DISCUSSION

Multidrug resistance in bacteria has become a major concern since the treatment of the infections caused by the drug resistant bacteria is problematic. It is well known that *Staphylococcus aureus* is most persistent infectious microorganism which develops resistance against antibiotics. The rise of multidrug resistant in *S. aureus* has become one of the most common causes in the treatment and control of staphylococcal infections (Livermore, 2000; Zapun et al., 2008). The role of the efflux mechanisms in bacterial multidrug resistance is an increasing clinical problem and has rendered many antibiotics ineffective (Piddock et al., 2006a; Stavri et al., 2007; Li et al., 2004; Poole et al., 2005). Efflux pumps are used as first-line defense mechanism by most multidrug resistant bacteria by decreasing intracellular drug concentrations to an ineffective level (Costa et al., 2013). MDR in *S. aureus* is primarily the result of over-expressed efflux pumps, which extrude antibiotics prior to reaching the intended targets (Sun et al., 2014). More than ten multidrug efflux pumps have been described, either encoded by the chromosome or plasmids in *S. aureus*. MDR efflux mechanisms studies in *S. aureus* indicated that NorA is predominant protein efflux pump and main protein target that cause multidrug resistance (Sabatini et al., 2012). NorA can reduce the intracellular concentration of drugs by the extrusion of chemically and structurally dissimilar compounds, namely hydrophilic fluoroquinolones (norfloxacin and ciprofloxacin), dyes (EtBr) and biocides (quaternary ammonium compounds) (Kaatz et al., 1993). Novel antimicrobials with new mode of action are required to suppress the rise of MDR. An alternative approach of efflux pump inhibitor and antimicrobial combination would be useful that can interfere with the process of efflux. Phytochemicals are of significant interest which may act as potent efflux pump inhibitors (EPIs) and may inhibit bacterial efflux pumps in combination with antimicrobials (Guz et al., 2001). A number of chemically synthesized and plant derived inhibitors which inhibit NorA efflux pump of *S. aureus* have been isolated. But unfortunately they are toxic at concentration required for activity (Handzlik, 2013). Therefore prospects for isolating new efflux pump inhibitors from medicinal plants with low toxicity could be clinically encouraging. Therefore, the focus of the study was to isolate phytochemicals from medicinal plants that act as EPIs and enhance the bioavailability of the drugs against NorA expressing *S. aureus* strains.
5.1 Confirmation of *S. aureus* strains

All the three *S. aureus* strains procured from Northeastern University, Boston were confirmed by culturing on selective media, gram staining, and biochemical assays. All the strains showed similar phenotypic characteristics and results were in conformity with Murray *et al.* (2003) and Bergey’s manual of determinative bacteriology, (1939).

5.2 Cartwheel assay for efflux pump presence

Earlier studies on efflux pump presence among *S. aureus* strains were restricted, laborious and required specialized instrumentation. But Patel *et al.* (2010) and Martins *et al.* (2011) have proposed the simple EtBr-agar cartwheel method for rapid screening of efflux-mediated MDR bacteria. EtBr, broad range efflux pump substrate which is effuxed out by NorA pump of *S. aureus* and thus is used as an marker for detection of efflux pump activity in this organism (*Costa et al.*, 2013). This method allows the comparison between different strains on the basis of their capacity to extrude EtBr and fluorescence emitted by strains is inversely proportional to their capacity to extrude EtBr.

In the present study, EtBr-agar cartwheel method was applied for direct assessment of efflux activity among three *S. aureus* strains (2378 NorA over-expressive, 8325-4 wild type and 1758 NorA knockout). This method demonstrated that 2378 NorA over-expressive strain had a higher efflux activity than 1758 NorA knockout strain. The presence and absence of efflux pump in the 2378 NorA over-expressive strain and 1758 NorA knockout strain was determined by the fluorescence emitted at the highest (2.5 mg/l) and lowest (0.5 mg/l) concentrations of EtBr respectively. However 8325-4 wild type strain emitted fluorescence at intermediate (1.5 mg/l) concentrations demonstrating less efflux than 2378 over-expressing strain. This method proved to be a differentiating tool to screen efflux activity in three different *S. aureus* strains.

5.3 Synergistic activity of plant extracts and antibiotics

One of the most effective approaches to overcome bacterial multidrug resistance is restoration of antibiotic activity through the synergism. Synergism is most desirable effects of combination in which two different compounds are combined to enhance their individual activity and is beneficial to treat bacterial infections. Synergism between known resistant antibiotics and
bioactive plant extracts is a novel approach. The synergistic mechanism becomes the apparent strategy employed by plants and hence the improved efficacy was demonstrated by combining plant extracts with antibiotics in this study. In the present study, methanolic, ethyl acetate and aqueous extracts of 23 selected medicinal plants were screened for synergistic activity with resistant antibiotics (ciprofloxacin and norfloxacin) which are effluxed by NorA pump of *S. aureus*. Methanolic and ethylacetate extract showed significant synergistic activity, while aqueous extract did not show any synergistic activity. In this study, many plant extracts exhibited synergistic activity, but the results obtained were variable depending upon the *S. aureus* strains (2378 NorA over-expressive, 8325-4 wild type and 1758 NorA knockout) and the antibiotic. Out of three *S. aureus* strains, maximum synergism was observed in 2378 NorA over-expressive strain, followed by 8325-4 wild type strain. Least synergism or additive interaction was observed in case of 1758 NorA knockout strain. The variable results among the three tested strains indicate the inhibition of NorA efflux pump in the *S. aureus* strains. This study appears to be the first to report synergistic effect of *Angeliica glauca, Dactylorhiza hatagirea, Ficus carica, Girardinia diversifolia, Heracleum lanatum, Malva neglecta, Picrorhiza kurrooa, Rheum australe, Rubia cordifolia, Sedum glaucophyllum, Sinopodophyllum hexandrum, Solena amplexicaulis, Valeriana jatamansi, Verbascum thapsus* and *Viola canescens* with ciprofloxacin and norfloxacin against *S. aureus* strains. The synergistic effect of plant extract and antimicrobial interaction could be due to the active phytoconstituents in the plant, which acts synergistically with ciprofloxacin and norfloxacin.

Oliveira *et al.* (2013) investigated synergistic activity of norfloxacin, ciprofloxacin, tetracycline and erythromycin with extracts of *Mangifera indica* against *S. aureus* strains. It was observed that individual extract did not show any antibacterial activity, but modulated the activity of antibiotics in combination. Eze *et al.* (2013) investigated synergism between extracts of *Chromolaena odorata* and *Aloe africana* with norfloxacin, ciprofloxacin against antibiotic resistant *S. aureus*. They concluded that *Chromolaena odorata* and *Aloe africana* plant extracts enhanced the activity of antibiotics and could be good sources of multidrug resistance inhibitors. Various other authors have also reported the antimicrobial and synergistic activity of many different plant (*Psidium guajava, Rosmarinus officinalis, Salvia fruticosa, Majorana syriaca, Ocimum basilicum, Syzygium aromaticum, Laurus nobilis and Rosa damascene*) extracts against
S. aureus strains (Betoni et al., 2006; Esimone et al., 2006; Darwish et al., 2002; Aquil et al., 2005; Braga et al., 2005; Yam et al., 1998; Yang et al., 2005).

5.4 Screening of efflux pump inhibitory activity

Inhibition of efflux pumps decrease the level of intrinsic resistance significantly, reverse the acquired resistance and resulted in a decreased frequency of emergence of S. aureus strains that are highly resistant to fluoroquinolones (Lomovskaya and Watkins et al., 2001). In the present study, crude plant extracts were evaluated in order to obtain new compounds with enhanced activity as EPIs. Twelve plants extracts exhibiting maximum synergistic activity with selected antibiotics against S. aureus strains were further screened for efflux pump inhibitory activity by berberine uptake assay and EtBr efflux inhibition assay.

5.5 Berberine uptake assay

To detect the EPI activity, berberine uptake assay was performed. Berberine (mild antibacterial) is effluxed from the cells by NorA multidrug pump of S. aureus. Overexpression of NorA increases bacterial resistance to berberine and deletion of NorA renders bacterial cells more sensitive to berberine. Crude plant extracts were tested in presence and absence of sub-inhibitory concentrations of berberine. Extracts that inhibited cell growth in the presence of berberine as compared to berberine alone, were likely to contain multidrug EPI in the extract. Combination of NorA inhibitor with beberine potentiates the antibacterial activity of berberine (Fiamegos et al., 2011). In the present study, Angelica glauca (ME), Ficus carica (EE), Heracleum lanatum (ME) and Viola canesiens (ME) improved inhibitory action of berberine by reducing the MIC of berberine by four-fold and two fold by Malva neglecta (EE), Sedum glaucophyllum (ME), Valeriana jatamansi (ME), Verbascum thapsus (EE) against 2378 NorA over-expressive strain of S. aureus. For wild type strain 8325-4, Ficus carica (EE), Malva neglecta (EE), Sedum glaucophyllum (ME), Valeriana jatamansi (ME), Verbascum thapsus (EE), Viola canesiens (ME) reduced the MIC by two fold, while Picrorrhiza kurrooa (EE), Rheum australe (EE), Sinopodophyllum hexandrum (EE), Solena amplexicaulis (ME) did not exhibit any reduction in the MIC of berberine in the presence of these extracts. No reduction in the MIC of berberine in combination with plant extracts were observed against 1758 NorA knockout strain. This data suggest that MIC reduction in 2378 NorA over-expressive strain and 8325-4 wild type strain is NorA inhibition dependent. This also indicates that the plant extracts acts as active EPIs. Two-
fold reduction in the MIC was observed with extracts of *Picrorrhiza kurrooa* (EE) and *Solena amplexicaulis* (ME) against 1758 NorA knockout strain, which indicates that other undefined mechanism of reduction in MIC of berberine. Stermitz *et al.* (2001) experimented on EPI activity of *Berberis trifoliolata* and *Berberis fendleri* and observed potentiation in antibacterial activity of berberine against *S. aureus* strains possessing NorA multidrug efflux pump. Belfosky *et al.* (2004) observed that berberine alone exhibited no antimicrobial activity but enhanced the effect of weak antimicrobial berberine, when tested with plant extract of *Dalea spinosa* (Smoke tree) against NorA possessing *S. aureus* strains. Chloroform extract of leaves of *Artemisia absinthium* showed EPI activity by potentiating the activity of berberine against NorA *S. aureus* strains, but showed no EPI activity against NorA knockout strains of *S. aureus* (Fiamegos *et al.*, 2011).

### 5.6 EtBr efflux inhibition assay

The ability of the EPIs to directly inhibit the efflux of EtBr from *S. aureus* strains was evaluated by EtBr fluorometric method (Brenwald *et al.*, 1997). In this fluorometric method, fluorochrome EtBr has a low fluorescence signal outside the bacterial cells, which increases once inside the cell in a concentration-dependent manner and is widely recognized as the best candidate for monitoring EPI activity (Rodrigues *et al.*, 2008). The increased accumulation of EtBr in the presence of the plant extracts suggests an inhibition of MDR transporter. In the current study, a well-known EPI, Carbonyl Cyanide m-chlorophenylhydrazone (CCCP) was used as positive control and dimethyl sulfoxide (DMSO) as negative control to evaluate the extent of possible efflux inhibition in the test strains. In 1758 NorA knockout strain, the accumulation of EtBr was maximum for all the 12 tested plant extracts than 2378 NorA over-expressive and 8325-4 wild type strain. *Angelica glauca* (ME), *Ficus carica* (EE), *Heracleum lanatum* (ME), *Malva neglecta* (EE), *Picrorrhiza kurrooa* (EE), *Rheum australe* (EE), *Sedum glaucophyllum* (ME), *Sinopodophyllum hexandrum* (EE), *Solena amplexicaulis* (ME), *Valeriana jatamansi* (ME), *Verbascum thapsus* (EE), *Viola canesiens* (ME) was found potent efflux pump inhibitors, but the potency to accumulate EtBr varied from highest to modest with each plant extract. Highest accumulation of EtBr was observed with *Angelica glauca* (ME) and *Heracleum lanatum* (ME) and maximum EtBr efflux was observed with *Picrorrhiza kurrooa* (EE). The decreased EtBr efflux suggests that the tested plant extracts inhibits the efflux of EtBr in the same manner as that of CCCP (positive control) and could be a potential source of EPI compound.
Several groups have reported many extracts that have shown EPI activity against *S. aureus* strains. Oluwatuyi *et al.* (2004) observed that chloroform extract of *Rosmarinus officinalis* inhibit EtBr efflux against NorA possessing *S. aureus* strain. Braga *et al.* (2005) concluded that methanolic extract of *Punica granatum* caused an increase in EtBr uptake in *S. aureus*, having a NorA dependent EtBr efflux mechanism. Dickson *et al.* (2006) showed that extracts of *Mezoneuron benthamianum* and *Securinega virosa* exerted a potentiation activity of fluoroquinolones against efflux resistant strains of *S. aureus* possessing NorA multidrug pump. Ethanolic extracts of *Mangifera indica*, *Callistemon citrinus* and *Vernonia adoensis* were observed as a potential source of EPIs against *S. aureus* (Chitemerere and Mukanganyama, 2011).


### 5.7 Isolation of bioactive compound

Bioactive compounds provide unlimited opportunities for new drug discoveries, because of the unmatched availability of chemical diversity (Cosa *et al.*, 2006). Isolation and identification of bioactive compounds present in a crude plant extract serve as the building block for the development of new type of therapeutics with more potent way to treat various human ailments (Lee *et al.*, 2008). To isolate pure compounds, several steps such as isolation, purification, separation, detection of the bioactive compounds and quantitative data analyses (Abidi, 2001) need to be standardized. In the present study, plant extracts of *Angelica glauca* (ME) and *Heracleum lanatum* (ME) showed maximum EPI activity and selected for analysis of bioactive compound present in the plant extracts by the applications of phytochemical screening assays, bioassay guided fractionation, chromatographic technique (TLC) and structural elucidation of bioactive compound by LC/MS.
5.8 Phytochemical analysis of crude plant extracts

Preliminary phytochemical screening is a valuable step in the detection of bioactive compounds present in medicinal plants and may lead to novel drug discovery (Ndam et al., 2014). Plant cells produce two types of metabolites (Pal, 2007). Primary metabolites (carbohydrates, lipids and proteins) are involved directly in growth and metabolism. Secondary metabolites includes alkaloids, phenolics, essential oils, terpenes, sterols, flavonoids, lignins, tannins, etc. and are considered products of primary metabolism and are the major source of pharmaceuticals, food additives, fragrances and pesticides and herbicides (Okwu, 2005; Ramawat and Dass, 2009; Ramu and Mohan, 2012).

In the present study, principal phytoconstituents of crude extracts of medicinal plants selected on the basis of EPI activity were identified in order to relate their presence with bioactivities of the plants. Screening of the crude plant extracts was performed using standard methods and resulted in the detection of carbohydrates, reducing sugars, glycosides, amino acids and proteins, terpenoids, tannins and phenolics, flavonoids and saponins in the root extract of Angelica glauca (ME). In root extract of Heracleum lanatum (ME), the major phytoconstituents present were carbohydrates, reducing sugars, glycosides, amino acids and proteins, steroids, terpenoids, tannins and phenolics, flavonoids, alkaloids and saponins. Both the extracts showed absence of phlobatannins. It is evident from the study that Angelica glauca (ME) and Heracleum lanatum (ME) registered the highest therapeutic efficacy, possessing majority of phytochemical classes of compounds. Nakata et al. (1982) isolated phenolic compound from the root extract of Heracleum lanatum. Additionally, there are no reports on the phytochemical screening of the methanolic extracts of Angelica glauca and Heracleum lanatum.

5.9 Bioassay guided fractionation

Crude plant extracts may contain a number of metabolites. The metabolites present in crude plant extracts can be separated by simple fractionation using solvents of different polarity. The separation techniques for isolation of bioactive compounds are crucial in the natural drug discovery process. Liquid-liquid fractionation was adopted for fractionating the methanolic root extract of Angelica glauca and Heracleum lanatum and yielded twelve fractions. The separated fractions were further processed for the efflux pump inhibitory activity and acetone fraction of methanolic root extract of Angelica glauca was found to be most potent efflux pump inhibitor.
5.10 Thin layer chromatography and silica column chromatography

TLC and column chromatography are most widely used techniques for the separation and purification of bioactive molecules present in the plant extracts. Separation and purification of bioactive compounds presents a considerable challenge. For purification, the sample was extracted with solvents of different polarity (Wang et al. 2014; Jyothy et al. 2011). Qualitative chromatographic analysis using TLC was used for analyzing the isolated fractions and to select the mobile phase for column chromatography. The phytochemical present in the active fraction were separated using chloroform: methanol (9:1) as a mobile phase. We observed six isolated spots with R_f values of 0.3, 0.5, 0.8, 1, 1.3, 1.8. LC-MS of bioactive acetone fraction showed the various phytocompounds with different molecular weight (m/z) ranging from 144.5-610. Most bioactive acetone fraction was subjected to silica gel column chromatography and eluted with chloroform: methanol (9:1) as a mobile phase. A total of 26 fractions were collected. Efflux pump inhibitory activity of 26 fractions by EtBr efflux inhibition assay revealed that fraction number 10 showed the maximum efflux pump inhibitory activity. Bioactive fraction 10 was analysed by thin layer chromatography and revealed the presence of single spot with R_f value of 0.5.

5.11 Structure elucidation of bioactive compound by LC/MS

LC-MS refers to the combination of liquid chromatographic separation with mass spectrometric detection. The combination of these two techniques gives the chemical analyst the ability to analyze virtually any molecular species; including, non-volatile, thermally labile and high molecular weight species (Arpino and Patrick, 1982). The bioactive fraction showing EPI activity was then characterized to know the molecular weight by LC/MS. The molecular weight of bioactive compound showed by LC/MS was 194.12. The mass spectrum of compound showed a molecular ion at m/z 194.12(25%). The compound was therefore found to be 4-Hydroxy-3-methoxycinnamic acid (Ferulic acid, C_{10}H_{10}O_{4}). Jong et al. (2006) reported similar results by LC/MS for ferulic acid isolated from Chinese medicinal preparation (Shiau-feng-saan and Dang-guei-nian-tong-tang).
5.12 Ferulic acid as efflux pump inhibitor

In the present study, ferulic acid isolated from root extract of *Angelica glauca* showed potent efflux pump inhibitory activity against the NorA strains of *S. aureus*. There are no reports on the isolation of ferulic acid and efflux pump inhibitory activity of ferulic acid isolated from methanolic extracts of *Angelica glauca*. Ferulic acid was isolated from various species of *Angelica* such as *Angelica archangelica* (Kimura *et al*., 2011), *Angelica sinensis* (Sun *et al*., 2006) and *Angelica acutiloba* (Fukuda *et al*., 2009). Ferulic acid, a phenolic phytochemical was reported to reduce the formation of free radicals (Gong *et al*., 2016). Ferulic acid is a phenolic acid of low toxicity and can be absorbed and easily metabolized in the human body. Ferulic acid has been reported to have many physiological functions such as antioxidant, antimicrobial, antiallergic, hepatoprotective, anti-thrombosis, anti-inflammatory and anti-cancer activities (Srinivasan *et al*., 2007). It also protects against coronary disease, increases sperm viability, antiviral, lowers cholesterol, vasodilatory actions, metal chelation, modulation of enzyme activity, activation of transcriptional factors, gene expression and signal transduction. Because of its properties, its low toxicity and structure (phenolic nucleus with extended conjugated side chain), ferulic acid is now widely used in cosmetic industries and food industry to prevent lipid peroxidation (Ou and Kwok, 2004). Huang *et al*. 1988 reported inhibitory effect of ferulic acid (low molecular weight natural phenolic compound) on tumor promotion of mouse skin.

5.13 Synergistic effect of ferulic acid with antibiotics (ciprofloxacin and norfloxacin)

Synergism is defined as a positive interaction when two agents are combined and together they exert an inhibitory effect that is greater than the sum of their individual effects. The synergism is a new concept in developing antibacterial agents, reversing drug resistance and also for efflux pump inhibitory activity (Blesson *et al*., 2015). In the present study, synergistic effect of ferulic acid with ciprofloxacin and norfloxacin was evaluated against three strains of *S. aureus* (2378 over-expressive, 8325-4 wild type, 1758 NorA knockout strain) and observed that ferulic acid exhibited synergistic activity with ciprofloxacin and norfloxacin against 2378 NorA over-expressive and 8325-4 wild type strain. No interaction was observed with the 1758 NorA knockout strain. Maximum reduction in the MIC of antibiotics was observed against 2378 NorA over-expressive strain, followed by 83254 wild type strain. No reduction in MIC of antibiotics was observed against 1758 NorA knockout strain, confirming ferulic acid as potent efflux pump
inhibitor of NorA pump of *S. aureus*. Many other authors have isolated compounds from natural sources or synthesized chemically, which reduced the MIC of antibiotics and potentiates the activity of ciprofloxacin and norfloxacin. Some of the compounds are INF240 (Penelope *et al*., 2002), INF55 (Ambrus *et al*., 2008), 2-aryl-5-nitroindole (Samosorn *et al*., 2006), indirubin (Ponnusamy *et al*., 2010), aryl alkanide amide (piperine, piperic acid) (Kumar *et al*., 2008; Wani *et al*., 2016), flavonoids (biochannin, tiliroside, baicalein, rhamnoside, genistein, catechin) (Morel *et al*., 2003; Falcao-Silva *et al*., 2009; Chan *et al*., 2011; Holler *et al*., 2012a; Wang *et al*., 2014; Hamilton-Miller *et al*., 2000), quinolines (German *et al*., 2008b; Sabatini *et al*., 2013; Doleans-Jordheim *et al*., 2013), diterpenes (carnosic acid, carnosol, totarol, ferruginol) (Roberts, 2005; Smith *et al*., 2007), phenolic metabolites (salicylic acid, dicafeoylquinic acid) (Price *et al*., 2002; Fiamegos *et al*., 2011), phenylpipridines (pentasubstituted pyridine) (Marquez *et al*., 2005), benzosulfurheterocycles (arylated benzo thiophene, COX-2 inhibitors, phenothiazines and thioxanthenes) (Charbert *et al*., 2007; Liger *et al*., 2016; Maia *et al*., 2011; Thorsing *et al*., 2013), cumarin derivatives (Abulrob *et al*., 2004), boronic acids (Fontaine *et al*., 2015), resin glycosides (Cherigo *et al*., 2008), spinosan A acetate (Belofsky *et al*., 2006), capsaicin (Kalia *et al*., 2012), ginsenoside (Zhang *et al*., 2014), GG918 (Gibbons *et al*., 2003). Results in the present study are in agreement with these compounds isolated by various authors in reduction of MIC values of ciprofloxacin and norfloxacin.

5.14 **Synergistic effect of ferulic acid with quaternary ammonium compound (Cetrimide)**

Cetrimide is a useful antiseptic and disinfectant used in consumer products, animal husbandry and clinical settings (Zhang *et al*., 2011). Conceicao *et al*., (2016) reported high prevalence of quaternary ammonium compounds resistant genes for NorA pump of *S. aureus*. They also reported significant association between resistance caused by quaternary ammonium compounds and antibiotic. In the present study, synergistic activity of ferulic acid with cetrimide was evaluated which showed 4-fold reduction in the MIC of cetrimide against 2378 NorA over-expressive strain and 8325-4 wild type strain. No reduction in MIC of cetrimide was observed against 1758 NorA knockout strain. The results of the present study revealed the association between cetrimide and antibiotic resistance is NorA dependent. Walsh *et al*., (2003) investigated linkage between bacterial resistance to antibiotics and different quaternary ammonium compounds.
A number of new compounds having NorA inhibitory activity have been investigated, but none of the efflux pump inhibitors in combination with antimicrobials have been used in the clinical settings to treat infections caused by multidrug resistant *S. aureus* possessing NorA multidrug pump (Zechini and Versace, 2009). The compounds from natural sources offer a number of novel and safe pharmacophores for further chemical modifications. In this study, three strains of *S. aureus* (2378 NorA over-expressive, 8325-4 wild type, 1758 NorA knockout strain) and antimicrobials (ciprofloxacin, norfloxacin and cetrimide) were experimented which confirmed the ferulic acid as potent NorA efflux pump inhibitor. Reports have shown that ferulic acid is of low toxicity and have many pharmacological properties, but further effort to evaluate toxicity profiling in a broad spectrum seems reasonable. Keeping in mind the importance of ferulic acid, *in vivo* studies are required to take this phytocompound to the next level in drug discovery and to combat MDR. Moreover, ferulic acid can be employed to formulate cetrimide to make them more effective and potent at lower concentrations.