

Spectroscopic Analysis and Antibacterial Activity of Partially Purified Dichloromethane Fraction of *Emilia praetermissa* Milne-Redhead (Asteraceae) Leaf

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Research Article

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Abstract

Purpose

The search for better antibacterial agents from plants has resulted in the identification of molecules with existing analogues. *Emilia praetermissa* is an herbaceous plant that is traditionally used to treat bacterial skin infections and diarrhea. This study aimed to identify compounds in partially purified *E. praetermissa* and investigate their antibacterial activity.

Methods

Gradient elution chromatography was used to achieve partial purification of the compounds. Gas chromatography–mass spectrometry (GCMS) and Fourier transform infrared spectroscopy were used to identify the phytochemicals from the eluent of interest (EP1). The agar well diffusion plate protocol enabled the assessment of the susceptibility profile of the eluents (EP1–EP11) (10 mg/mL) against clinical isolates: *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Results

EP1 revealed fourteen compounds: 4aS-3,4,5,9-tetramethyl-1,25,6,7,8-hexahydro-4a,8-epoxybenzo[7]annulene (15.74%), N-(3-allyl-2-oxo-2,3-dihydro-1,3-benzothiazol-6-yl) acetamide (5.52%), pentadecane (1.87%), heptacosane (3.52%), heptadecane (1.34%), nonadecane (1.09%), eicosane (3.56%), heneicosane (1.31%), di-sec-butyl phthalate (46.92%), bis(2-ethylhexyl) phthalate (6.62%), 2,4-di-tert-butylphenol (7.90%) and 2-ethyl-2H-benzotriazole (4.51%). The functional groups identified include O-H, N-H, C-H, C-C and C = C, which combine with the data from the GC–MS. Susceptibility testing revealed the inhibition of *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* by eluents EP1–EP11, excluding EP4, EP5 and EP10.

Conclusion

The results show that the identified compounds inhibit the pathogenic bacteria responsible for inducing skin infections and diarrhea, confirming their traditional use.

Introduction

Emilia praetermissa Milne Redhead (Asteraceae) is a non-persistent vascular plant with origin in West Africa and is widely distributed on other continents, such as the Caribbean and Asia [1–3], where it was initially misidentified as *Emilia forsbegii* [1]. It is habitually known for its invasive nature in both cultivated and fallowed lands. According to reports locally, *E. praetermissa* has been found to be very effective in the treatment of internal and external wounds, bacterial diarrhea, and respiratory infections and in preventing new-born skin infections [4].

Bacteria have been implicated in several skin infections and diarrheal-related diseases [5]. These entities are microscopic, ubiquitous, single-cell organisms that are broadly classified as gram-positive and gram-negative [6]. Monoderm bacteria are characterized by a thick peptidoglycan cell wall, whereas diderm bacteria possess an outer cell membrane [7]. Many of these bacteria can be found within and outside the body, where they have either beneficial or harmful effects. Some examples of monoderm include *Staphylococcus aureus* and *Bacillus subtilis*, whereas diderm contain *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* [8].

S. aureus are cocci measuring 1 μm in diameter and are distinguished from other species in the genus by their coagulase-positive nature, salt leniency and regular hemolysis [9]. It has been implicated in several forms of infection, extending from shallow skin lesions to profound seated and nosocomial infections. It has also been reported to cause spoilage in food by releasing enterotoxin into foodstuff and responsible for toxic shock syndrome in human through super antigens release into the blood [10–12]. *S. aureus* colonizes the skin and nasal passage while exerting its virulence by releasing surface proteins that promote the colonization of host tissue [13]. The release of capsules and immunoglobulin-binding protein A promote phagocytosis and toxin release, which damage host tissues [14–15].

E. coli are facultative anaerobic rods that inhabit the gastrointestinal tract in human, where it forms part of the normal microbiota [16–17]. It exists singly or in pairs measuring 0.5–1.0 μm $\times$ 1.0–4.0 μm in width and length, do not form protective covering after exposure to harsh conditions and non-acid fast [18]. Although many *E. coli* strains are considered safe, though some serovar may cause running stomach when taken into the gastrointestinal tract; others may cause anaemia, while infections may be observed in some body organs and tracts [18]. Virulence may be acquired in *E. coli* by bacteriophage and plasmid formation, with pathogenicity islands encoding virulence factors and transporons within a genome [19].

The search for better antibacterial agents has necessitated the study of plant materials because of their believed safety, low untoward effects and cost effectiveness. Extracts from plants are known to contain phytochemicals with similar or better analogues than existing drugs. The antibacterial nature of the leaf extract of *E. praetermissa* was established in a previous study by our group, and GC–MS analysis was also used to obtain a list of identified phytochemicals with likely antibacterial activities [20]. This study intends to narrow down the identified compounds by partial purification of the dichloromethane fraction from the extract that has previously shown antibacterial activity. This study provides information on compounds with antibacterial potential in *E. praetermissa*; thus, this research aims to identify compounds with antibacterial potential from the leaves of *E. praetermissa*.

Materials and methods

Solvents, Reagent, Drugs and Instruments

Solvent and reagents used in this study were of analytical grade, while drug used was gotten from manufacturer of repute. Methanol (99%) (BHD), dichloromethane (BHD), n-hexane (BHD), Silica gel (Himedia GRM7477), KBr (99 -100.2%) (Merck), Mueller-Hinton Agar (Babio), Ciprofloxacin (Cipla), GC (7890A Agilent US model) - MS (MSD5675C), FTIR (Perkin Elmer 3000 MX).

Collection, identification and preparation of the plants

Fresh aerial parts of *E. praetermissa* were collected in October within the hours of 7:00 am – 8:30 am at the University of Benin, Ugbowo Campus, with geo-references at latitude 6° 23' 30"N and longitude 5° 36' 30"E. Identification and authentication were performed by Professor Emmanuel Aigbokhan of the Plant Biology and Biotechnology Department. The herbarium voucher number was UBH-E407, while further authentication was performed by uploading the picture to Google image service. A specimen of *E. praetermissa* was placed in the herbarium for prospective reference.

The leaves were carefully separated from the stem and air dried away from direct sunlight for a duration of 25 days. The sample was reduced to a fine powder with the aid of an electric milling machine, and airtight container was used to store the pulverized sample until further work was carried out.

Extraction and Partial Purification

Extraction was performed via maceration using 2x2.5 L of methanol and 400 g of the powdered sample. This setup was left for 48 h with stirring at intervals of 12 h; this mixture was subsequently decanted into a 3 L beaker and passed through size 1 filter paper. An *in-vacuum* rotary evaporator maintained at a temperature of 50°C was used to concentrate the filtrate, and the weight of the extract obtained was noted (35.00 g) and subsequently kept in a refrigerator at 4°C until use.

The crude extract (26.00 g) was adsorbed to 25 g of silica gel (mesh size 60–120 µm) until it became powdery, after which it was transferred into a column (measuring 5 cm × 50 cm) packed with silica gel (200 g). N-hexane (600 mL) was isocratically used to elute the column, and the eluent was collected. This process was repeated by gradient elution using n-hexane-dichloromethane at different ratios: 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100. Each variation in solvent mixture resulted in a total volume of 300 mL and a total of 292 test tubes used to collect the eluent from the column. These samples were observed to have different colours ranging from yellow to deep green and pale green. Crystals were observed in test tubes labelled 41–67 (EP1); hence, they became our focus. The white crystals were subjected to gas chromatography–mass spectrometry (GC–MS) and Fourier transform infrared (FTIR) spectroscopic analysis, and the antibacterial potential of the eluents (EP1–EP11) was evaluated.

GC–MS analysis: This analysis was carried out via coupled GC–MS with a triple-axis detector and a 10 µL syringe autosampler. The GC system (7890A) was an Agilent USA model, while the MS was an inert MSD 5675C. A capillary column from Agilent (19091-433HP-5Ms) treated with 5% phenyl, 95% methyl polysiloxane and measuring 30 m (length), 0.2 µm (internal diameter), and 250 µm (thickness) was utilized for chromatographic separation, while helium was the carrier gas. The ion source and interface temperatures were set at 250°C and 300°C, 16.2 psi as the pressure and a 1:50 split ratio with a 1 µL injector in split mode, while 280°C was the injection temperature. The temperature of the column was started at 50°C for 2 min and adjusted at a rate of 20°C/min to 100°C. This mixture was elevated to 250°C at 20°C/min and maintained for 5 min. While acquiring data, software from the manufacturer was utilized to control the system, and compound qualification was achieved by mass spectra comparison with data from previous research and NIST [21].

FTIR Spectroscopy

Functional group determination in EP1 was performed via a Perkin Elmer 3000 MX spectrometer. This process involves mixing a few crystals of EP1 with potassium bromide and reducing them to a fine homogenous powder in a mortar (agate). This formed an appropriate pellet following the application of a pressure of 6350 kg. The spectra were recorded following scanning in the wavenumber range between 4000 cm⁻¹ and 650 cm⁻¹. The resolution was set at 4 cm⁻¹, and the scan per spectrum was 32 cm⁻¹. Analysis of the infrared spectra was performed via WIN-IR Pro Version 3.0, with a sensitivity of the peak at 2 cm⁻¹.

Antimicrobial activity

Preparation of media and agar plates

A total of 38 g of Mueller–Hinton agar was added to 1 L of distilled water in a conical flask, and this mixture was heated to 100°C while stirring. The mixture was covered with foil paper and autoclaved for 15 min at 121°C. The mixture was allowed to cool to 45°C and subsequently dispensed (30 mL) under a sterile lamp into Petri dishes, while the plates were allowed to set and solidify. The samples were dried in a hot air oven at 50°C for 10 minutes to remove excess surface moisture.

Collection and preparation of bacterial cultures

The drug-resistant clinical isolate of the bacteria used for this study was obtained from the University of Benin Teaching Hospital and was cultured overnight (24 h at 37°C) in sterile nutrient broth by inoculation. The bacteria used for this study included *S. aureus*, *K. pneumoniae*, *E. coli*, *B. subtilis* and *P. aeruginosa*. Identical colonies from these plates were inoculated into sterile nutrient broths. These mixtures were allowed to incubate for 12 h and subsequently adjusted with

the MacFarland standard (0.5%) to obtain an inoculum size of 108 cfu/mL. These concentrations were further adjusted to 106 cfu/mL by diluting the inoculum to 1:100.

The modified agar well diffusion method was used in the susceptibility test; this was achieved by thoroughly mixing 200 µL of standard inoculum with 30 mL of sterile Mueller-Hinton agar, which was allowed to solidify. A sterile cork borer (8 mm or 10 mm in diameter) was used to cut the wells into the agar plate. The base of each well was sealed with molten Mueller-Hinton agar, and a calibrated micropipette with a rubber teat was used to introduce 10 mg/mL of the eluent (EP1–EP11) into each well. The plates were allowed to stand for 30 min before being incubated upside down at 37°C for 24 h. Inhibition zone diameters (measured in millimetres) of each eluent were measured and recorded. A similar protocol was repeated for the control, ciprofloxacin (0.5 mg/mL) [22].

Statistical analysis

The antimicrobial test was performed in duplicate, and the data are presented as the means ± standard errors of the means. The data were analysed via one-way analysis of variance. A post hoc study was performed via Tukey analysis, and the level of significance was set at $p \leq 0.05$ (***), using version 5 of the GraphPad Prism.

Results and Discussion

Following extraction, the crude extract was adsorbed onto silica gel, and different solvent ratios (dichloromethane–hexane) were used to elute the prepared column. The eluents were collected in test tubes (Table 1), which were subsequently bulked (EP1–EP11) together on the basis of thin-layer chromatographic profiles (Table 2). Figure 1 show the scheme from the extraction of the powdered plant to the point where the eluents were obtained.

Table 1
partially purified crude extract of *Emilia praetermissa*

Solvent mix	Solvent ratio	Volume (mL)	Test tubes
Hexane	100	600	1–40
Hexane-dichloromethane	90:10	300	41–67
Hexane-dichloromethane	80:20	300	68–91
Hexane-dichloromethane	70:30	300	92–118
Hexane-dichloromethane	60:40	300	119–142
Hexane-dichloromethane	50:50	300	143–169
Hexane-dichloromethane	40:60	300	170–193
Hexane-dichloromethane	30:70	300	194–219
Hexane-dichloromethane	20:80	300	220–242
Hexane-dichloromethane	10:90	300	243–269
Dichloromethane	100	300	270–292

Table 2
Coding of eluents from
test tubes

Coding	Test tube
EP1	41–67
EP2	68–80
EP3	81–96
EP4	97–111
EP5	112–143
EP6	144–157
EP7	158–197
EP8	198–224
EP9	225–240
EP10	241–268
EP11	269–292

Susceptibility test

The antibacterial potential of the eluents was evaluated against five bacterial species, *B. subtilis*, *E. coli*, *K. pneumoniae*, *S. aureus* and *P. aeruginosa*, via the agar well diffusion method previously described by Babaiwa and coworkers [22]. Ciprofloxacin was utilized as the reference drug at 0.5 mg/mL. *E. coli*, *B. subtilis* and *S. aureus* were susceptible to 10 mg/ml EP1-EP4, EP6-EP9 and EP11, respectively (Table 3). The zone of inhibition was 12 ± 0.12 mm (EP4) for *B. subtilis*, ranging from 12 mm to 14 mm (EP1-EP3, EP6-EP9 and EP11) for *E. coli* and 14 mm to 18 mm (EP1-EP3, EP6-EP9 and EP11) for *S. aureus*. The effects of the EP2, EP3, EP7 and EP10 strains tested against *E. coli* were significant compared with those of ciprofloxacin, with a level of significance at $p \leq 0.05$. Similarly, EP2, EP6 and EP11 were significantly different at $p \leq 0.05$ compared with the standard drug with *S. aureus*. Resistance of *B. subtilis*, EP4, EP5 and EP10 to both *E. coli* and *S. aureus* was observed with the EP1-EP3 and EP5-EP11 eluents. While maintaining similar concentrations, resistance was observed against *P. aeruginosa* and *K. pneumoniae* via EP1 - EP11. Eluents that are more susceptible than ciprofloxacin (0.5 mg/mL) are known, although our previous study revealed the susceptibility of the *E. praetermissa* dichloromethane fraction to *P. aeruginosa* [20]. This study further purified the dichloromethane fraction into various eluents (EP1 to EP11), and the activities against *S. aureus*, *B. subtilis* and *E. coli* were noted. Resistance may have been observed in gram-negative bacteria, but susceptibility to *E. coli* indicated that these eluents may possess specific related antibacterial actions. The specific mechanism by which these genes confer resistance could involve efflux pump action, permeability barrier induction, mutational or recombination changes in target enzymes and alterations in targets with decreased affinity for drug compounds [23]. Additionally, resistance could hypothetically be due to virulence [24–25]. The susceptibility of *S. aureus*, *E. coli* and *B. subtilis* to these eluents could imply their use in the treatment of skin infection and diarrhea caused by these bacteria, justifying their ethno-medicinal usage of this plant.

Table 3
Antibacterial activity

Bacteria	EP1	EP2	EP3	EP4	EP5	EP6	EP7	EP8	EP9	EP10	EP11	Ciprofloxacin
<i>Escherichia coli</i>	14 ± 0.20	13 ± 0.11	12 ± 0.11	NZ	NZ	13 ± 0.10	14 ± 0.10	13 ± 0.11	13 ± 0.12	NZ	13 ± 0.00	13 ± 0.11
<i>Klebsiella pneumoniae</i>	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	22 ± 0.20
<i>Pseudomonas aeruginosa</i>	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	30 ± 0.14
<i>Staphylococcus aureus</i>	16 ± 0.10	18 ± 0.11	15 ± 0.21	NZ	NZ	18 ± 0.10	18 ± 0.10	18 ± 0.08	18 ± 0.05	NZ	14 ± 0.20	26 ± 0.12
<i>Bacillus subtilis</i>	NZ	NZ	NZ	12 ± 0.12	NZ	NZ	NZ	NZ	NZ	NZ	NZ	30 ± 0.22

Keywords: NZ = No zone of inhibition; zones of inhibition were measured in millimetres.

Escherichia coli

EP1 vs all except EP7: ***

EP2 vs EP3, EP4, EP5, EP7 and EP10: ***

EP3 vs EP6, EP7, EP8, EP9, EP10, EP11 and ciprofloxacin: ***

EP4 vs EP6, EP7, EP8, EP9, EP11 and ciprofloxacin: ***

EP9 vs EP10: ***

EP5 vs EP6, EP7, EP8, EP9, EP11 and ciprofloxacin: ***

EP6 vs EP7 and EP10: ***

EP7 vs EP8, EP9, EP10, EP11 and ciprofloxacin: ***

EP8 vs EP10: ***

EP10 vs EP11 and ciprofloxacin: ***

Staphylococcus aureus

EP1 vs all: ***

EP2 vs EP3, EP10, EP11 and ciprofloxacin: ***

EP3 vs all: ***

EP7 vs EP10, EP11 and ciprofloxacin: ***

EP8 vs EP10, EP11 and ciprofloxacin: ***

EP9 vs EP10, EP11 and ciprofloxacin: ***

EP4 vs EP6, EP7, EP8, EP9, EP11 and ciprofloxacin: ***

EP5 vs EP6, EP7, EP8, EP9, EP11 and ciprofloxacin: ***

EP6 vs EP10, EP11 and ciprofloxacin: ***

EP10 vs EP11 and ciprofloxacin: ***

EP11 vs ciprofloxacin: ***

FTIR analysis

The functional groups in the EP1 eluent were determined from the absorption spectrum from the FTIR data. The absorption spectrum is provided in Fig. 2, while a summary of the absorption range and specific functional groups in EP1 are provided in Table 4. The main functional groups include the O–H bond of the hydroxyl group and the N–H medium bond of the secondary amine and amide. C–H and C–C stretches were observed for saturated alkanes and rings. The C = C bonds and C ≡ C stretches are indicative of alkene and alkyne groups. The H–C = O stretch for the aldehydic group and overtones was observed at 1919.60 cm⁻¹, although it was weak. Towards the end of the group frequency region, N–O symmetric stretching and C–O stretching were observed, representing nitrile and aldehydic, amide and ester groups, respectively.

Table 4
FTIR spectra interpretation

S/N	Wavenumber (Reference)	Wavenumber (Test Sample)	Functional group assignment	Inference
1	3650 – 3200	3336.00	O-H, N-H	N-H medium, H-bonded
2	3000 – 2850	2974.40	C-H	C-H stretching bond
3	3000 – 2850	2929.70	C-H	C-H stretching bond
4	2830 – 2695	2832.80	H-C = O	H-C = O stretch
5	2260 – 2100	2124.60	-C $\equiv$ C-	C $\equiv$ C stretch
6	2000 – 1665	1919.60		Overtones, weak from this range
7	1680 – 1620	1636.30	C = C	C = C bonds
8	1500 – 1400	1449.90	C-C	C-C stretch in ring
9	1360 – 1290	1326.90	N-O	N-O symmetric stretch
10	1260 – 1050	1088.40	C-O	C-O stretch
11	1300 – 1000	1028.40	C-O	C-O stretch
12	1000 – 650	879.70	=C-H	=C-H bend

GC–MS analysis

The chromatogram of EP1 revealed fourteen compounds (peaks) (Fig. 2), with retention times ranging from 4.792 min to 19.944 min. The prominent compounds were observed at 9.319 min, 9.719 min, 13.662 min, 13.902 min, and 19.944 min, with a total percentage area of 82.70%, while the individual percentage areas were as follows: 15.74%, 7.90%, 5.52%, 46.92%, and 6.62%, respectively.

These fourteen compounds are mainly triazole, straight-chain alkanes, epoxide, amide and phthalate (Table 3). The prominent compounds are 2-ethyl-2H-benzotriazole (4.51%), N-(3-Allyl-2-oxo-2,3-dihydro-1,3-benzothiazol-6-yl) acetamide (5.52%), bis(2-ethylhexyl) phthalate (6.62%), 2,4-di-tert-butylphenol (7.90%), (4aS)-3,5,5,9-tetramethyl-1,2,5,6,7,8-hexahydro-4a,8-epoxybenzo[7]annulene (15.74%) and di-sec-butyl phthalate (46.92%). These findings indicate a high level of phthalates in EP1 (53.54%). In a previous study, bis-(2-ethylhexyl) phthalate was shown to have antibacterial activity against *E. coli* and *S. aureus* [27]. In contrast, di-sec-butyl phthalate, which is isolated from the whole plant of *Begonia malabarica* (Begoniaceae), has shown antibacterial potential against *K. pneumoniae*, *P. aeruginosa* and *E. coli* at 100 mg/mL [28]. 2,4-Di-tert-butylphenol purified from the *Streptomyces* species KCA1 from *Phyllanthus niruri* inhibited *S. aureus* and *E. coli* at 0.78 μ g/mL and 50 μ g/mL [29]. These findings contradict those of this study, especially with respect to the inhibition of *K. pneumoniae* and *P. aeruginosa*.

Table 5
Compounds identified from EP1 from the retention time, percentage area and molecular weight

S/N	Retention Time (min)	Percentage Area	Base peak	Molecular weight	Compound
1	4.792	4.51	91.0	147.0	2-ethyl-2H-Benzotriazole
2	7.013	1.09	57.0	268.5	Nonadecane
3	9.319	15.74	161.0	218.3	(4aS)-3,5,5,9-Tetramethyl-1,2,5,6,7,8-hexahydro-4a,8-epoxybenzo[7]annulene
4	9.427	1.87	57.0	212.4	Pentadecane
5	9.719	7.90	191.0	206.3	2,4-Di-tert-butylphenol
6	11.539	1.77	57.0	280.7	Heptacosane
7	11.951	1.34	57.0	240.5	Heptadecane
8	13.427	1.85	57.0	280.7	Heptacosane
9	13.662	5.52	231.0	248.3	N-(3-Allyl-2-oxo-2,3-dihydro-1,3-benzothiazol-6-yl) acetamide
10	13.902	46.92	149.0	278.3	Di-sec-butyl phthalate
11	15.138	2.33	57.0	282.5	Eicosane
12	15.464	1.23	57.0	282.5	Eicosane
13	17.026	1.31	57.0	296.6	Heneicosane
14	19.944	6.62	149.0	390.6	Bis(2-ethylhexyl) phthalate

Savelli and coworkers [30] reported the use of derivatives of 2-ethyl-2H-benzotriazole as antimicrobial agents, whereas Nuvole *et al.* [31] reported the susceptibility of *E. coli* to acidic derivatives of benzotriazole. This compound is made of a benzene ring fused to a triazole; this triazole ring possesses antimicrobial activity, which can be partially or completely lost following annulation of the triazole ring at any point [32]. Pentadecane, heptadecane, eicosane, heneicosane and nonadecane, with a percentage area of 12.79%, are straight alkanes; some have previously been reported in *E. coccinea* [33] to have antimicrobial activity [34]. Derivatives of nonadecane have been reported to have moderate antibacterial effects against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis* [35–36]. The presence of these compounds in the eluent EP1 may be responsible for the antibacterial activity observed and could be synergistic in action against these bacteria.

Conclusion

Gradient eluents from hexane-dichloromethane of *E. praeterrmissa* were shown to possess antibacterial activity against selected clinical isolates. Antibacterial activity was observed in the EP1 eluent because of the presence of long-chain alkanes, benzotriazole, phthalate and butylphenol derivatives. These findings validate the use of *E. praeterrmissa* in the treatment of skin infection and diarrhea, since some of the bacteria involved in these conditions are inhibited.

Declarations

Contribution of Authors

Beauty Ighomena was involved in data collection and editing of the manuscript, whereas Emmanuel Eimiomodebheki Odion conceptualized the study and wrote and edited the manuscript.

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Figures

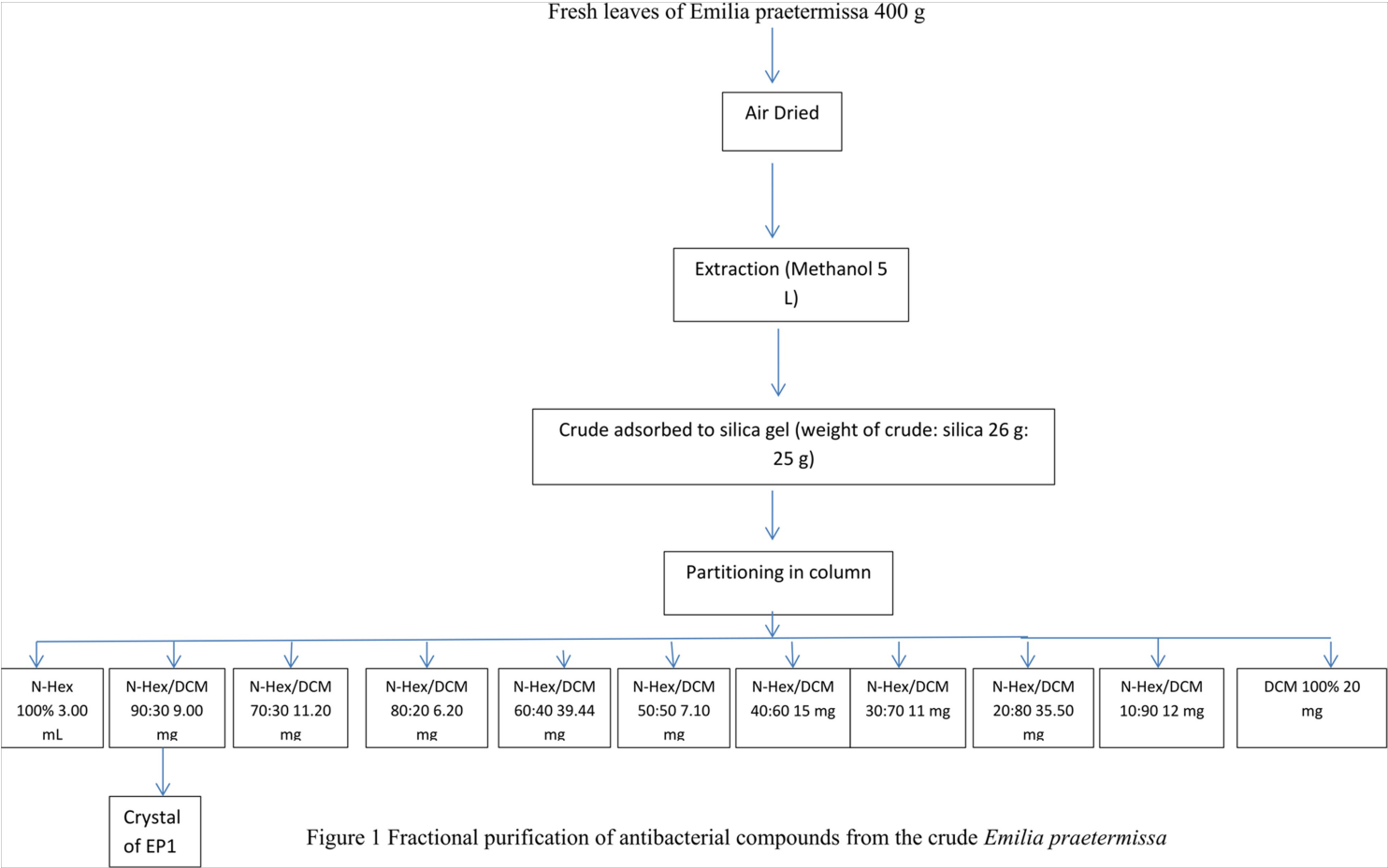


Figure 1

See image above for figure legend.

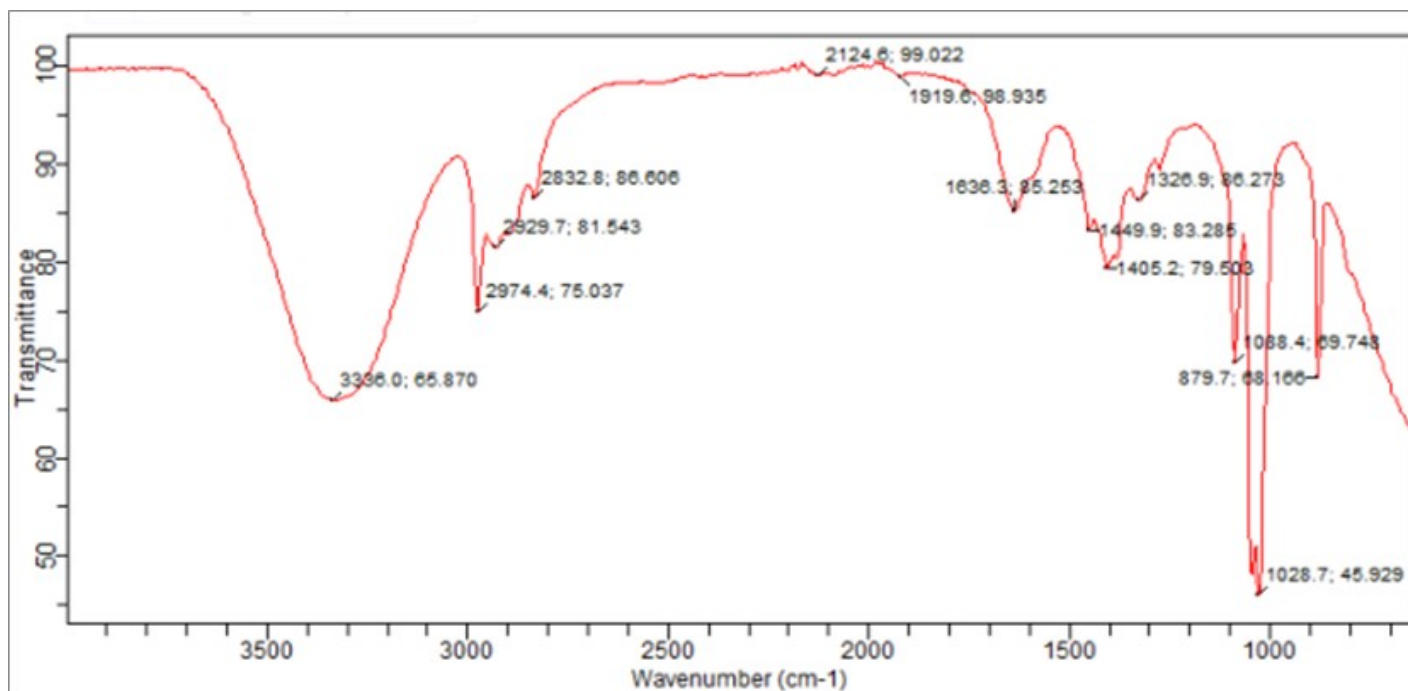


Figure 2

FTIR spectra of EP1

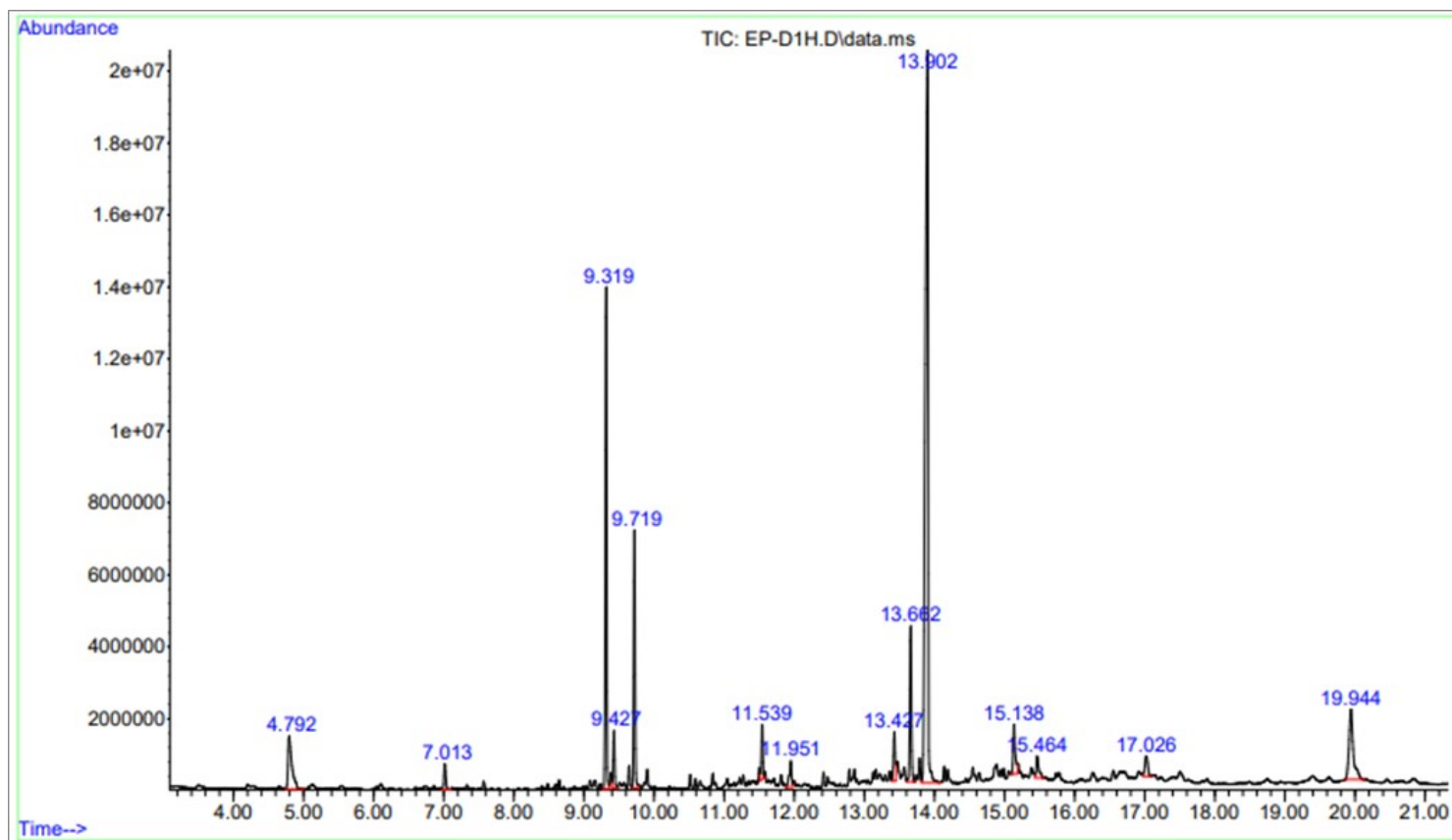


Figure 3

Chromatogram of EP1