0.76	9.67±0.57	10.67±0.57
Zone of inhibition of negative control (in mm)							
0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

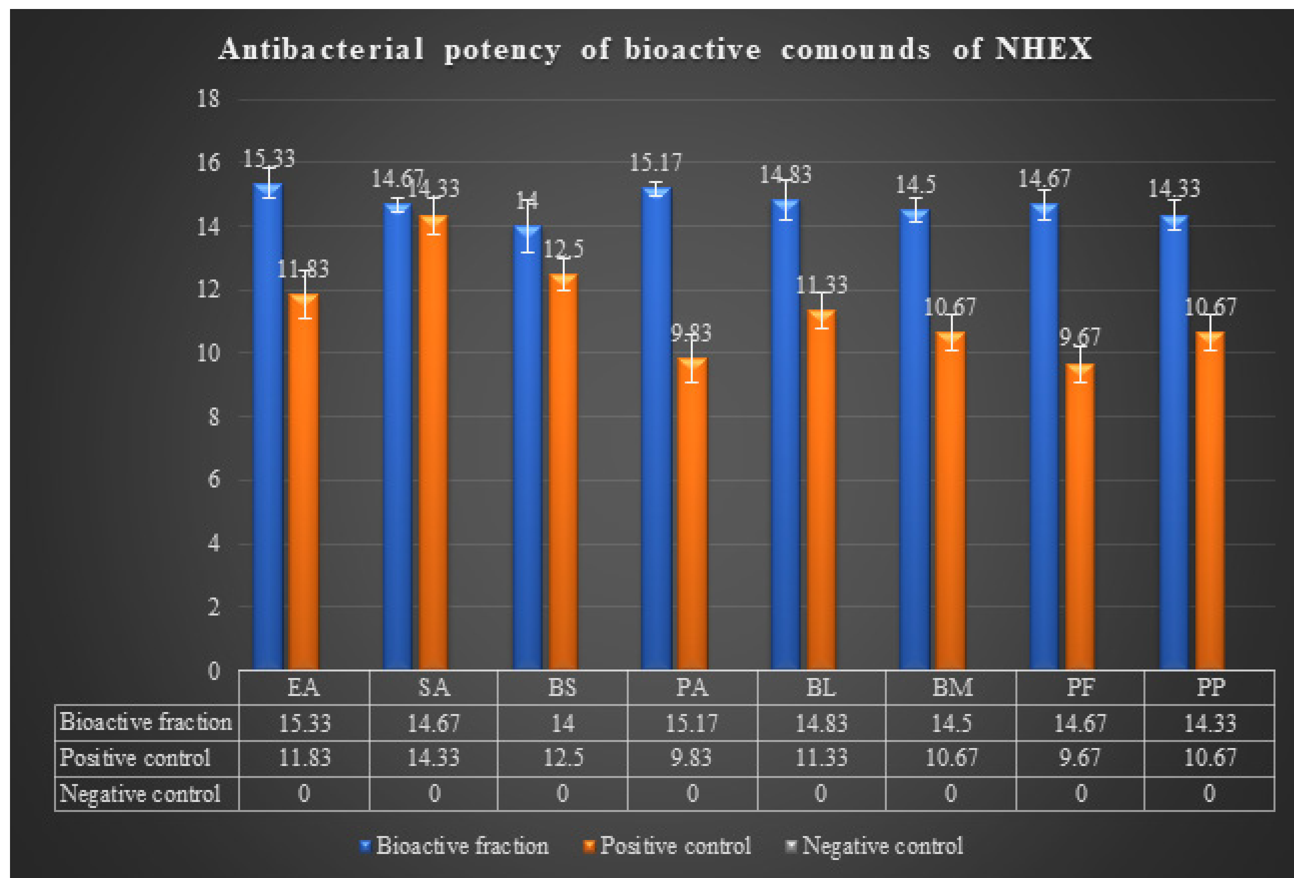

Figure 1: Antibacterial potency of bioactive ingredients of *Callistemon linearis* flower's n-hexane fraction (NHEX) of ethanol extract against selected bacteria (EA= *Escherichia coli*, SA= *Staphylococcus aureus*, BS= *Bacillus subtilis*, PA= *Pseudomonas putida*, BL= *B. licheniformis*, BM= *B. mycoides*, PF= *P. fluorescens* and PP= *P. putida*).

Table 2: MIC, MBC and MBC/MIC value of bioactive ingredients of *Callistemon linearis* flower's n-hexane fraction (NHEX) of ethanol extract (SA= *Staphylococcus aureus*, BL= *B. licheniformis*, BM= *B. mycoides*, BS= *Bacillus subtilis*, EA= *Escherichia coli*, PA= *Pseudomonas putida*, PF= *P. fluorescens* and PP= *P. putida*)

	Bacterial strain							
	SA	BL	BM	BS	EA	PA	PF	PP
MICs (in µg/mL)	23.33±2.88	25.00±0.00	21.67±2.88	21.67±2.88	18.33±2.88	13.33±2.88	15.00±0.00	11.67±2.88
MBCs (in µg/mL)	48.33±2.88	46.67±2.88	40.00±0.00	41.67±2.88	36.66±2.88	26.67±2.88	28.33±2.88	26.67±2.88
MBC/MIC	2.14	1.86	1.84	1.92	2.00	2.00	1.88	2.28
Nature of Extract (Bacteriostatic/ Bactericidal)	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal

Table 3: FTIR analysis of n-hexane fraction (NHEX) of ethanol extract of *Callistemon linearis* flower.

Sl. No.	Peak value (in cm^{-1})	Range (in cm^{-1})	Classification	Bonds	Groups
1	2958.27	2972-2952	Alkanes	CH	$CH\text{-}(CH_3)_2$
2	2925.48	2936-2916	Alkanes	CH	$R(CH_2)_4\text{-}OR$
3	2870.52	2900-2800	Aldehydes	CH (Stretching in Fermi resonance)	C=CC=C-CHO
4	1715.37	1740-1715	Esters	C=O	C=C-COOR
5	1595.81	1620-1560	Amine Salts	NH_2 (Secondary amine salt)	NH_2^+
6	1462.74	1480-1410	Alcohols	OH	$R\text{-}CH_2\text{-}OH$
7	1267.97	1300-1160	Esters	C-O (Usually multiple bands)	C=C-COOR
8	1174.44	1300-1160	Esters	C-O (Usually multiple bands)	C=C-COOR
9	1015.34	1030-1010	Aromatics	CH (In-plane H bend)	1,2,3-trisubstituted
10	950.73	965-950	Aromatics	Rings (Out-of-plane ring bending)	1,2,3-trisubstituted

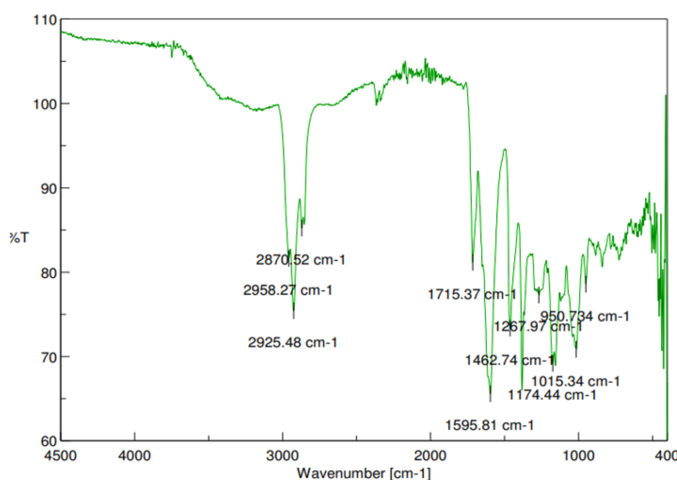

Figure 2: FTIR graph of n-hexane fraction (NHEX) of ethanol extract of *Callistemon linearis* flower.

which reported to have great antibacterial activity due to cell leakage.[40] Another compound, €-2-methyl-5-((1S,2R,4R)-2-methyl-3-methylbocyclo[2,2,1]-heptane-2-yl)-pent-2-enoic acid was identified through NIST library, which showed promising antibacterial activity as mentioned in literature.[41] Third compound, identified through GC-MS, 3-(1-Benzyl-1H-imidazol-2-yl)-5-methylisoxazole, is an aromatic aldehyde which also displayed antibacterial activity as per the report cited in literature.[42] These three compounds individually exhibit good antibacterial activity, however presence of these three compounds combinely in a single plant may increse its antibacterial activity.

Plants are rich source of secondary metabolites and it is very difficult to compare our data with previously documented data because of various factors like seasonal variation in phytocompounds, fractionation process, solvents used for isolation of compounds, etc. Compounds isolated from GC-MS analysis of our present study (R_f value = 0.278) are somewhat

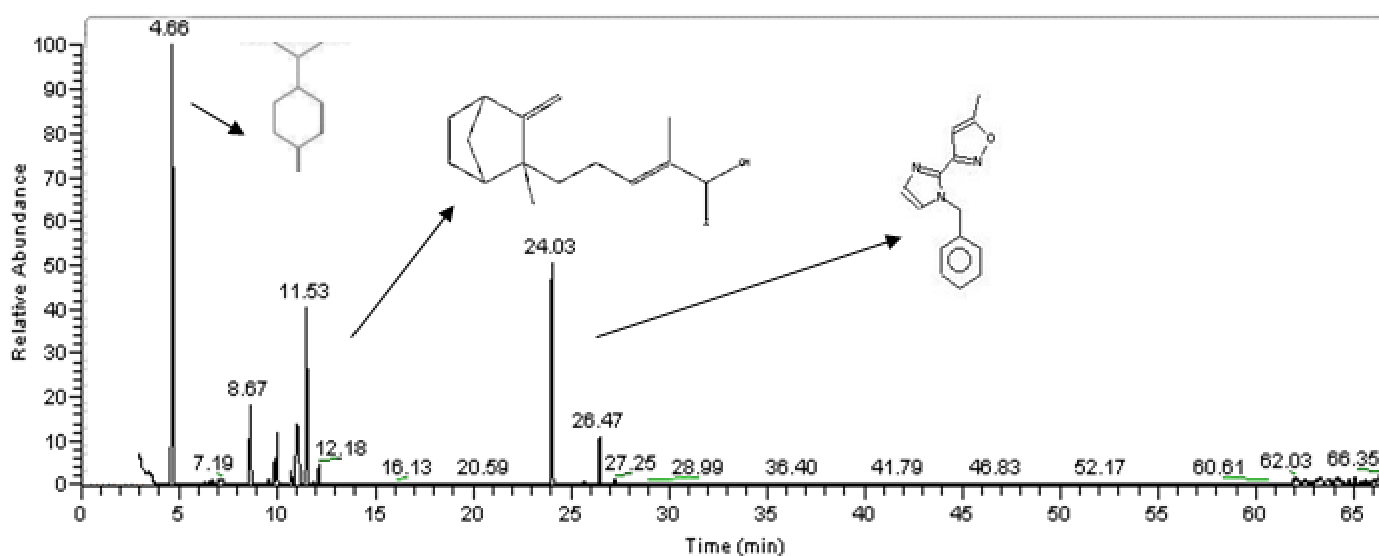

Figure 3: GC-MS graph of bioactive compounds of n-hexane fraction (NHEX) of ethanol extract of *Callistemon linearis* flower.

Table 4: Isolation of bioactive compounds from GC-MS analysis of n-hexane fraction of ethanol extract of *Callistemon linearis* flower.

Sl. No.	Compound name and chemical formula	Retention time	Percentage area	Molecular weight
1	ϭ-Phellandrene, $C_{10}H_{16}$	4.66	29.53	136
2	€-2-methyl-5-((1S,2R,4R)-2-methyl-3-methylbo cyclo[2,2,1]-heptane-2-yl)-pent-2-enoic acid, $C_{15}H_{22}O_2$	11.53	10.73	234
3	3-(1-Benzyl-1H-imidazol-2-yl)-5-methylisoxazole, $C_{14}H_{13}N_{30}$	24.03	15.72	239

different because those exhibiting best inhibition zone in *E. coli* followed by *P. aeruginosa*, both of which are Gram-negative bacteria.

Mechanism of Antibacterial Action

Time-kill assay

Time-kill analysis of tested bacterial strains treated with isolated bioactive compounds present loss of bacterial cells. Time kill assay graphs of different bacteria are shown in Figure 4. During the first few hours, each bacterial strain treated with MIC and 2×MIC shows decrease in bacterial concentration, which clearly indicates that bioactive compounds have good antibacterial activity. The bioactive compounds of NHEX of ethanol flower extract of *C. linearis* show various kinetics. Compare to the treated cells untreated bacterial strains shows increase in bacterial inoculum concentration with times which is clear in each graph. Test bioactive compounds are considered as bactericidal if the inoculum size of bacterial strains is reduced to>3 log CFU/mL as per literature.[43] Thereafter, our study showed that bioactive fraction of NHEX of *C. linearis* flower show good bactericidal potency.

Loss of Cellular Constituents

Table 5 shows results of protein and nucleic acid leakage assays of selected bacteria (Figure 5) treated with bioactive compounds. Highest protein leakage was found in case of Gram-negative bacteria compared to Gram-positive bacteria. Scenario of nucleic acid leakage is quite different, highest leakage was found in case of Gram-positive bacteria. The absorbance values were increased for both protein leakage and nucleic acid leakage with concentrations. Macromolecules like protein, nucleic acids etc. are the key structural components of bacterial cell, which have been released from the bacterial cells, after treatment with bioactive compounds isolated indicating the loss of integrity of cell wall and cell membrane causing bacterial cell damages. Control bacterial strains which do not treated with bioactive fraction shows a lower value of protein and nucleic acid concentration compared to treated strains.

FESEM

Alteration in the morphology of *B. subtilis* and *P. aeruginosa* are observed by FESEM study after treatment with bioactive compounds (Figure 6). Figure 6a and 6c show rod shaped cells are flocking together, and their cell surface are smooth and whole cell surface are present. Figure 6b and 6d show distorted deformed

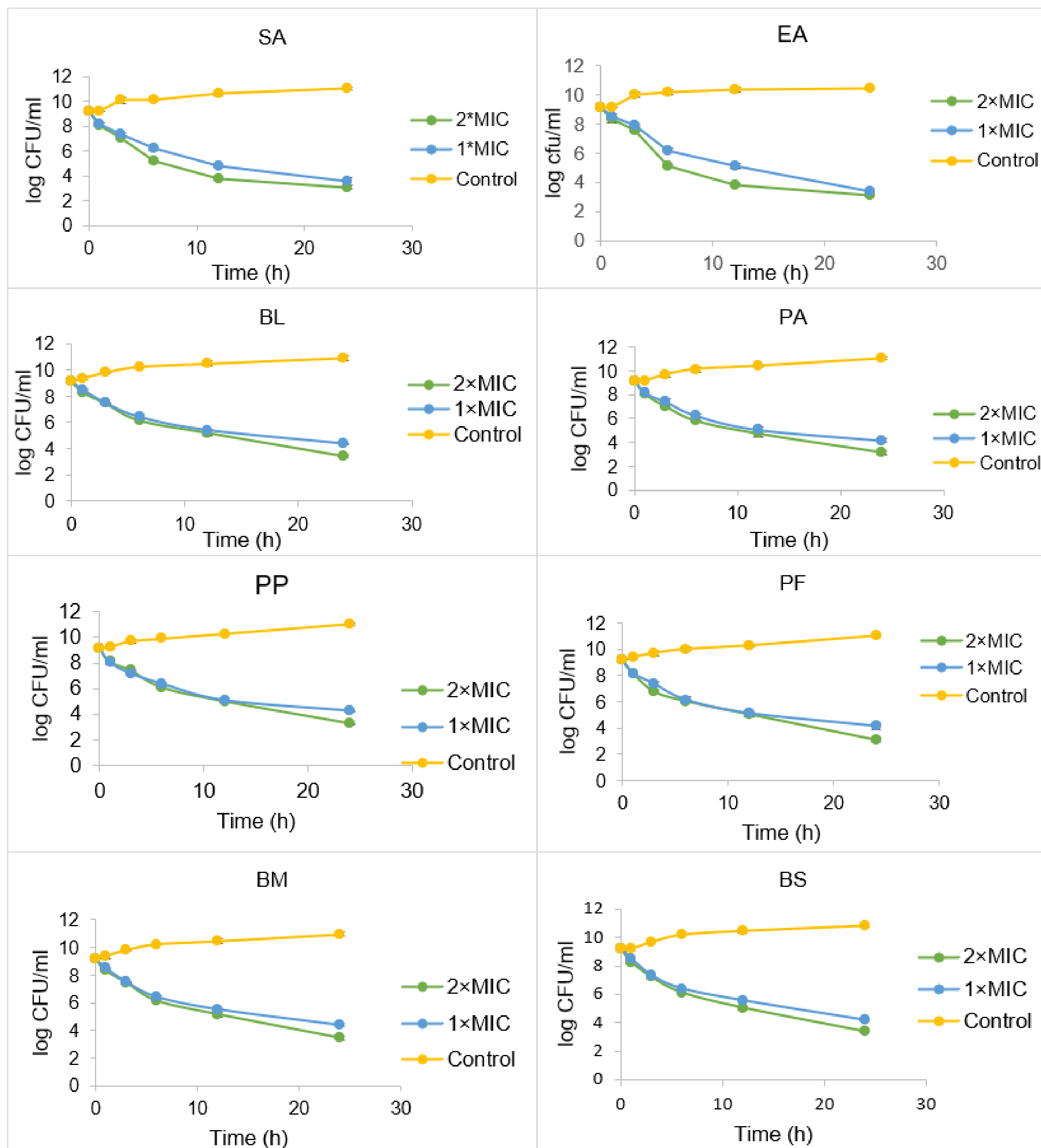

Figure 4: Time kill assay graph of bacterial strains after treated with n-hexane fraction (NHEX) of ethanol flower extract of *Callistemon linearis* (EA= *Escherichia coli*, SA= *Staphylococcus aureus*, PA = *Pseudomonas aeruginosa*, PF = *Pseudomonas fluorescens*, PP = *Pseudomonas putida*, BS= *Bacillus subtilis*, BL = *Bacillus licheniformis*, BM = *Bacillus mycoides*)

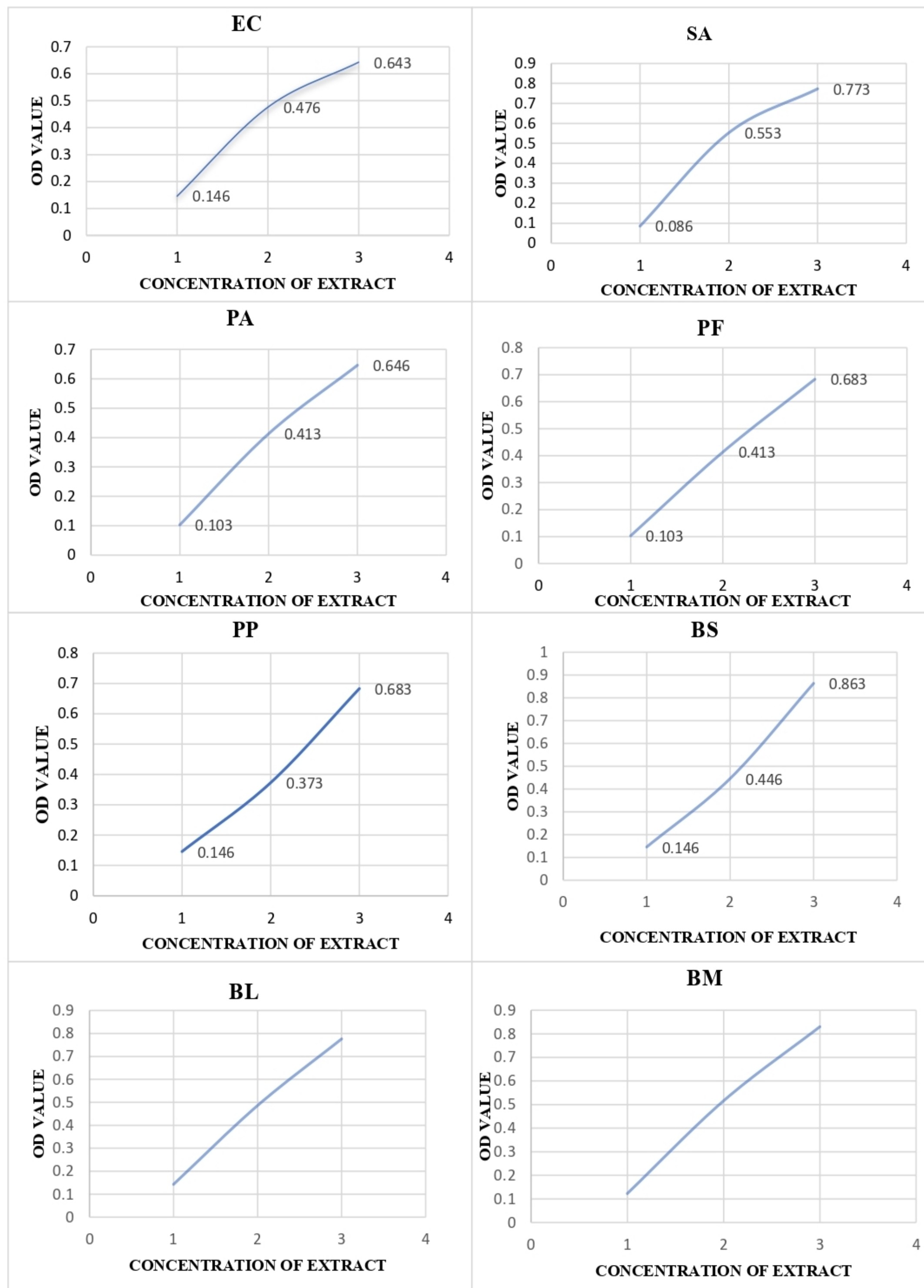

Figure 5: Cellular nucleic acid constituents (OD_{260} value) of bacterial strains treated with n-hexane fraction (NHEX) of ethanol flower extract of *Callistemon linearis* (EA= *Escherichia coli*, SA= *Staphylococcus aureus*, PA = *Pseudomonas aeruginosa*, PF = *Pseudomonas fluorescens*, PP = *Pseudomonas putida*, BS= *Bacillus subtilis*, BL = *Bacillus* licheniformis, BM = *Bacillus mycoides*)

Table 5: Leakage of Protein and Nucleic acid of tested pathogens by n-hexane fraction (NHEX) of ethanol extract of *Callistemon linearis* flower.

	Name of Bacterial strain							
Leakage of Protein in µg/mL	***Escherichia coli* (MTCC 739)**	***Pseudomonas aeruginosa* (MTCC 2453)**	***Pseudomonas fluorescens* (MTCC103)**	***Pseudomonas putida* (MTCC 1654)**	***Bacillus subtilis* (MTCC 441)**	***Bacillus mycoides* (MTCC7343)**	***Bacillus licheniformis* (MTCC530)**	***Staphylococcus aureus* (MTCC 2940)**
Control	7.33±0.57	8.33±0.57	9.33±0.57	8.33±0.57	7.33±1.52	6.33±1.15	5.33±0.57	8.67±0.57
1×MIC	26.33±1.15	30.33±0.57	31.33±2.51	36.67±1.52	37.33±1.52	28.33±1.52	31.67±2.08	27.67±0.57
2×MIC	68.33±1.15	59.67±3.21	72.67±2.51	75.33±0.57	57.33±0.57	56.67±1.52	64.67±1.52	66.67±1.15
Nucleic acid leakage (OD_{260}) measurement	*Escherichia coli* (MTCC 739)	*Pseudomonas aeruginosa* (MTCC 2453)	*Pseudomonas fluorescens* (MTCC103	*Pseudomonas putida* (MTCC 1654)	*Bacillus subtilis* (MTCC 441)	*Bacillus mycoides* (MTCC7343)	*Bacillus licheniformis* (MTCC530)	*Staphylococcus aureus* (MTCC 2940)
Control	0.146±0.005	0.103±0.005	0.103±0.011	0.146±0.005	0.146±0.005	0.123±0.005	0.143±0.005	0.086±0.005
1×MIC	0.476±0.005	0.413±0.015	0.413±0.015	0.373±0.005	0.446±0.011	0.516±0.005	0.486±0.005	0.553±0.005
2×MIC	0.643±0.025	0.646±0.005	0.683±0.011	0.683±0.011	0.863±0.011	0.830±0.017	0.776±0.005	0.773±0.011

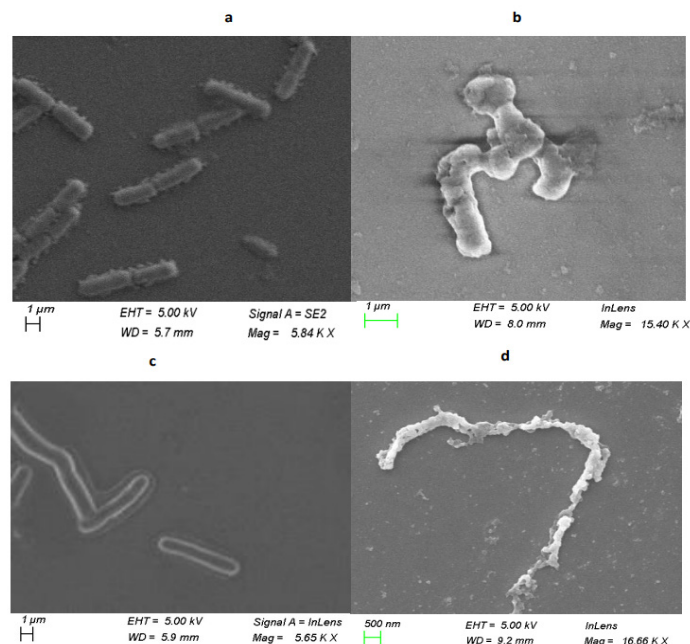

Figure 6: FESEM images of treated and untreated bacteria cells (a = normal *Bacillus subtilis*, b= distorted *B. subtilis* after treatment, c = normal *Pseudomonas aeruginosa*, and d = distorted *P. aeruginosa* after treatment).

shrank cells with clear morphological changes as compared to untreated cells. Our study shows that without the inoculation of bioactive compounds bacterial cells are normal and *C. linearis* bioactive fraction caused most serious damage to the bacterial cell. Thus, it clearly concludes that, bioactive compounds of NHEX ethanol extract are a rich source of secondary metabolites which show broad spectrum antibacterial potency. Therefore, *C. linearis* flower extract can provide an alternative source to treat microbes either in purified bioactive fraction or in combination with other antibiotics.

CONCLUSION

Our investigation is carried out to evaluate isolation of bioactive compounds and determination of antibacterial activity of different fractions of ethanol extract of *C. linearis* flower against Gram-positive and Gram-negative bacteria in *in vitro* condition. Among all the fractions, NHEX shows the best bactericidal mechanism. To the best of our knowledge, till date no proper investigation on flower extract of *C. linearis* have been carried out with the isolation of bioactive phytocompounds. Mechanism of action of the isolated phytocompounds is also done for the first time in our present experiment. Time kill assay, Protein Leakage Assay and FESEM study of treated and untreated bacteria help to understand the mechanism of bioactive compounds, which in near future give a new ray of hope for usefulness of this compound. Further, *in vivo* toxicity study, cytotoxic study etc. of bioactive compounds are need to be investigated.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

NHEX: n-hexane fraction; **EA:** Ethyl acetate fraction; **DCM:** Dichloromethane fraction; **AQ:** aqueous fraction; **DMSO:** Dimethylsulphoxide; **MBC:** Minimum Bactericidal Concentration; **MIC:** Minimum Inhibitory Concentration; **FT-IR:** Fourier Transform Infrared Spectroscopy; **TLC:** Thin Layer Chromatography; **GC-MS:** Gas Chromatography Mass Spectroscopy; **FESEM:** Field emission scanning electron microscopy.

SUMMARY

Bacteria become resistant to available antibiotics due to improper use of them. This resistance can be look over by searching of plant based active compounds because they have less side effects, low cost, etc. *C. linearis* floral extracts possess several secondary metabolites which show good broad spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria. Time kill assay, Protein leakage, Nucleic acid leakage, FESEM study etc. confirms that *C. linearis* floral extracts should be an effective antibacterial extract which can be help to produce new drug for bacteria in near future.

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