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, ceftriaxone, 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 (Moble 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 (Tarig 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 (Oyedeni 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 carboxyl group on cinnamaldelyde 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 effectively (Chesman 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 sturdily 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 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 cleansed 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 inoculating them in a sterile Petri dish containing Agar (NA) and Sabouraud’s Agar (SAB) plate respectively (HemaSairaya 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 OD650) and poured into sterile petri plates. After solidification, wells were punched in duplicate with 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 with an interval of 0.5 mg/ml). 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-letal (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 of ampicillin with an interval of 100 μg/ml (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 24 h 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 | 95:5  |
| 3.      | Toluene: Methanol: Acetic acid | 14:1:1 |
| 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 300°C, followed by 90°-280°C at a rate of 6°C/min, then held at 280°C for 5 min. The injection port temperature was 240°C. 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-letal 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](image)

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 (mg mL⁻¹) |
|----------------|-------------------|--------------------------------------------------|--------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| *E. coli* (10) | 2                 | 1.5                                              | 300                                                                      | 500                                                                             |
| *K. pneumoniae* (10) | More than 10mg/mL | 3.5                                              | 2.5                                                                      | 300                                                                             |
| *Citrobacter* spp. (10) | 5               | 4                                                | 4                                                                        | 300                                                                             |
| *Proteus* spp. (5) | 3.5              | 2.5                                              | 300                                                                      | 300                                                                             |
| *Pseudomonas* spp. (10) | 4.5              | 3                                                | 3                                                                        | 500                                                                             |

Our previous study carried out with ethanolic ajwain extract also showed similar results (Tarig et al., 2014). Similar findings have also been reported by Youkeng et al. (2012), where a synergistic effect was observed between 11 different Cameloon 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.
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, antibacterial 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 (RTₐ) 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 RTₐ 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.

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.

| Sr. no. | Sample | Retention time (mins) | Compounds |
|--------|--------|-----------------------|-----------|
| 1.     | Chloroform extract | 7.6                   | Caprylene |
|        |         | 8.0                   | Beta-Myrcene |
|        |         | 15.5                  | 2-allylphenol |
|        |         | 18.1                  | m-Eugenol |
|        |         | 28.0                  | Phthalic acid |
|        |         | 29.4                  | Crocetane |
|        |         | 29.5                  | Dibutyl phthalate |
|        |         | 38                    | 1,2 benzene dicarboxylic acid diisocyl ester |
| 2.     | Separated band on TLC plate used for bioautography | 7.15 | Dodecane 2, 6, 1 trimethyl |
|        |         | 8.45                  | Eugenol, Iso-Eugenol |
|        |         | 10.52                 | Dodecane, 2, 6, 11 trimethyl |
|        |         | 13.63                 | Tridecanol,2,ethyl-2- methyl |
|        |         | 16.96 13.63           | Hexadecane, 1-iodo |
|        |         | 23.37 16.96 13.63     | (i) 1,2-Benzene dicarboxylic acid, diisocyl ester |
|        |         | 24.36 23.37           | (ii) 1,2-Benzene dicarboxylic acid, monoo(2-ethylhexyl) ester |
|        |         |                       | Dibutyl phthalate |
A similar study carried out by Kamonwannasit et al. (2013) reported swelling and distortion of bacterial cells on treatment with extracts of Agaricia crassa 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 activity 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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