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Original article

The first report on the *in vitro* antimicrobial activities of extracts of leaves of *Ehretia serrata*

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ABSTRACT

The increasing resistance of infectious microbial organisms to the existing arsenal of antibiotic drugs is on the rise. There is a growing demand for the new antibiotics that are cost effective and easily available to the common people. In search of new antimicrobial entities, this report deals with the *in vitro* antimicrobial activities of the crude extracts of leaves of *Ehretia Serrata*. The methanolic extract and its sub-fractions namely *n*-hexane, chloroform, ethyl acetate, *n*-butanol and residual water fraction were screened against a range of 30 different bacterial strains and their zones of inhibition (ZI) and minimum inhibitory concentration (MIC) were subsequently evaluated. Methanolic extract has shown activity against all the tested microorganisms such as *Azospirillum lipoferum, Escherichia coli, Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Enterococcus* sp. with ZOI ranged from 10.3 to 29.0 mm. Moreover, the MIC values of methanolic extract and its sub-fractions against the tested bacteria ranged from 0.8 to 5.1 mg/mL. GC–MS analysis of sub-fractions revealed the presence of mono (2-ethylexyl) 1,2-benzenedicarboxylate, diisooctyl-1,2-benzenedicarboxylate, 3,5-dehydro-6-methoxypivalate-cholest-22-ene-21-ol, and 3,5-bis (1,1-dimethylethyl)-4-hydroxybenzene-propanoic acid. This is the first report on the *in vitro* antimicrobial activities of leaves of *E. Serrata.*

1. Introduction

Antibiotics are crucial in the treatment of several severe infections such as pneumonia, tuberculosis and meningitis. Moreover, they are used in the prevention of surgical site infections and management of immunocompromised individuals (Herbst et al., 2009; Finch, 2007). However, the increasing resistance of infectious microbial organisms to the existing arsenal of antibiotic drugs is considered to be responsible for more than 25,000 deaths in the European Union annually and 23,000 in the United States (Antibiotic Resistance Threats in the United States, 2013; ECDC/EMEA Joint Technical Report: The bacterial challenge: time to react). Thus, the emergence of the multi drug resistant strains poses a huge challenge for humanity to combat (Pallavali et al., 2017). Therefore, there is an ever growing demand for the new antibiotics that are cost effective and easily available to the common people.

The genus *Ehretia* of the Boraginacae family has about 50 species, distributed in tropical areas of Africa and Asia. *Ehretia serrata* Roxb. (syn. *E. acuminata* var. *serrata* (Roxb.) is a deciduous tree found in Pakistan and India that is spread in the sub-Himalayan and outer Himalaya from Indus eastward to Sikkim (Johnston, 1951). The plants of genus *Ehretia* are known for rich folkloric medicinal applications including in the treatment of abdominal and chest pains (Mncwangi et al., 2012), syphilis, toothache, cachexia, cough, diarrhea, stomach diseases and eczema (Torane et al., 2011; Khare, 2007). Moreover, they have been used to treat venereal diseases, pneumonia, epilepsy, dry cough, asthma, tonsils, mental problems, malaria, typhoid, wounds, aphrodisiac (Jeruto et al., 2008), nervous disorder and kidney inflammation (Arenas et al., 2013). A quinonoid xanthene, ehretianone, isolated from the root bark of *Ehretia buxifolia* has been found to possess anti-snake venom activity (Gomes et al., 2010).

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Motivated by the medicinal importance of plants of genus *Ehretia*, herein we wish to disclose antimicrobial studies of the crude extracts of leaves of *E. serrata*. Based on the available literature, this plant has been rarely used for the phytochemical and biological screening studies in the past. Earlier, we have reported a comparative study on the antioxidant capacity of different parts of the same plant (Zara, et al., 2012). Our present study is directed to determine the antimicrobial activities of the methanolic extract and its sub-fractions of *E. serrata* against a range of bacterial strains. In addition, phytochemical screening and GC–MS analysis was also performed to identify the chemical constituents responsible for therapeutic effects.

2. Materials and methods

2.1. Chemicals and instruments

Solvents used for extraction and fractionation were of commercial grade. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific and Mueller-Hinton agar from Merck. Standard antibiotic drugs amoxylin, cefixime and levofloxacin were obtained from Pharmagen, Lahore, Pakistan. GC–MS analysis was performed on Agilent Technologies GCMS system GC-7890A/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column ((30 m in length × 250 μm in diameter × 0.25 μm in thickness of film). The ionization mode was electron ionization with high energy electron (70 eV). The injection volume of the sample (prepared by dissolving 0.5 mg/mL of respective solvents) was 1 μL and flow pressure for helium carrier gas (99.999%) was maintained at 1.0 mL/min. The split mode was used having 10:1 ratio. The sample was injected at 200 °C while the initial temperature of oven 35 °C and was gradually increased to 270 °C with an increase rate of 5 °C per minute followed by raising temperature to 320 °C with the rate of 10 °C per minute. The mass spectrometer was set to scan mode ranging from *m*/*z* 50–600 with ion source temperature of 250 °C and interface temperature of 150 °C. The analyses were performed in triplicate and a blank run was run after each analysis.

2.2. Microorganisms and growth conditions

The microorganisms used for this study were both gram negative and gram positive bacteria. The gram negative bacteria were: *Achromobacter xylosoxidans, Stenotrophomonas maltophilia, Klebsiella pneumonia, Enterobacter cloacae, pseudomonas* sp., *Escherichia coli, Salmonella typhi, Azospirillum lipoferum, Rhizobium* sp., *Citrobactor freundii* whereas gram positive bacteria include *Staphylococcus epidermidis, Staphylococcus aureus, Bacillus* sp., *Bacillus subtilis, Bacillus megatherium, Enterococcus* sp. The bacterial strains were obtained from the Department of Biotechnology, Forman Christian College, University, Lahore, and were subsequently grown at 37 °C and maintained on nutrient agar (Merck, Germany). All the clinical isolates were identified by standard morphological, cultural and biochemical profile (API-20E, bioMerieux, France). Serological identification was performed by using antisera (BD Difco, USA). The isolates were preserved in micro bank tubes containing beads (Pro-Lab Diagnostics, UK) and 16% (v/v) glycerol in brain heart infusion (Oxoid Ltd, UK) and were stored at -70 °C.

2.3. Plant material

The leaves of *Ehretia serrata* were collected from the campus of Forman Christian College University Lahore, Pakistan. The collected leaves were washed with distilled water and dried under shade for 15 days. The air-dried leaves were pulverized into powdered form by using heavy duty blander.

2.4. Extraction and fractionation

500 g of powdered leaves were soaked in 2000 mL of methanol (100%) at a ratio of 1:4 (powder/solvent) for 7 days. The mixture was agitated from time to time during soaking period in order to ensure homogeneity and to maximize the extraction of the plant constituents. The mixture was first filtered by cheese cloth and then by Whatman No.1 filter paper. The filtrate was concentrated under reduced pressure by using rotary evaporator at 40 °C to complete dryness to yield 42.6 g (8.5%) of crude methanolic extract (Zara et al., 2012). In order to obtain *n*-hexane, chloroform, ethyl acetate and *n*-butanol fractions, 32 g of methanolic extract was dissolved in 100 mL of distilled water and then sequentially extracted three times each with 150 mL of *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The combined organic extractions were evaporated to dryness under vacuum to obtain the corresponding extracts of *n*-hexane (3.02 g), chloroform (4.01 g), ethyl acetate (7.03 g) and *n*-butanol (6.05), respectively. Finally, the aqueous residue (10.9 g) was also obtained by drying the aqueous layer under vacuum at 45 °C for 1 h. The methanolic extract and its sub-fractions were subjected to antimicrobial activity, phytochemical screening and GC–MS analysis.

2.5. Antimicrobial assays

2.5.1. Sample preparation

The stock solutions were prepared by dissolving dried methanolic extract and its fractions in DMSO (50 mg/mL). Stock solutions were then diluted into five different concentrations of 10, 20, 30, 40, and 50 mg/mL in DMSO. Solutions of standard drugs were also prepared in the same manner.

2.5.2. Determination of zones of inhibition (ZOI)

A literature known agar well diffusion assay was used according to the method recommended by Clinical and Laboratory Standards Institute (CLSI) (Njeru et al., 2015; Al Akeel et al., 2014). A well-mixed nutrient agar was prepared by dissolving 32 g of NA (Nutrient Agar) in 1 L of distilled water which was autoclaved at 121 °C for 1 h. For the preparation of bacterial suspension, a lyophilized bacterial culture was diluted before it was streaked onto a slant made of tryptic soya agar and placed in an incubator at 32.5 °C for 24 h. Bacterial growth was observed in the incubated slant, which was then diluted carefully by mixing 3 mL of normal saline in the slant. 2 mL of the diluted culture was then taken and its absorbance was standardized with 0.5% McFarland standard solution at 550 nm. In order to make seed agar, 2 mL of the diluted suspension were added into100 mL of the liquid nutrient agar which was kept at 50 °C. Since, *B. subtilis* is a spore forming bacterial strain which required extended period of incubation of 7 days for maximum spore formation on the slant. Then 20 mL of the 0.9% of saline solution was added and the resulting mixture was heated at 70 °C for 30 min to ensure complete killing of all vegetative cells of the *B. subtilis*. In addition, 2 mL of the suspension was taken and standardized with 0.5% McFarland standard solution at 550 nm. The rest of the procedure was same. Then 20 mL of NA was poured into each petri plate of 90 mm diameter and allowed to solidify. After solidification, 4 mL of the seed agar was poured onto the base agar to make a thin layer of seed agar in the petri plate, which was then placed in refrigerator for 1 h for cooling. Holes were made into the petri plates at equal distances with a sterilized agar borer (9 mm). Each of holes was filled with 140 μL of a plant extract prepared in DMSO at a concentration of 40 mg/L. The plates were then kept in an incubator at 37 °C for overnight. The clear ZOI of the

samples were measured in millimeters by using digital Vernier's clippers. The same method was used for all plant samples, DMSO was used as negative standard whereas known antibiotics i.e. amoxicillin, cefixime and levofloxacin were employed as positive standard (Mohapatra et al., 2011). All tests were performed in triplicate.

2.5.3. Determination of minimum inhibitory concentration (MIC)

The MIC of a sample was determined by agar well dilution method (Wiegand, Hilpert, and Hancock, 2008). Mueller-Hinton agar was dissolved in distilled water with concentration 32 g/L, and autoclaved at 121 °C for 1 h. The agar was allowed to cool to 50 °C and poured into Petri plates for sample preparation. Different dilutions of each extract/fraction were prepared in this agar with concentrations 10–50 μg/20 mL. The content of each plate was mixed well and allowed to solidify. Likewise, stock solution of each extract/fraction was prepared in DMSO (5 mg/mL) and dilutions were made. For inoculum, 4–5 colonies of a pure culture were selected and introduced into a test tube containing 5 mL of 5% sterilized NS solution and the mixture was compared with 0.5% McFarland solution to adjust the density of bacterial suspension at 10^6 CFU/mL. The inoculum for each bacterium was poured into the wells. Then these thirty bacterial strains (3 μL) were transferred on to the MHA medium. The plates were kept in an incubator at 37 °C for overnight. The growth of the bacterial strains was observed according to the template and the MIC values were recorded. All tests were performed in triplicate.

2.5.4. Statistical analysis

In order to keep up the reproducibility of the results statistical analysis was also performed. Mean values of the results were calculated as all of the experiments were done in triplicate. The data was subjected to one-way analysis of variance (ANOVA) and results were expressed (where appropriate) as mean ± standard deviation.

2.5.5. Identification of chemical constituents

The chemical compounds isolated from the extracts of *E. serrata* were identified by GC–MS analysis. The known compounds were identified by using database library NIST05 comparing them with their known GC–MS profile.

3. Results

3.1. Antimicrobial activity

3.1.1. Zones of inhibition (ZI)

The antimicrobial properties of the methanolic extract were studied against 30 bacterial strains and the results in zones of inhibition (mm) are displayed in Table 1. For comparison, three antibiotics were used in the study namely amoxicillin, cefixime and levofloxacin. The methanolic extract showed a broad range of antimicrobial properties against the tested microorganisms including human pathogens. It showed notable activity at a concentration of 40 mg/mL against *Azospirillum lipoferum* (29.0 ± 2.64), *Pseudomonas aeruginosa* (1) (25.7 ± 0.57) and *Staphylococcus aureus* KSR1 (20.3 ± 0.57). The methanolic extract also showed considerable activity at concentration of 20 mg/mL against *Escherichia coli* (28.0 ± 0.00), *Bacillus megatherium* (19.6 ± 2.08), *Bacillus subtilis* (32.5 ± 2.12) and at concentration of 10 mg/mL against *Pseudomonas aeruginosa* (1) (30 ± 0.000). In addition, these inhibition

Table 1
Comparison of antimicrobial activity* of the methanolic extract of leaves of *E. serrata* with standard antibiotics against different bacterial pathogens in terms of ZOI (mm).

Sr. No.	Bacterial strains	Gram +/−	Standard antibiotics (40 mg/mL)			Methanolic extract (mg/mL)				
			A[a]	C[b]	L[c]	40	20	10	5	2.5
1	*Staphylococcus epidermidis*	+	48.5 ± 2.12	41.5 ± 0.70	48.0 ± 2.82	15.5 ± 0.70	13.5 ± 0.70	12.5 ± 0.70	11.0 ± 0.00	10.5 ± 0.00
2	*Staphylococcus aureus* KSR1	+	17.5 ± 2.12	24.5 ± 0.50	32.0 ± 1.00	20.3 ± 0.57	19.5 ± 2.12	17.5 ± 0.70	15.5 ± 0.70	14.5 ± 0.70
3	*Staphylococcus aureus* KSR2	+	24.0 ± 1.41	32.0 ± 1.41	32.0 ± 1.41	11.5 ± 0.70	32 ± 0.000	31 ± 1.212	29.0 ± 1.41	27.0 ± 1.41
4	*Staphylococcus aureus* KSR3	+	46.0 ± 0.00	53.0 ± 2.82	54.5 ± 0.70	17.0 ± 0.00	12.0 ± 0.00	00.0 ± 0.00	00.0 ± 0.00	00.0 ± 0.00
5	*Bacillus subtilis*	+	15.0 ± 1.41	24.5 ± 2.12	26.0 ± 0.00	20.3 ± 0.50	32.5 ± 2.12	29.0 ± 5.65	28.5 ± 0.70	26.0 ± 0.00
6	*Bacillus megatherium*	+	12.0 ± 0.00	23.5 ± 0.50	31.0 ± 1.00	19.6 ± 2.08	20.5 ± 7.77	19.0 ± 5.65	18.5 ± 6.63	17.5 ± 6.36
7	*Bacillus* sp. KSR325	+	19.0 ± 0.00	25.5 ± 1.50	30.0 ± 2.50	19.5 ± 0.50	26.0 ± 1.41	24.0 ± 1.41	22.0 ± 0.00	19.5 ± 3.53
8	*Bacillus* sp. KSR356	+	15.5 ± 1.50	24.5 ± 1.00	27.5 ± 0.50	15.5 ± 0.58	22.5 ± 2.12	18.0 ± 1.41	17.0 ± 0.00	15.5 ± 1.12
9	*Bacillus* sp. KSR357	+	23.0 ± 3.00	27.0 ± 1.50	43.0 ± 0.70	13.6 ± 0.57	20.0 ± 1.41	18.0 ± 0.00	15.0 ± 4.24	14.5 ± 2.12
10	*Enterococcus* sp.	+	16.0 ± 1.00	24.5 ± 2.12	30.0 ± 0.00	23.00 ± 1.0	21.0 ± 8.34	17.5 ± 3.53	11.0 ± 1.41	10.5 ± 0.70
11	*Achromobacter xylosoxidans*	−	40.0 ± 1.41	46.5 ± 0.70	51.0 ± 1.41	17.5 ± 0.70	17.0 ± 0.00	16.5 ± 0.70	15.5 ± 0.70	15.0 ± 0.00
12	*Stenotrophomonas maltophilia*	−	26.5 ± 1.41	32.5 ± 0.70	47.0 ± 1.41	21.5 ± 0.70	18.0 ± 0.00	15.0 ± 0.00	13.0 ± 0.00	12.5 ± 0.70
13	*Klebsiella pneumonia*	−	13.5 ± 2.12	21.0 ± 0.00	38.5 ± 0.70	12.0 ± 0.00	11.5 ± 0.00	11.0 ± 0.00	10.5 ± 0.70	00.0 ± 0.00
14	*Enterobacter cloacae*	−	19.0 ± 1.41	39.0 ± 2.82	41.0 ± 2.82	12.0 ± 1.41	12.0 ± 0.00	11.0 ± 1.41	10.5 ± 0.70	00.0 ± 0.00
15	*Enterobacter aerogenes*	−	11.5 ± 0.70	23.0 ± 0.00	38.0 ± 0.00	14.5 ± 0.70	13.5 ± 0.00	13.0 ± 0.00	12.0 ± 1.41	11.0 ± 0.00
16	*Pseudomonas aurantiaca*	−	17.5 ± 2.12	25.0 ± 1.00	30.5 ± 2.50	16.1 ± 0.76	19.5 ± 0.70	21.5 ± 0.70	19.5 ± 2.12	17.5 ± 0.70
17	*Pseudomonas aeruginosa*	−	16.5 ± 0.70	24.5 ± 0.50	29.0 ± 0.50	25.7 ± 0.57	28 ± 0.000	30 ± 0.000	24.5 ± 0.70	23.5 ± 0.70
18	*Pseudomonas aeruginosaKSR125*	−	16.5 ± 0.50	24.0 ± 0.00	35.0 ± 2.00	19.6 ± 0.57	22 ± 0.000	20.0 ± 1.41	19 ± 2.828	17 ± 0.000
19	*Pseudomonas aeruginosa KSR360*	−	38.0 ± 0.00	44.0 ± 1.41	43.5 ± 0.70	22.0 ± 1.41	20.0 ± 0.00	18.5 ± 0.70	18.00 ± 0.0	16.0 ± 0.00
21	*Pseudomonas* sp.	−	27.0 ± 0.00	39.5 ± 0.70	33.0 ± 1.41	21.0 ± 1.41	19.5 ± 0.70	18.0 ± 0.00	17.5 ± 0.70	16.0 ± 0.00
22	*Escherichia coli*	−	17.0 ± 1.41	27.0 ± 3.00	35.0 ± 2.00	15.3 ± 0.57	28 ± 0.000	26.0 ± 0.00	23.5 ± 3.53	22.5 ± 0.70
23	*Escherichia coli KSR2345*	−	16.0 ± 1.41	26.0 ± 0.00	30.0 ± 1.00	23.00 ± 1.0	21 ± 1.414	20.0 ± 0.00	18.5 ± 0.70	17.5 ± 0.70
24	*Escherichia coli* KSR3245	−	38.0 ± 0.00	43.5 ± 2.12	47.0 ± 0.00	27.0 ± 1.41	23.5 ± 2.12	20.5 ± 0.00	19.0 ± 0.00	18.0 ± 0.00
25	*Escherichia coli* KSR2346	−	24.0 ± 1.41	30.0 ± 1.41	30.0 ± 1.41	18.5 ± 0.70	22.0 ± 0.00	19.0 ± 0.00	17.0 ± 0.00	16.5 ± 0.70
26	*Salmonella typhi*	−	18.5 ± 0.70	26.5 ± 0.50	43.0 ± 3.00	15.3 ± 0.57	20 ± 1.414	19.5 ± 0.70	18.5 ± 0.70	18.0 ± 4.21
27	*Salmonella typhi* KSR2347	−	15.0 ± 1.41	24.0 ± 0.00	38.5 ± 1.41	18.5 ± 0.50	20 ± 0.000	19.5 ± 0.70	18.0 ± 0.00	16.0 ± 4.24
28	*Salmonella typhi* 3 KSR2349	−	26.5 ± 0.70	36.5 ± 2.12	36.5 ± 2.12	18.5 ± 0.70	23.5 ± 2.21	21.0 ± 1.41	18.0 ± 1.41	16.0 ± 2.82
29	*Azospirillum lipoferum*	−	19.0 ± 1.41	25.5 ± 0.70	34.5 ± 1.50	29.0 ± 2.64	21.5 ± 1.41	19.0 ± 2.82	18.5 ± 2.12	17.0 ± 1.41
30	*Rhizobium* sp.	−	19.5 ± 1.50	27.0 ± 0.00	35.5 ± 0.50	10.33 ± 0.5	27.0 ± 0.00	24.5 ± 4.94	23.5 ± 3.53	22.0 ± 0.00
31	*Citrobactor freundii*	−	16.0 ± 0.00	25.0 ± 1.00	35.5 ± 1.50	11.68 ± 0.5	24.0 ± 4.94	23.0 ± 0.00	13.5 ± 0.70	15.0 ± 0.00

* Negative control = DMSO (dimethyl sulfoxide), showed no activity.
[a] A = Amoxylin.
[b] C = Cefixime.
[c] L = Levofloxacin.

zones demonstrated by methanolic extracts are higher than the zones of inhibition of standard antibiotic amoxicillin (Fig. 1). Moreover, amoxicillin turned out to be highly active against *Staphylococcus epidermidis, Staphylococcus aureus KSR3, Achromobacter xylosoxidans, Pseudomonas aeruginosa KSR360* and *Escherichia coli KSR3245* whereas in other cases the methanolic extract has either equal or higher activity then amoxicillin (Fig. 1).

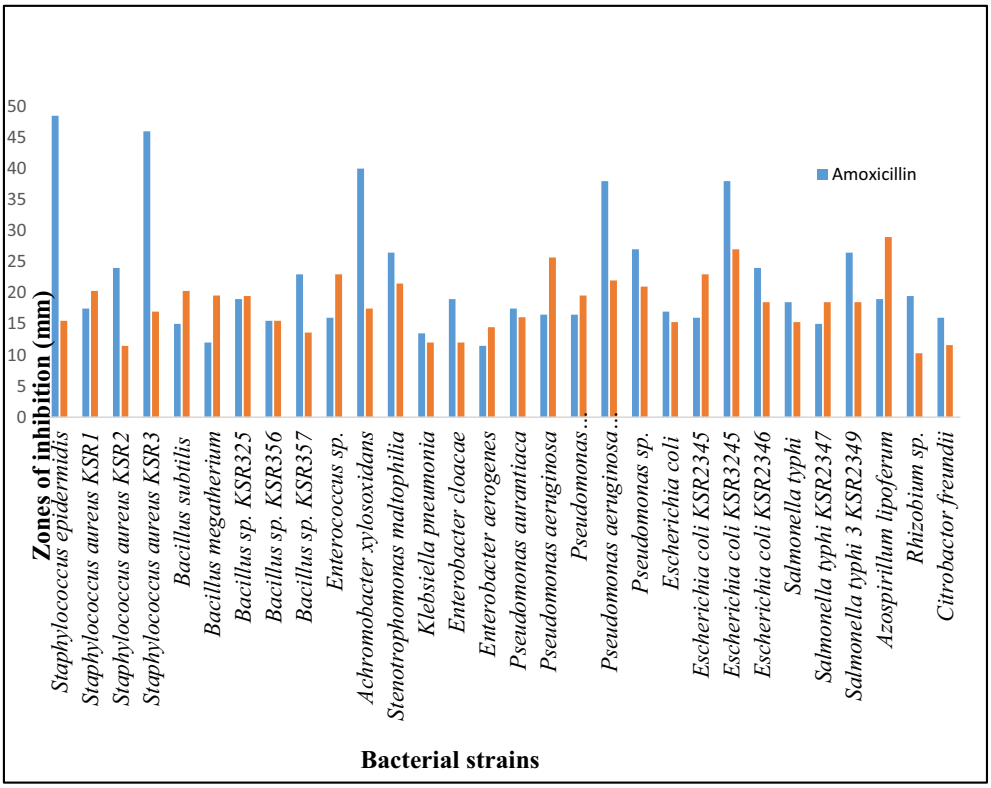

Fig. 1. Comparison of antimicrobial activity of the methanolic extract of leaves of *E. serrata* with standard amoxicillin against different bacterial pathogens in terms ZOI (mm).

Table 2
Antimicrobial activity of sub-fractions of leaves of *E. serrata* against different bacterial pathogens in terms of ZOI (mm).

Sr. No.	Bacterial stains Gram +/−		*n*-Hexane sub-fraction Concentrations (mg/mL)		Chloroform sub-fraction Concentrations (mg/mL)		Ethyl acetate sub-fraction Concentrations (mg/mL)		*n*-Butanol sub-fraction Concentrations (mg/mL)		Aqueous residue Concentrations (mg/mL)	
			40 ZI (mm)	20 ZI (mm)	40 ZI (mm)	20 ZI (mm)	40 ZI (mm)	20 ZI (mm)	40 ZI (mm)	20 ZI (mm)	40 ZI (mm)	20 ZI (mm)
1	*Staphylococcus epidermidis*	+	13.5 ± 0.7	12.0 ± 0.00	14.0 ± 0.00	13.5 ± 0.70	10.5 ± 0.70	0.00 ± 0.00	20.0 ± 1.41	18.5 ± 0.70	00.0 ± 0.000	00.0 ± 0.00
2	*Staphylococcus aureus* KSR1	+	18.5 ± 0.70	15.0 ± 0.00	17.5 ± 0.70	16.0 ± 0.00	23.5 ± 0.70	22.0 ± 0.00	18.5 ± 2.12	17.5 ± 2.12	20.0 ± 1.414	19.0 ± 0.00
3	*Pseudomonas aeruginosaKSR125*	+	16.0 ± 1.41	14.5 ± 0.70	17.5 ± 2.12	14.5 ± 0.70	14.0 ± 1.41	13.0 ± 1.41	14.5 ± 0.70	13.5 ± 0.70	14.0 ± 1.414	13.5 ± 0.70
4	*Bacillus* sp.	+	12.5 ± 0.70	10.0 ± 0.00	14.5 ± 0.70	13.5 ± 0.70	15.0 ± 1.21	12.0 ± 0.00	14.0 ± 1.21	12.0 ± 0.0	14.5 ± 0.707	12.0 ± 0.000
5	*Bacillus subtilis*	+	12.5 ± 0.70	10.0 ± 0.00	16.0 ± 1.41	13.0 ± 0.00	19.5 ± 0.70	18.5 ± 0.70	20.0 ± 1.41	18.5 ± 0.70	22.0 ± 1.414	20.5 ± 0.70
6	*Enterococcus* sp.	+	14.5 ± 0.70	13.0 ± 0.00	14.0 ± 0.00	12.5 ± 0.70	18.0 ± 1.41	16.5 ± 2.12	18.0 ± 1.41	16.5 ± 2.12	18.5 ± 2.121	17.5 ± 2.12
7	*Achromobacter xylosoxidans*	–	12.5 ± 0.70	10.0 ± 0.00	17.5 ± 0.70	16.0 ± 1.41	14.0 ± 1.41	12.5 ± 0.70	17.0 ± 1.41	15.0 ± 0.00	13.5 ± 0.707	13.0 ± 0.00
8	*Stenotrophomonas maltophilia*	–	19.5 ± 0.70	17.5 ± 0.70	16.0 ± 0.00	15.0 ± 0.00	13.5 ± 0.70	11.0 ± 0.00	15.5 ± 0.70	14.0 ± 1.41	00.0 ± 0.00	00.0 ± 0.00
9	*Klebsiella pneumonia*	–	12.0 ± 0.00	10.0 ± 0.00	13.0 ± 1.41	12.5 ± 0.70	15.5 ± 0.70	12.5 ± 0.70	13.0 ± 0.00	12.0 ± 1.41	12.0 ± 0.000	9.0 ± 0.000
10	*Salmonella typhi* KSR2347	–	17.5 ± 0.70	14.0 ± 0.00	14.0 ± 0.00	13.5 ± 0.70	16.0 ± 0.00	14.5 ± 0.70	16.0 ± 0.00	14.5 ± 0.70	13.0 ± 1.141	12.5 ± 0.70
11	*Escherichia coli KSR2345*	–	15.5 ± 0.70	12.5 ± 0.70	15.0 ± 0.00	13.5 ± 0.70	28.5 ± 0.70	27.0 ± 1.41	28.5 ± 0.70	27.0 ± 1.41	00.0 ± 0.000	00.0 ± 0.00
12	*Azospirillum lipoferum*	–	16.0 ± 0.00	13.0 ± 0.00	14.5 ± 0.00	12.5 ± 0.00	26.0 ± 0.00	19.0 ± 1.41	26.0 ± 0.00	19.0 ± 1.41	12.0 ± 0.000	10.0 ± 1.41

3.1.2. Antimicrobial activity of fractions of methanolic extracts

Driven by the promising antimicrobial activities of the methanolic extract of leaves of *E. serrata*, the activity of the sub-fractions of the crude extract against highly sensitive bacterial strains was also explored. The results are exhibited in Tables 2. The *n*-hexane fraction at a concentration of 40 mg/mL showed considerably high activities against against *Stenotrophomonas maltophilia* (19.5 ± 0.707), *Staphylococcus aureus KSR1* (18.5 ± 0.707) and *Salmonella typhi KSR2347* (17.5 ± 0.70 mm). Likewise, the chloroform fraction at a concentration of 40 mg/mL showed good activ-

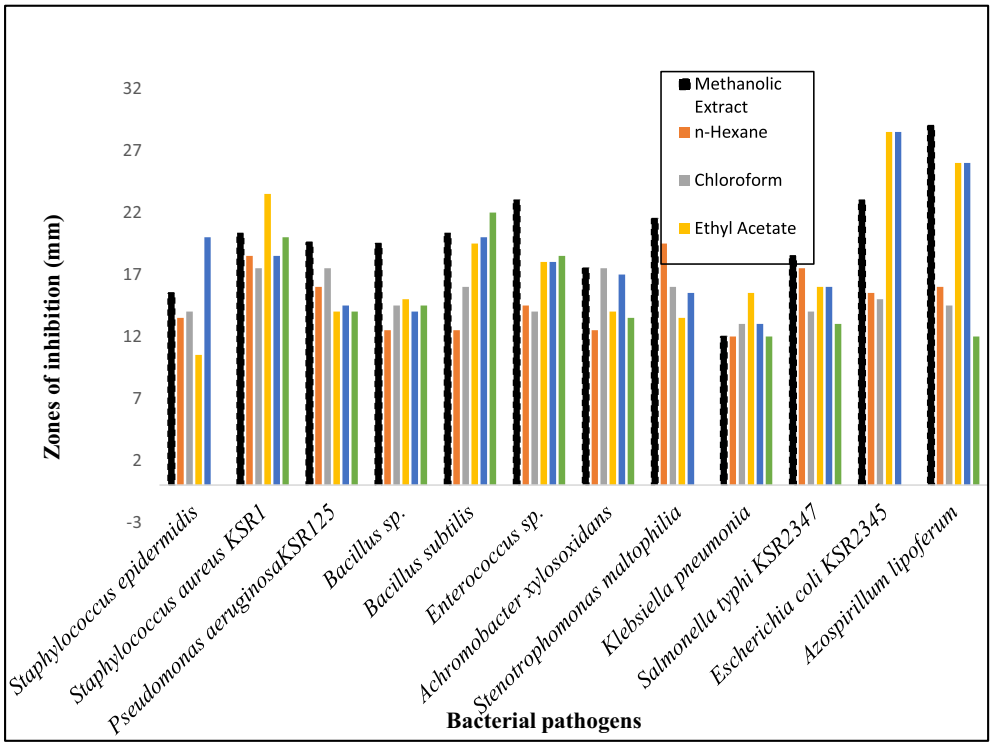

Fig. 2. Comparison of antimicrobial activity of the methanolic extract with its sub-fractions of leaves of *E. serrata* against different bacterial pathogens in terms of ZOI (mm).

Table 3
MICs of the extracts of *E. serrata* (mg/mL) against various bacterial strains.

S. No.	Bacterial strains	Gram +/–	Methanolic	*n*-hexane	Chloroform	Ethyl acetate	*n*-butanolic	Aqueous
1	*Staphylococcus epidermidis*	+	1.6	0.8	3.2	1.6	2.0	3.2
2	*Staphylococcus aureus* KSR1	+	1.6	0.8	3.2	3.3	0.8	0.8
3	*Staphylococcus aureus* KSR2	+	3.2	0.8	3.2	2.4	1.6	4.0
4	*Staphylococcus aureus* KSR3	+	1.6	3.6	2.4	2.0	2.0	3.2
5	*Bacillus subtilis*	+	0.8	1.2	1.2	4.1	1.6	3.2
6	*Bacillus megatherium*	+	0.8	1.2	3.2	2.4	2.8	1.2
7	*Bacillus* sp. KSR325	+	0.8	1.2	4.4	5.0	0.4	4.0
8	*Bacillus* sp. KSR356	+	0.8	2.4	4.0	4.1	2.8	3.2
9	*Bacillus* sp. KSR357	+	1.6	3.6	4.0	3.3	3.6	3.6
10	*Enterococcus* sp.	+	1.6	2.4	1.2	2.4	2.0	2.8
11	*Achromobacter xylosoxidans*	–	2.0	0.8	3.6	2.4	3.6	3.6
12	*Stenotrophomonas maltophilia*	–	1.6	0.8	3.2	2.0	3.2	3.6
13	*Klebsiella pneumonia*	–	4.0	3.6	3.6	3.6	3.2	3.6
14	*Enterobacter cloacae*	–	3.6	1.2	3.6	2.0	2.0	3.6
15	*Enterobacter aerogenes*	–	2.0	1.2	3.6	3.6	3.6	3.2
16	*Pseudomonas aurantiaca*	–	0.8	2.8	2.0	2.4	1.6	1.2
17	*Pseudomonas aeruginosa*	–	0.8	1.2	3.6	4.1	1.6	4.0
18	*Pseudomonas aeruginosaKSR125*	–	0.8	1.2	3.6	3.3	2.0	3.6
19	*Pseudomonas aeruginosa KSR360*	–	3.2	2.8	2.0	1.2	0.4	1.2
20	*Pseudomonas* sp.	–	2.0	0.8	3.6	2.0	2.8	3.6
21	*Escherichia coli*	–	1.6	3.6	2.0	0.8	2.8	3.2
22	*Escherichia coli KSR2345*	–	1.6	1.2	1.6	4.1	1.6	1.2
23	*Escherichia coli* KSR3245	–	2.0	0.8	3.6	2.4	2.0	4.0
24	*Escherichia coli* KSR2346	–	1.6	1.2	2.8	3.3	3.6	0.8
25	*Salmonella typhi*	–	2.4	3.6	3.6	1.6	2.4	3.6
26	*Salmonella typhi* KSR2347	–	1.6	0.8	3.6	5.0	1.6	3.2
27	*Salmonella typhi* 3 KSR2349	–	2.4	1.2	1.6	4.1	2.0	0.8
28	*Azospirillum lipoferum*	–	1.6	1.2	2.0	2.4	2.4	3.6
29	*Rhizobium* sp.	–	1.6	2.4	1.6	2.4	2.0	3.2
30	*Citrobactor freundii*	–	2.4	1.6	1.6	2.4	1.6	1.2

ities against *Staphylococcus aureus* KSR1 (17.5 ± 0.707), *Stenotrophomonas maltophilia* (16.0 ± 0.000) and *Achromobacter xylosoxidans* (17.5 ± 0.707) (Table 2). Similarly, ethyl acetate fraction (40 mg/mL) also exhibited significant activity against *Staphylococcus aureus* (23.5 ± 0.707), *Escherichia coli* (1) (28.5 ± 0.707), *Azospirillum lipoferum* (26.0 ± 0.000) (Table 2). The *n*-butanol extract (40 mg/mL) displayed significant activities against *Staphylococcus epidermidis* (20.0 ± 1.414), *Bacillus subtillis* (*20.0* ± 1.414), *Escherichia coli KSR2345* (28.5 ± 0.707) and *Azospirillum lipoferum* (26.0 ± 0.000) (Table 2). In addition, the aqueous fraction at a concentration of 40 mg/mL also showed high value of ZOI against *Staphylococcus aureusKSR1* (20.0 ± 1.414) and *Bacillus subtilis* (22.0 ± 1.414) (Table 2).

Fig. 2 shows a comparison of the efficacy of the methanolic extract with its sub-fractions at 40 mg/mL which reveals that all the fractions have activities against the given bacterial strains. However, *n*-butanol extract was found to be more active against *Staphylococcus epidermidis* whereas ethyl acetate fraction turned out to be more active against *Staphylococcus aureusKSR1* compared to other fractions. Moreover, both *n*-butanol and ethyl acetate fractions were found to be very active against *Escherichia coli KSR3245* in comparison to other fractions. Likewise, methanolic extract has shown higher efficacy against all of the remaining tested bacterial strains compared to its sub-fractions (Fig. 2).

3.1.3. The MIC values

The results of antimicrobial screening by agar well diffusion assay prompted us to calculate the MIC. The methanolic extract and its fractions showed notable results (Table 3). The methanolic extract was found to be the most effective among all the samples. The MIC values were ranged between 0.8 and 4.0 mg/mL against different strains (Table 3 and Fig. 3). The methanolic extract at 0.8 mg/mL concentration inhibited the growth of most of the bacterial strains including *Bacillus subtilis, Bacillus megatherium*, *Bacillus* sp. KSR325, *Bacillus* sp. KSR356, *Pseudomonas aurantiaca, Pseudomonas aeruginosa, Pseudomonas aeruginosaKSR125*. The *n*-hexane fraction also displayed significant MIC values ranging from 0.8 to 3.6 mg/mL. Likewise, the MIC values of chloroform fraction were found between 1.2 and 4.4 mg/mL. At a concentration of 3.6 mg/mL, it inhibited the growth of most of the bacterial strains.

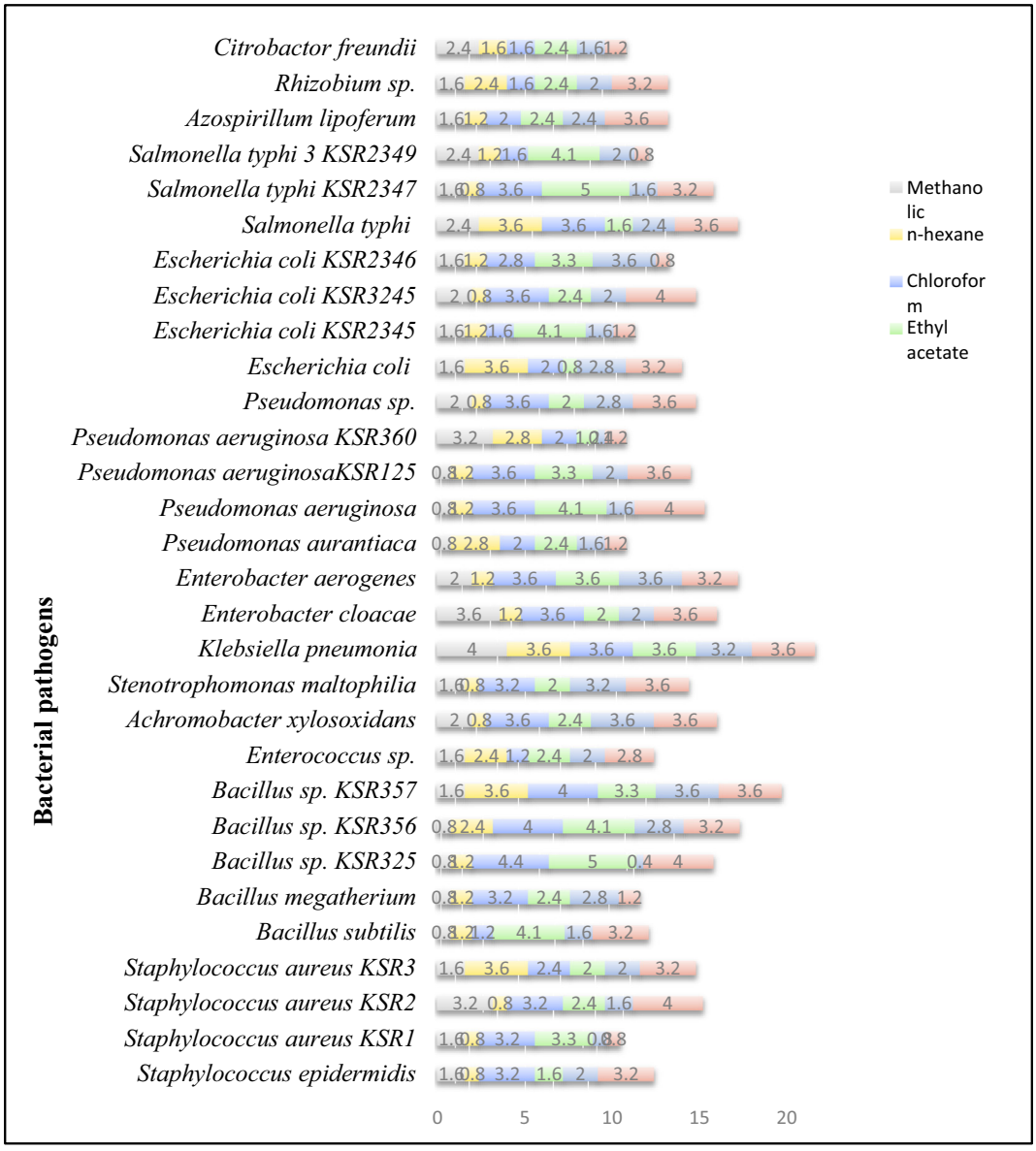

Fig. 3. MICs values of extracts of *E. serrata* against various bacterial pathogens (mg/mL).

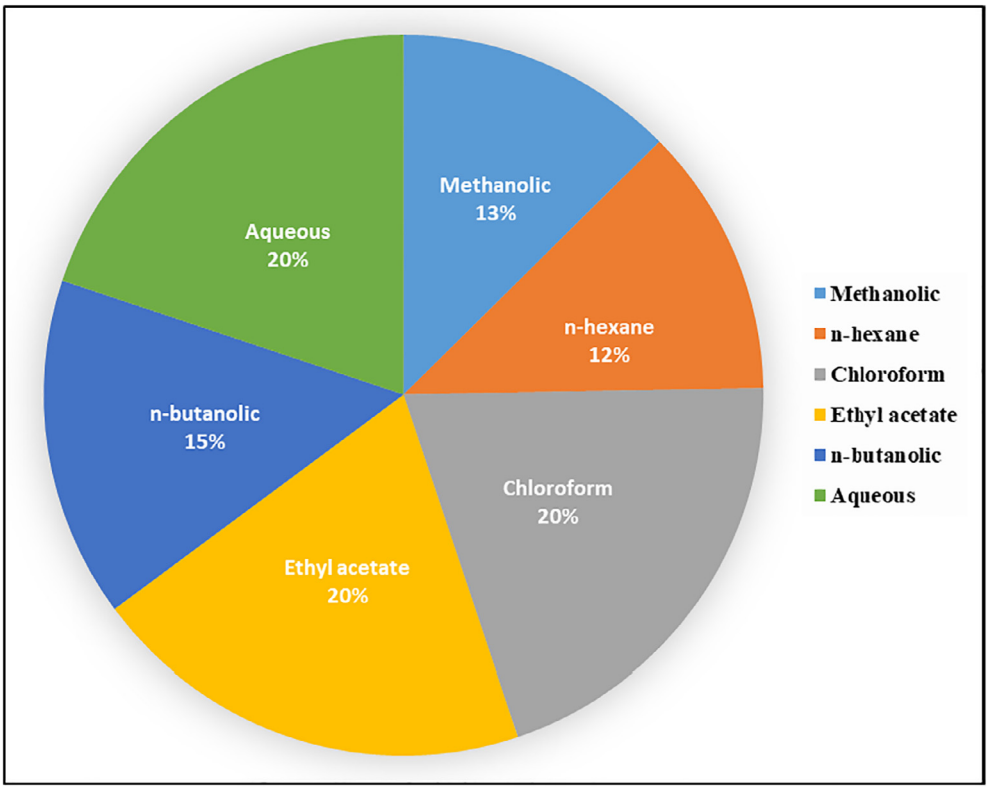

Fig. 4. The dose dependent efficiency of extracts of *E. serrata* against various bacterial pathogens.

Moreover, the MIC values of ethyl acetate and *n*-butanol fractions were ranged between 0.8 and 5.0 and 0.8 to 3.6 mg/mL, respectively. On the other hand, ethyl acetate fraction at a concentration of 2.4 mg/mL inhibited the greater number of bacterial strains whereas in case of *n*-butanol fraction the 2.0 mg/mL was found to be the most effective concentration for the inhibition of the growth of large number of bacteria (Table 3). Likewise, the aqueous fraction also showed considerable activity against the tested microorganisms, MIC values were ranged between 0.8 and 4.0 mg/mL (Table 3). The pie chart shown in Fig. 4 indicates that fractions were effective in the following descending order *n*-hexane (12%) > methanolic (13%) > *n*-butanol (15%) whereas the remaining fraction including chloroform, ethyl acetate and aqueous fractions were equally effective (20%).

3.2. GC–MS analysis

The GC–MS analysis was performed to explore the volatile chemical constitutes in different fractions (Table 4). Compound 2,2′-Methylenebis[6-(1,1-dimethylethyl)-4-methyl phenol was present in hexane (2.126%), ethyl acetate (2.586%) and chloroform (16.026%). Similarly, phytol existed in hexane (4.420%), chloroform (8.439%) and ethyl acetate (2.410%). Likewise, *n*-hexadecanoic acid was found in chloroform (14.806%), ethyl acetate (10.048%) and *n*-butanol (17.853%). Compounds such as Diisooctyl ester-1,2-benzenedicarboxylic acid (84.260%) heptacosane (3.148%) and 3-ethyl-5-(2-ethylbutyl)-octadecane (3.320%) were also found in hexane fraction. Moreover, di-*n*-octyl phthalate (12.950%) and 3,5-dehydro-6-methoxy-, pivalate, cholest-22-ene-21-ol (10.570%) found in chloroform fraction are believed to render activity to kill all the tested microorganisms (Frank et al., 2016). Furthermore, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-benzenepropanoic acid (38.810%) was present in the *n*-butanol fraction. In short, the presence of variety of these metabolites was responsible for the different activities against the tested bacterial strains.

4. Discussion

The higher activities of the plant extracts have been attributed to the presence of different types of secondary metabolites (Joanne et al., 2007). Further, the secondary metabolites like flavonoids, polyphenols, coumarins, tannins, triterpenes, saponins, cardiac glycosides and alkaloids have been reported to have considerable antimicrobial activities (Teke et al., 2011). GC–MS analysis of sub-fractions of methanolic extract revealed the presence of *n*-hexadecanoic acid and mono(2-ethylhexyl) ester of 1,2-benzenedicarboxylic acid that are known to have antimicrobial activity (Agoramoorthy et al., 2007).

The higher activity of *n*-hexane fraction at a concentration of 40 mg/mL is attributed to the presence of compounds such as phytol and 2,2′-Methylenebis(6-*tert*-butyl-4-methylphenol) (Hernández-Villegas et al., 2012). Similarly, other fractions including chloroform, ethyl acetate, *n*-butanol and aqueous fractions were found active against the tested microbes indicating that both polar and non-polar fractions were active against the microbes (Rocha et al., 2011).

The methanolic extract proved to be highly efficient in terms of lowest dose needed to kill the tested microbes. Compared to methanolic extract, *n*-hexane fraction required higher dose (3.6 mg/mL) to inhibit the growth of the same bacterial strains since at concentration of 0.8 mg/mL it did not inhibit the growth of any of the bacteria killed by methanolic extract (Table 3). This suggested that the composition of the natural products in *n*-hexane fraction is quite different from that of methanolic fraction (Khan et al., 2016). However, *n*-hexane fraction showed the best performance in killing the tested microbes since most of the bacterial

Table 4
GC–MS analysis of the different extracts of leaves of *E. serrata*.

Sr. No	Retention time (min)	% of total	Compounds	Molecular formula
n-hexane fraction				
1	23.022	4.420	Phytol	$C_{20}H_{40}$
2	25.749	2.126	2,2′-Methylenebis(6-*tert*-butyl-4-methylphenol)	$C_{23}H_{32}O_2$
3	26.700	84.260	Diisooctyl ester-1,2-benzenedicarboxylic acid	$C_{24}H_{38}O_4$
4	29.127	3.148	Heptacosane	$C_{27}H_{56}$
5	30.672	3.320	3-Ethyl-5-(2-ethylbutyl)- octadecane	$C_{26}H_{54}$
6	33.468	2.726	2-Hexadecanol	$C_{16}H_{34}O$
Chloroform fraction				
1	21.564	14.806	*n*-Hexadecanoic acid	$C_{16}H_{32O2}$
2	23.022	8.439	Phytol	$C_{20}H_{40}$
3	25.749	16.026	2,2′-Methylenebis(6-*tert*-butyl-4-methylphenol)	$C_{23}H_{32}O_2$
4	26.687	112.950	Di-*n*-octyl phthalate	$C_{24}H_{38}O_4$
5	29.127	10.570	Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy, pivalate	$C_{33}H_{54}O_3$
6	30.666	23.480	3-Ethyl-5-(2-ethylbutyl)-octadecane	$C_{26}H_{54}$
Ethyl acetate fraction				
1	19.838	1.292	(E)-5-Eicosene	$C_{20}H_{40}$
2	21.664	10.048	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$
3	23.022	2.410	Phytol	$C_{20}H_{40}O$
4	23.309	3.072	4-Chloro-3-*n*-hexyltetrahydropyron	$C_{11}H_{21}ClO$
5	25.749	2.586	2,2′-Methylenebis(6-*tert*-butyl-4-methylphenol)	$C_{23}H_{32}O_2$
6	26.694	181.884	Di-*n*-octyl phthalate	$C_{24}H_{38}O_4$
n-butanol fraction				
1	21.445	7.421	Cyclohexanol, 5-methyl-2-(1-methylethyl)	$C_{10}H_{20}O$
2	21.621	17.853	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$
3	23.022	12.888	1-Cyclohexylnonene	$C_{15}H_{28}$
4	25.743	23.028	Diisooctyl phthalate	$C_{24}H_{38}O_4$
5	26.687	38.810	3,5-Bis(1,1-dimethylethyl)-4-hydroxy-benzenepropanoic acid	$C_{35}H_{62}O_3$
Aqueous fraction				
1	21.564	26.358	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$
2	26.700	73.642	Mono(2-ethylexyl) ester-1,2-benzenedicarboxylic acid	$C_{16}H_{22}O_4$

strains were killed at very low dosages (Fig. 3). This fact was further confirmed by evaluating overall dosage required by all the fractions to kill germs in the MIC experiment. The *n*-hexane fraction required the lowest dosage to inhibit the growth of 32 bacterial strains. The behavior of the remaining fractions was also appreciable in terms of their MIC values. The antimicrobial properties of methanolic extract and its sub-fractions were attributed to the presence of various metabolites confirmed by GC–MS analysis. These compounds contain different functional groups such as hydroxyl and carboxylic groups, which are required for antimicrobial activity (Omosa et al., 2016).

5. Conclusion

The antimicrobial activity of methanolic extract and its sub-fractions of leaves of *Ehretia serrata* were evaluated against a wide range of pathogens. Both the methanol and *n*-hexane extracts have shown considerably promising results against the tested multi drug resistant human pathogens like *Escherichia coli*, *Staphylococcus aureus* and *Azospirilum lipoferum*. In some cases, crude methanolic extract and *n*-hexane fraction have shown superior activity than the existing antibiotics. This manifests the medicinal importance of *E. serrata*. This is the first report to explore the potential of this plant against pathogens which warrants further extensive studies on the phytochemical studies, which in turn may result in the discovery of new and more potent antibiotics.

Competing interests

The authors have no competing interests.

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