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ANTIMICROBIAL ACTIVITY OF *PIMENTA DIOICA* (L.) Merr. LEAVES AND ITS SYNERGISTIC ACTIVITY WITH AMPICILLIN AGAINST ESBL PRODUCING CLINICAL ISOLATES

*Meghana R. Gore, Darshana Raut, Aruna K**

Address(es): Dr. Aruna K.,
Department of Microbiology, Wilson College, Girgaum Chowpatty, Mumbai 400007, Maharashtra.

*Corresponding author: arunasam2000@yahoo.co.in

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ABSTRACT

The intriguing discovery of antibiotics that led to innumerable accomplishments in the past is slowly losing its efficiency in the clinical society. The emergence of antibiotic-resistant pathogens, such as those which can hydrolyze β-lactam antibiotics with the help of enzymes such as Extended Spectrum β-lactamases (ESBLs) has created an urgent necessity to witness innovation in the current drug therapy, and develop new antimicrobial agents. Keeping the current clinical adversity in mind, the present study was carried out to investigate the antibacterial activity of *Pimenta dioica* (L.) Merr. extracts, prepared in various chemical solvents with the help of soxhlet apparatus, and its synergistic activity with ampicillin. The antibacterial activity of these extracts was verified against 45 ESBL producing bacteria. Our study revealed chloroform extract to exhibit maximum antibacterial activity evident with the zones of inhibition (12-17 mm) showed by agar diffusion method, and their observed Minimum Bactericidal Concentrations (MBC) in the range of 2-5 $mg.ml^{-1}$. The MBC of ampicillin was found to decrease from 10 $mg.ml^{-1}$ to 300-500 $\mu g.ml^{-1}$ in presence of chloroform extracts of *P. dioica* (L.) Merr. leaves, indicating a synergistic activity between them. In addition, the TLC bioautography of chloroform extract was carried out using 2, 3, 5-Trimethyl tetrazolium chloride as a chromogen, and the separated bioactive components were analyzed with the help of GC-MS which showed eugenol as a major constituent. Moreover, the scanning electron microscope analysis confirmed a visible deformation in the cell membrane of *E. coli* treated with chloroform extracts of *P. dioica* (L.) Merr., indicating stress and cellular damage in presence of these extracts.

Keywords: ESBL, *Pimenta dioica* (L.) Merr., Bioautography, MBC, Soxhlet

INTRODUCTION

The growing complications associated with Multiple Drug Resistant (MDR) strains of pathogens are clearly evident in the thousands of literature available online. The gradual exposure of pathogens to antibiotics, coupled with its indiscriminate use and consequent antibiotic selection pressure, has led to an enrichment of MDR strains not only in the hospitals but also in the general community (**Selim, 2012**). The progressive ineffectiveness of current antibiotics to treat major infectious diseases emanates from the long-term drug abuse by profit-seeking organizations (i.e., healthcare and pharmaceutical industries); and little awareness amongst the masses (**Aruna and Mobashshera, 2012; Shriram *et al*., 2010**). The complexity of this situation is further enhanced by the constantly evolving nature of drug resistance among pathogens, and the contemporaneous decline observed in drug discoveries in the last few decades (**Ventola, 2015**).
The emergence of Extended-Spectrum β-Lactamases (ESBLs) connotes one of the best examples in this situation. ESBLs are enzymes produced by MDR pathogens that confer a high degree of resistance to most of the commonly used β-lactam antibiotics including the advanced 3rd generation cephalosporins viz., cefotaxime, ceftazidime, ceftriaxone etc. (**Giske *et al*., 2008**). The choice of treatment under such circumstances generally include administering a high dose of a suitable antibiotic from other classes, or the use of combination therapy. However, they are commonly linked to severe side-effects; sometimes irreversibly damaging the liver and kidneys (**Scarpignato *et al*., 2016**). The host of other ill consequences of antibiotic over-use like drug dependence and correspondingly lowered immunity (**Mohle *et al*., 2016**), allergies (**Garcia *et al*., 2012**) etc., apart from the proven transformation of harmless bacteria to superbugs, elicits tapping alternative treatment protocols to confront these difficulties. In the past few decades, the attempts to safe clinical approaches have made scientist more keen towards other branches of medicine like Ayurveda, Unani, acupuncture, and phytotherapy to name a few (**Chaudhury and Rafei, 2001**).

The profound side-effects of high dose antibiotic therapies associated with effective treatment of infections are as much responsible for shifting our focus from the allopathic pharmacopeia, as it is for the safety and competency promised by the phytotherapeutic approaches. Phytotherapy is an indispensable branch of herbal medicine practiced exclusively in ancient times. Its fundamentals are deep-rooted in the undiscovered laws of nature that have greatly benefitted the humankind; among which, synergistic activity of biochemical compounds is one of the best understood unraveled phenomena. With the advent of antibiotics, it was believed that humans will revolutionize the clinical world and conquer over the health disasters, yet the traditional therapies have found its way back into the current era with promising solutions to the problem of antibiotic resistance. This can be apparently manifested by numerous research studies published previously by several authors (**Tariq *et al*., 2014; Freitas *et al*., 2013**).
Herbal medicines are a major source of the raw materials used alone and in combination with conventional antibiotics, and have shown promising activities against many pathogens (**Belofsky *et al*., 2004; Beg *et al*., 2004**). For instance, eugenol- a common constituent present in majority of the spices can induce cell lysis and leakage of protein and lipid content within 120min of exposure in *Listeria monocytogenes*, *Streptococcus pyogenes*, *Proteus vulgaris* and *Escherichia coli* (**Oyedemi *et al*., 2009**). It has also shown synergistic activity with several antibiotics (viz., penicillin, chloramphenicol, ampicillin, polymyxin B, norfloxacin, tetracycline, rifampicin and vancomycin) against *E. coli, Enterobacter aerogenes, P. vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium* as well as cariogenic and periodonto-pathogenic bacteria (**Hemaiswarya and Doble, 2009; Moon *et al*., 2011**).
The essential oils produced by plants during the process of secondary metabolism act as a concentrated mixture of bioactive components that can be used for medicinal purposes. The diverse mechanisms of these compounds can exhibit anti-bacterial, anti-fungal as well as anti-viral properties at the same time, as against most of the antibiotics that we use in general practice. The essential oils are also observed to negatively affect the pathogenicity of several organisms. Studies carried out on *Staphylococcus aureus* have shown that oregano oil can

significantly reduce its lipase and coagulase activity (**Carneiro de Barros *et al*., 2009**). In addition, eugenol oil reduces the production or activity of enterotoxin A and B, toxic shock syndrome toxin 1 and a-haemolysin in *S. aureus* (**Qiu *et al*., 2010**). Moreover, the carbonyl group on cinnamaldehydes may bind to proteins, thus inhibiting the function of bacterial amino acid decarboxylases (**Wendakoon and Sakaguchi, 1993**). The inhibition of ATPase activity due to disruption of cellular membrane, and blocking of efflux pumps is also reported (**Bolla *et al*., 2011; Di Pasqua *et al*., 2007**). Hence, the identification, purification, and use of bioactive components from parts of medicinal plants may end our struggle to fight serious infections cost-effectively (**Cheesman *et al*., 2017**).
One such plant; and the basis of our current investigation, is *Pimenta dioica* (L.) Merr., commonly known as clove pepper, which is used as a spice. It is a sturdy perennial tree belonging to the family Myrtaceae. It possesses the characteristic flavor and aroma of clove, nutmeg, cinnamon and black pepper, all combined in this one spice, hence also named allspice (**Kamble *et al*., 2012**). The traditional literature, along with several recently published articles, verifies the use of *P. dioica* (L.) Merr. leaves and oil as an ailment against complications of the gastrointestinal tract, rheumatism, arthritis, stress and neuralgia. In some regions of India, it is also used as a means of relieving symptoms like pain, fever, indigestion and nausea (**Khandelwal *et al*., 2012; Agrawal, 1997**). The leaf and bark extracts of *P. dioica* (L.) Merr. have shown antimicrobial activity against clinical isolates of *Streptococcus mutans* and *S. aureus* obtained from dental caries and burn exudates, respectively (**Manasa *et al*., 2013**; **Al-Harbi *et al*., 2017**). The essential oil extracted from this plant has also shown inhibitory effect on pathogens like *Pseudomonas putida*, *E. coli*, *S. typhimurium*, *L. monocytogenes* and *S. aureus* (**Al-Harbi *et al*., 2017; Oussalah *et al*., 2007**). However, to our knowledge, no data is available on the efficacy of this plant against multi-drug resistant pathogens like ESBL producers.
Hence, considering the increasing antibiotic resistance among pathogens, the objective of our study was to investigate the efficacy of *P. dioica* (L.) Merr. leaf extract as a possible alternative source of medicine by exploring its antibacterial as well as synergistic activities against ESBL producing clinical isolates.

MATERIAL AND METHODS

Plant material used in the study

The leaves were collected from the *P. dioica* (L.) Merr. plant, maintained in a local garden, and authenticated by an expert botanist from Botany department, Wilson College, Mumbai, before use.

Test organisms

A total of 45 gram-negative ESBL producing pathological isolates, screened and characterized in our previous studies, were used in the current investigation (**Tariq and Aruna, 2015; Tariq and Aruna, 2016**). We selected 10 representative isolates each from the genera *Klebsiella*, *Escherichia*, *Pseudomonas* and *Citrobacter*, and 5 isolates from the genera *Proteus* for checking the antibacterial efficacy of *P. dioica* (L.) Merr. leaf extracts. All isolates were maintained on Luria-Bertani (LB) agar slants supplemented with 100 µg.ml^{-1} of ampicillin and stored at 4°C until use in our laboratory.

Preparation of the extracts

The leaves of *P. dioica* (L.) Merr. plant were thoroughly cleaned with distilled water, dried in shade for 10 days, and powdered using a grinder before commencing the extraction procedure. The bioactive components were extracted from 15 g of powdered leaves in 200 ml of chemical solvents viz., methanol, butanol, chloroform, petroleum ether and acetone for a duration of 8 h with the help of Soxhlet apparatus. The obtained extracts were concentrated by allowing the solvents to evaporate at Room Temperature (RT). The concentrates thus obtained, yielding 500 mg - 1.5 g, were stored at 4°C until further use.

Sterility testing of solvent extracts

The sterility of the extracts was confirmed by checking for bacterial or fungal growth after spot inoculating them on a sterile Nutrient Agar (NA) and Sabouraud's Agar (SAB) plate respectively (**Hemaiswarya and Doble, 2009; Rao *et al*., 2010**). The NA plates were incubated at 37°C and SAB plates at RT for an extended duration of 7 days to confirm the absence of contaminants.

Qualitative assay for determination of antibacterial activity of solvent extracts of *P. dioica* (L.) Merr. leaves

The antibacterial efficacy of each extract was analyzed qualitatively by agar well diffusion method. Sterile molten NA butt was seeded with 0.4 ml of 24 h old test pathogens (0.1 OD_{540nm}) and poured into sterile petri-plates. After solidification, wells were punched into the medium using a sterile cork-borer and inoculated with 50 µl of solvent extracts. It was then allowed to diffuse through the wells during its incubation at 37°C for 24 h, after which the resulting zones of inhibition were measured. The solvent extract showing the maximum zone of inhibition against test pathogens were selected for further study (**Rao *et al*., 2010**).

Determination of Minimum Bactericidal Concentration of *P. dioica* (L.) Merr. leaf extracts

The Minimum Bactericidal Concentration (MBC) of *P. dioica* (L.) Merr. leaf extracts was carried out with the help of agar dilution method using sterile Brain Heart Infusion (BHI) agar medium. Multiple plates of BHI agar were prepared by supplementing it with different concentrations of solvent extracts of *P. dioica* (L.) Merr. leaves (1-5 mg.ml^{-1} with an interval of 0.5 mg.ml^{-1}). The test pathogens were spot inoculated on these plates after solidification of media and incubated at 37°C for 24 h. The lowest concentration of *P. dioica* (L.) Merr. leaf extract that inhibited the growth of pathogens was reported as MBC (**Lorian, 1991**).

Determination of synergistic activity

The agar dilution method was similarly used to determine the synergistic activity of solvent extracts of *P. dioica* (L.) Merr. leaves in presence of ampicillin. It was carried out by incorporating sub-lethal (½MBC) concentrations of *P. dioica* (L.) Merr. leaf extracts into molten NA butt which were cooled to around 40°C along with 100-500 µg.ml^{-1} of ampicillin with an interval of 100 µg.ml^{-1} (**CLSI, 2006**).

Bioautography

Thin layer chromatography (TLC) was carried out by spotting 25 µl of *P. dioica* (L.) Merr. leaf extract on silica gel sheet (2 x 15 cm) and immersing it in the solvent chamber. The solvent system was allowed to run until it reached around a 3/4th length of the plate (**Himanshu and Pradeep, 2012**). After separation of components on silica gel, the sheets were dried, cut into two halves and placed in sterile petri-plates. It was then over-layed with sterile molten NA containing 24h old culture of test pathogen and 0.03 % of 2, 3, 5-Trimethyl tetrazolium chloride (TTC) which was used as the chromogen. The plates were incubated at 37°C for 24 h, and the zones of inhibition were reported.

Table 1 Solvent systems used in Bioautography

Sr. no.	Solvent systems	Ratio
1.	Butanol: Acetic acid	4:1
2.	Toluene: Ethyl Acetate	93:7
3.	Toluene: Methanol: Acetone: Acetic acid	14:4:1:1
4.	Toluene:Acetic acid	70:30
5.	Chloroform: Ethyl acetate: Formic acid	7.5: 6: 0.6
6.	Ethyl Acetate: Formic Acid: Acetic Acid	100:11:27

Gas Chromatography-Mass Spectrophotometry analysis

The bioactive component from the chloroform extract of *P. dioica* (L.) Merr. leaves was analyzed with the help of Gas Chromatography-Mass Spectrophotometry (GCMS). The GC-system was equipped with a capillary column of dimensions 30m X 0.25mm X 0.25µm. The program used for GC oven temperature was 5 min isothermal at 300ºC, followed by 90º-280ºC at a rate of 6ºC/min, then held at 280ºC for 5 min. The injection port temperature was 240ºC. Along with that a Joel, AccuTOF GCV MS system, with a time of flight analyzer, was used (**Tariq *et al*., 2014**).
In addition, the active components exhibiting anti-bacterial activity, identified qualitatively with the help of above mentioned bioautography technique was also analyzed by GCMS. In this case, the separated components were scraped from silica plates with the help of sterile scalpel and dissolved in chloroform which was used as a sample for GCMS.
Both analyses were carried out at IIT Bombay, Mumbai 400076 and the compounds in the crude extract were identified by comparing their retention indices (RI) and mass spectra fragmentation with those in the stored library available with IIT, Bombay.

Scanning Electron Microscopy

The effect of the chloroform extract of *P. dioica* (L.) Merr., on the cell membrane of pathogenic *E. coli*, was investigated by using Scanning Electron Microscopy (SEM). The *E. coli* cells treated with sub-lethal concentrations of the chloroform extract of *P. dioica* (L.) Merr. leaves were considered as a test sample and untreated cells were used as a control sample in the current study. After 24 h incubation, the test and control cells were suspended in 1ml of Phosphate buffered saline and fixed onto clean grease free cover-slips (**Kim *et al*., 2015**). They were allowed to dry and then analyzed by SEM at SAIF, IIT Powai.

Statistical analysis

All the experiments were performed in triplicates and reported as mean ± Standard Deviation (SD).

RESULTS AND DISCUSSION

Sterility testing of solvent extracts

The extracts of *P. dioica* (L.) Merr. leaves showed the absence of bacterial and fungal contaminants even after 7 days of incubation. The extended incubation time confirmed the absence of slow-growing contaminants and stressed cells that may have survived the processing of solvent extracts.

Qualitative assay for determination of antibacterial activity of solvent extracts of *P. dioica* (L.) Merr. leaves

Figure 1 represents the antibacterial activities of chloroform and petroleum ether extracts of *P. dioica* (L.) Merr. leaves carried out by agar well diffusion method. It showed zones of inhibition in the range of 12-17 mm. The acetone and methanol extracts did not show any activity against the test pathogens. Consequently, no further studies were carried out using these extracts. The solvent controls, in our study, did not show any zone of inhibition against test pathogens except for butanol. Hence, further studies with butanol extract were also discontinued.

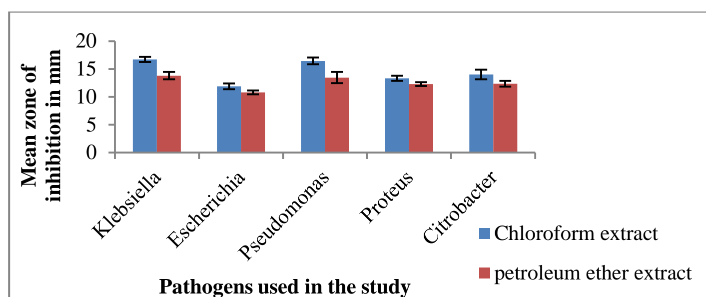

Figure 1 Antibacterial activity of chloroform and petroleum ether extract of *P. dioica* (L.) Merr. leaves against ESBL producers

Other researchers have also reported the effective antibacterial activity of *P. dioica* (L.) Merr. extracts against *Pseudomonas aeruginosa, Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium* and *Staphylococcus aureus* (**Marzouk *et al*., 2007; Oussalah *et al*., 2006**). A similar study carried out with allspice oil reported zones of inhibition in the range of 22-44 mm against *Candida* species. **Khandelwal *et al.* (2012)** reported significant antibacterial activity of methanol extract of *P. dioica* (L.) Merr. leaves when compared to acetone and an aqueous solvent, against test isolates of *E. coli*, *Bacillus cereus*, *S. typhimurium*, and *S. aureus*, carried out in their study. The zones of inhibition of methanol extract were observed in the range of 11-13 mm. Another study carried out using alcohol and hexane extracts of *P. dioica* leaves showed zones of inhibition of 32.1 ± 0.26 mm for *Bacillus megaterium* and 20.3 ± 0.16 mm for *Pseudomonas fluorescens* (**Boyd and Benkeblia, 2014**). A similar study was carried out to study the efficacy of the leaf and bark extracts of *P. dioica* (L.) Merr. against clinical isolates of *S. aureus* and *S. mutans* isolated from burn and dental caries patients respectively. They reported zones of inhibition in the range of 14-21 mm against the test pathogens (**Asha *et al*., 2013**).

Determination of MBC by Agar dilution method

The ineffectiveness of acetone and methanol extracts, and the antibacterial activities of chloroform and petroleum ether extracts, of *P. dioica* (L.) Merr. against the test pathogens, were further confirmed by determination of it MBCs. Table 2 represents the mean MBC values of the chloroform extract of *P. dioica* (L.) Merr. carried out by agar dilution method. It was found to be in the range of 2-5 mg.ml^{-1}. Other solvent extracts of *P. dioica* (L.) Merr. leaves showed very high MBC as compared to that of chloroform extract. As a result, further studies were carried out using only chloroform extracts of *P. dioica* (L.) Merr. leaves.

Table 2 MBC of chloroform extract against ESBL pathogens

Test organisms	No. of isolates	Mean MBC of chloroform extract of *P. dioica* (L.) Merr.
Klebsiella pneumoniae	10	3.5 mg.ml^{-1}
Escherichia coli	10	2 mg.ml^{-1}
Pseudomonas spp.	10	4.5 mg.ml^{-1}
Proteus spp.	05	3.5 mg.ml^{-1}
Citrobacter spp.	10	5 mg.ml^{-1}

Kang *et al.* (2011) studied the MBC of methanol extracts of 8 different medicinal herbs that are native to South Korea. The study was carried out against 15 standard ATCC and CDC strains and their reported MBC ranged between of 1.22-5000 µg.ml^{-1}. A similar study reported MBC of essential oils extracted from *P. dioica* (L.) Merr. leaves against *S. aureus* and *B. cereus* to be 2.5 mg.ml^{-1} and 20 mg.ml^{-1} respectively (**Vazquez *et al*., 2013**). The extensive variations observed in the zones of inhibition and MBC values in several studies including ours can be attributed to several underlying factors which cannot be controlled by researchers in certain cases. For example, it is a well-known fact that growth conditions of plants severely affect its biochemical composition. Hence, the same plant collected from different areas may give rise to variations in the study. Other factors that may contribute to variations include the type of solvent used for extraction of bioactive components, extraction protocol, type of media and inoculum size among others.

Determination of synergistic activity

Table 3 provides an overview of the synergistic activity observed between the sub-lethal concentration of chloroform extract of *P. dioica* (L.) Merr. leaves and ampicillin. An impressive reduction was observed in the MBC value of ampicillin i.e., from 10mg.ml^{-1} to 300-500 µg.ml^{-1} when used in combination with chloroform extracts of *P. dioica* (L.) Merr.

Table 3 Synergistic activity of Chloroform extract of *P. dioica* (L.) Merr leaves and ampicillin

Test Pathogens	MBC of Ampicillin	Mean MBC of *P. dioica* (L.) Merr. Extract (mg.ml^{-1})	Sub-lethal concentration of *P. dioica* (L.) Merr. extract used (mg.ml^{-1})	Synergy observed - MBC of ampicillin in presence of *P. dioica* (L.) Merr. extract (µg.ml^{-1})
E. coli (10)	More than 10mg/ml	2	1.5	500
K. pneumoniae (10)		3.5	2.5	300
Citrobacter spp. (10)		5	4	300
Proteus spp. (5)		3.5	2.5	300
Pseudomonas spp. (10)		4.5	3	500

Our previous study carried out with ethanolic ajwain extract also showed similar results (**Tariq *et al*., 2014**). Similar findings have also been reported by **Voukeng *et al.* (2012)**, where a synergistic effect was observed between 11 different Cameroon spices and erythromycin in 56.25% of the tested bacteria. Another study by **Noumedem *et al.* (2013)** reported synergistic activities of *Piper nigrum* and *Telfairia occidentalis* in presence of 7 different antibiotics used in their study. Since, the pathogens have developed resistance to most of the existing antibiotics, discovering a new antibiotic is probably not an effective solution to the problem. Instead, the modification of existing therapies, although challenging, may be more practical and productive in terms of treatment options. To this effect, our current study holds immense value in the screening of valuable medicinal plant like *P. dioica* (L.) Merr., which is not only antibacterial in nature but also shows potential in reversing the already developed resistance to common antibiotics like ampicillin.

Bioautography

In our study, two TLC plates were run simultaneously, out of which one of them was placed in a petri-plate and over-layed with NA medium containing test isolate and TTC. The separated constituent showing antibacterial activity, after the incubation period, was scraped from the second plate and analyzed using GCMS. Among the six solvent systems used, Toluene: Ethyl acetate showed a maximum zone of inhibition around the isolated component. Figure 2 represents the TLC of chloroform extract which was developed in Toluene: Ethyl acetate solvent system. Figure 3 represents the TLC plate used to carry out bioautography.

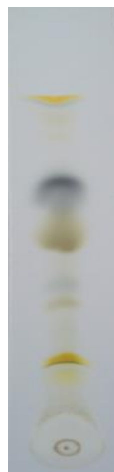

Figure 2 TLC of chloroform extract of *P. dioica* (L.) Merr. developed in Toluene: Ethyl acetate solvent system

Figure 3 Bioautography of the chloroform extract of *P. dioica* (L.) Merr. leaves

Bioautography is a simple and effective method that can be applied to studies related to natural compounds since the antibacterial activity of all separated constituents can be qualitatively determined at once. It is for this reason that bioautography method is preferred by many researchers. A study carried out by **Vazquez *et al*., (2013)** reported two separated fractions of *P. dioica* (L.) Merr. showing antibacterial activity. In addition, they also carried out bioautography using extracts of *M. arboreus*, *B. crassifolia,* and *P. guajava.* However, anti-bacterial activity was observed only from the fractions of *P. dioica* and *P. guajava.* Similarly, another study reported 4 different fractions obtained from the methanolic extract of *Ricinus communis* to show antibacterial activity against *P. aeruginosa* and *K. pneumoniae* (**Sandam and Ponamma, 2015).**

GC-MS analysis

The chromatogram showing Retention Time (RT_m) of several constituents identified with the help of GC-MS analysis of a crude extract of *P. dioica* (L.) Merr. leaves, obtained in chloroform are shown in figure 4. It showed 7 distinct peaks and the highest peak observed at RT_m 18.1 mins was identified as eugenol, making it a major constituent of chloroform extract. In addition, 2-allylphenol, dibutyl phthalate, and crocetane were found to be present in a significant concentration as compared to other constituents (Table 4). Figures 5 represents the chromatogram of the separated band of chloroform extract on TLC plate showing antibacterial activity, determined by bioautography. The retention times and their corresponding constituents of crude chloroform extract and TLC separated bioactive constituents identified by GC-MS analysis in our study is listed in Table 4.

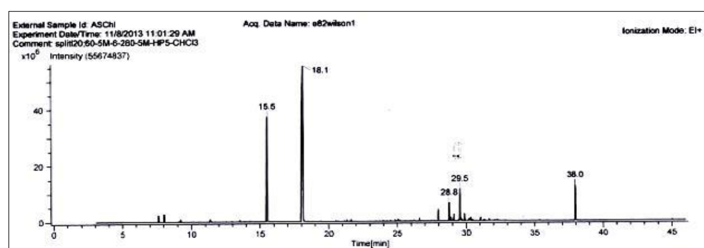

Figure 4 Chromatogram of chloroform extract of *P. dioica* (L.) Merr. leaves

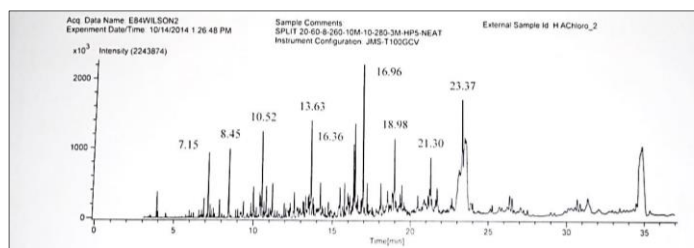

Figure 5 Chromatogram of separated band on TLC plate used in bioautography

Table 4 Identified constituents from chloroform extracts of *P. dioica* (L.) Merr. leaves using GC-MS analysis

Sr. no.	Sample	Retention time (mins)	Compounds
1.	Chloroform extract	7.6 8.0 15.5 18.1 28.0 29.4 29.5 38	Caprylene Beta-Myrcene 2-allylphenol m-Eugenol Phthalic acid Crocetane Dibutyl phthalate 1,2 benzene dicarboxylic acid disoctyl ester
2.	Separated band on TLC plate used for bioautography	7.15 8.45 10.52 13.63 16.96 23.37 24.36	Dodecane 2,6,11trimethyl Eugenol, Iso-Eugenol Dodecane, 2, 6, 11-trimethyl Tridecanol,2-ethyl-2-methyl Hexadecane, 1-iodo (i) 1,2-Benzene di-carboxylic acid, diisooctyl ester (ii) 1,2-Benzene di-carboxylic acid, mono(2-ethylhexyl) ester Dibutyl phthalate

In our study, Eugenol was found to be the major constituent of the chloroform extract of *P. dioica* (L.) Merr. leaves, however, the separated band on TLC plate exhibiting antibacterial activity, showed a lower concentration of eugenol comparatively. Although the most probable anti-bacterial activity of the above-mentioned compounds may be attributed to the most abundant constituent in the extract, it cannot be claimed with absolute certainty. In certain cases, the bioactivity of constituents present in smaller concentration may attenuate the significance of other constituents present in higher concentrations. The antibacterial activity of eugenol is reported in several studies. A recent review published by **Marchese *et al.* (2017)** describes the extensive reports associating the antioxidant and anti-inflammatory activities of eugenol to health benefits. In addition, several published records of the efficacy of eugenol against antibiotic sensitive as well as resistant pathogens are highlighted in their study. Another study carried out to investigate the mechanism of action of eugenol oil indicated that it is very efficient in inactivating *S. typhi* within 60 mins post exposure. They indicated that the chemo-attractant and bactericidal properties of eugenol can work more efficiently when given in vivo. In their study, eugenol was found to increase the permeability of the cell membrane, which was confirmed by the crystal violet assay (**Devi *et al*., 2013**). Other phenolic compounds and organic acids identified in table 4 are also proven for its antibacterial activities in several previously published studies (**Alves *et al*., 2013; Maldonado *et al*., 2011**).

Scanning Electron Microscopy

The effect of the chloroform extract of *P. dioica* (L.) Merr. leaves on ESBL producing *E. coli* was investigated by SEM analysis. The *E. coli* cells treated with (test) and without (control) *P. dioica* (L.) Merr. extracts are shown in figures 6a and b respectively. The test sample shows distortion of shape and swelling in structure. In addition, the prominent white outline evident in the control sample also appears to be broken and uneven when observed in test samples. These results clearly indicate cell wall damage and stress induced in the ESBL producing *E. coli* test cells used in our study, hence confirming the potency of chloroform extract of *P. dioica* (L.) Merr. leaves.

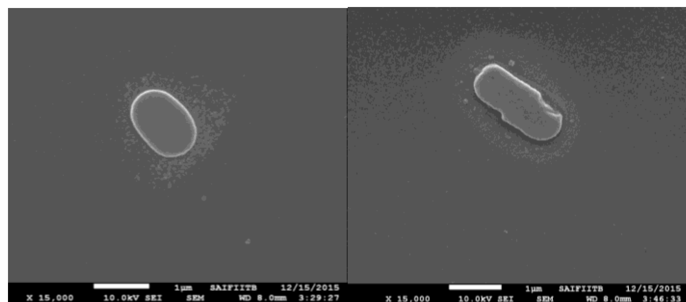
Figure 6 SEM analyses of pathogenic *E. coli* cells

A similar study carried out by **Kamonwannasit *et al.* (2013)** reported swelling and distortion of bacterial cells on treatment with extracts of *Aquilaria crassna* and inhibition of bacterial biofilm formation. In addition, a rupture in bacterial cell wall was observed after treatment of bacterial isolates with the extract for 24h. Both results in their study was confirmed by SEM analysis. Another study by **Kaya *et al.* (2008)** reported shrinking of bacterial cells and cell wall degradation of bacterial cells on treatment with *Ocimum basilicum* extracts.

CONCLUSION

The present study confirms antibacterial activitiy of *P. dioica* (L.) Merr. leaf extracts and its synergistic activity with ampicillin. The distortion and cellular damage caused by *P. dioica* (L.) Merr. leaf extract on bacterial cells are also evident by SEM analysis carried out in our study. Moreover, the organic acids and phenolic compounds identified by GC-MS along with eugenol can be the basis of future studies aimed at in-depth analysis of activities of these compounds. All these findings consistently indicate the potential of *P. dioica* (L.) Merr. leaf extracts to be potential chemotherapeutic agents for the treatment of infections caused by drug-resistant pathogens.

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