CHAPTER V

DISCUSSION

Most of the traditionally used antibiotics are on the verge of becoming obsolete due to its misuse or overuse, conferring resistance to microbes. Therefore it is necessary to develop alternative methods for management of infectious diseases (Carson et al. 2006). The main aim of this research is to understand the antibacterial potential of different plants used in ethno medicine by local people of Assam and to elucidate the active component responsible for antibacterial activity.

The use of plants in infectious disease treatment is popular in present times because of their low cost, easy availability and public acceptance. Proper exploration of plants could lead to the betterment of primary healthcare, especially in the treatment of infectious diseases. Therefore, medicinally active ingredients must be isolated and studied keeping in mind that overexploitation of valuable plants might lead to extinction. This may in turn pose risk on chemical synthesis of useful potent phytochemicals.

The North Eastern region of India, which is considered as one of the mega biodiversity hotspots harbours many rare and endemic plant species. There are different ethnic communities in the North Eastern region of India. The tribal communities in Assam such as Boro, Dimasa, Karbi, Deori, Missing, Kachari, Sonowal, Rabha and many others residing in rural and hilly areas are known to practice ethnic traditions for treatment of the common diseases. The survey in this study involved interviewing of traditional herbal
healers from some parts of Assam. Most of the traditional healers in this study gained traditional medicinal knowledge from the elders in their family. Different plant species used for treatment of bacterial infections with special emphasis on pneumonia were recorded. The survey revealed that the local people and the traditional herbal healers used different parts depending on the disease. Medicine preparation involved various methods like boiling with water, macerating, grinding or blending to make paste or was given raw for consumption if required. Similar methods are also used for herbal medicine preparations in other parts of India and also outside India (Xavier et al. 2014, Upadhya et al. 2012, Das et al. 2012, Giday et al. 2010). In skin infections mostly the plant parts were applied externally. Some treatment involved combination of different plant species. Sometime, same plant was used for treatment of different infections by the traditional healers of these areas. Leafy parts were the most commonly used parts and the most frequently used preparation was decoction. This result is similar to the results reported by Xavier et al. (2014) for ethno- medicinal use of kani tribes of Kerala, South India. Khan et al. (2015) reported that the Khasi tribes of Garo hills of Durgapur, Bangladesh used leaves most commonly in their ethnomedicinal practice. Some of the plants recorded from this survey were reported to be used in traditional medicine of other parts of the country. There are published reports on ethno- medicinal uses of Alstonia scholaris, Clerodendrum viscosum, Nyctanthes arbor –tristis, Oroxylum Indicum, Piper longum and few other plants collected through this survey (Pratyush et al. 2011, Nandi and Lyndem 2016, Lawania et al. 2010, Kamboj and Saluja 2010).

The plants collected based on ethnomedicinal use were screened for their antibacterial activity. In order to separate active components from the plants four different
solvents were used with their increasing polarity. Solvents with different polarity are generally known to extract slightly different phytochemicals. So different solvent extracts usually show different activity. But the activity may also vary due to the varying concentration of the active components in the solvent extracts (Eloff 2001, Masoko et al. 2008). In most of the reports previously published, organic solvents are considered more active than aqueous solvents (Eloff et al. 2005, 2008, Nkomo and Kambizi 2009). Our findings also showed lowest activity of aqueous extract than the organic solvent extracts.

In this study, some of the collected plants were failed to support scientific validation for their ethnomedicinal use whereas others showed scientific validation for their use. A. calamus, P. caespitosum, C. flexuosus, S. torvum, M. jalapa and L. camara did not show antibacterial activity against any of the test bacterium. There are reports on antibacterial activity of most of these above mentioned plants against gram positive and gram negative bacteria (Haghighi et al. 2014, Kakarla and Ganjewala 2009) except for M. jalapa, L. camara and Polygonum caespitosum. These contradictory results may be due to the difference in extract doses used in this study, method of plant extraction or antibacterial screening, genetic variation of the plant, age of the plant or the environment (Gouvea et al. 2012, Gobalakrishnan et al. 2013). Results of disk diffusion method showed that Xanthium strumarium seed and L. salicifolia leaf extracts exhibited very high level of antibacterial activity against S. aureus and M. pruriens showed high degree of activity against S. pneumoniae. Clerodendrum viscosum also showed moderate antibacterial activity against S. aureus. X. strumarium leaf essential oil was reported to be active against S. aureus and P. aeruginosa (Rad et al. 2015, Rad et al. 2013). In a previous study, CHF extract of M. pruriens seeds was found to possess antibacterial activity against E. coli and S. aureus.
(Marimuthu et al. 2013). But there are no known reports on antibacterial activity of *M. pruriens* seeds against *S. pneumoniae* yet. This is the first report on the antibacterial activity of *L. salicifolia* leaf extract although there are previous reports on insecticidal activity of *L. salicifolia* leaf extract (Phukan and Kalita 2005). Out of the four bacteria tested *S. aureus* showed maximum sensitivity. This result supports previous studies of sensitivity of *S. aureus* against plant extracts (Dahiya and Purkayastha 2012, Abew et al. 2014).

Since *L. salicifolia* and *M. pruriens* showed broad spectrum antibacterial activity we carried out further analysis of these two plants. *Mucuna pruriens* belonging to the family fabaceae is widely distributed in tropical and sub-tropical regions of the world. The seeds of this plant are considered as a good source of food as it contains high concentration of dietary protein (Janardhanan et al. 2003, Pugalenthi et al. 2005). *M. pruriens* has been used in traditional Indian medicine system from long time for curing Parkinson’s disease (Sathiyaranayanan et al. 2007). L- Dopa one of the major constituents of *M. pruriens* is used in commercial drugs for treatment of Parkinson’s disease (Lorenzetti et al. 1998). There are reports on antimicrobial, antiepileptic and antineoplastic activity of *Mucuna pruriens* (Gupta et al. 1997, Rajeshwar et al. 2005). *L. salicifolia* is used from the ancient time in traditional medicine in different parts of North East India. There are few published reports on antibacterial activity of *Litsea* sp. (Mandal et al. 2000 and Zhang et al. 2012).

In this study both *L. salicifolia* and *M. pruriens* extracts showed antibacterial activity in dose dependant manner i.e. their activity was concentration dependant. This study is similar with the previous reports on dose dependant antibacterial activity of plant extracts (Jeyaseelan et al. 2012, Cheruiyot et al. 2009, Kajaria et al. 2012).
MIC and MBC study of *M. pruriens* using broth dilution method showed that out of the four extracts used, CHF extract showed lowest MIC (3.6±1.15 mg/ml) and MBC (11.33±0.57 mg/ml) values against *S. aureus* although preliminary screening by disk diffusion method showed highest activity against *S. pneumoniae*. This variation in activity may be due to inability of some non polar compounds of the extracts to diffuse properly in agar medium which resulted in smaller size of zone of inhibition (Thakare 2004). Additionally, other characteristics of the extract such as volatility, solubility can also influence the size of zone of inhibition (Scorzoni et al. 2007). The CHF extract of *L. salicifolia* also showed lowest MIC (0.076 mg/ml) and MBC (0.4 mg/ml) values. This study supports the previous works suggesting higher activity of CHF extract than other solvent extracts (Amabye and Shalkh, 2015, Yousufi 2012, Zaman et al. 2011, Kothari et al. 2011, Dhabl et al. 2012, Kamaraj et al. 2012). The possible reason for showing broad spectrum activity by CHF extract could be due to its ability to extract various active ingredients from plants. This indicates that CHF extract could serve as better solvent for the extraction of antibacterial compound from both the plants.

All the four extracts of *M. pruriens* and *L. salicifolia* were tested for presence of various phytochemical constituents such as terpenoids, flavonoids, alkaloids, phenols, tannins, carbohydrates and steroids. CHF extract of *M. pruriens* showed the presence of terpenoids, steroids, alkaloids, flavonoids, tannins and phenols. Antiviral, antiinflammatory, anticarcinogenic, antimicrobial, hypotensive and antioxidant activities of certain plants have been suggested to be due to the presence of these phytochemicals (Ogundare and Olorunfemi 2007, Mandal et al. 2005, Lampariello et al. 2012). The
antibacterial activity showed by the CHF extract in this study could be due to the presence of these phytochemicals present in the extract. The chemical composition of *M. pruriens* was determined. There are few reports on chemical composition of *M. pruriens* seeds (Bhaskar *et al.* 2011, Chinnamadasamy and Veerabahu 2011, Sidduraju *et al.* 2000). The results of these reports were similar to the chemical composition of seed CHF extract reported in our study. However, there are differences in quantity. In this study, a total of fifteen compounds were identified Linolenic acid (10.85%) as the major constituent of CHF extract. Linolic acid and palmitic acid were identified as the major components with quantitative percentage of 48-49 % in the work reported by Sidduraju *et al.* (2000). Most of the chemical components found in seed CHF extract of *M. pruriens* are reported to exhibit antimicrobial activity (Delaquis *et al.* 2005, Lee *et al.* 2002, Da Silva *et al.* 2002). It is evident from the literature that besides antimicrobial activity *M. pruriens* is also used in the treatment of various other illnesses such as in male impotency and other sexual problems and also used as nerve medicine (Misra and Wagner 2007, Lampariello 2012). The fact is that, there may be several chemical constituents in a single plant species, that can fight against various diseases (Akinpelu *et al.* 2009).

CHF extract of *L. salicifolia* showed positive results for terpenoids, steroids, alkaloids, flavonoids and carbohydrate. These phytochemicals present in medicinal plants are known to exhibit different biological activities (Tung *et al.* 2008, Goren *et al.* 2011, Silva *et al.* 2007, Kumar and Pandey 2013, Kaur and Arora 2015, Shahidi 2001). The chemical constituents of plants have therapeutic abilities that can be effective against various diseases (Cowan 1999). Various reported biological activities of *Litsea* sp. (Yang *et

Although plants antimicrobial activities have been widely studied (Silva and Fernades 2010), the mechanism of action of the plant extracts on bacterial cells is not very clear in most cases. In this study, we investigated the role of ROS on bacterial sensitivity to plant extracts. NBT dye was used to investigate the level of free radical production in treated bacterial cells. The cells growing aerobically naturally generate ROS as byproducts of oxygen. Cells have natural ability to defend oxidative stress induced by free radical up to a certain level, beyond which the oxidative stress could cause cell damage. Becerra et al. (2004) and Kohanski (2007) reported that the mechanism of ciprofloxacin action involved ROS production. In this work, CHF extract of *M. pruriens* and *L. salicifolia* showed high degree of antibacterial activity against *E. coli* and *S. aureus*. Therefore, CHF extract treated *E. coli* and *S. aureus* cells were incubated with NBT dye to study the role of oxidative stress in their antibacterial activity. There was significant rise in level of ROS production in the treated *E. coli* and *S. aureus* than that of control cells. Plants synthesize various phytochemicals continuously or in response to pathogen attack (Brantner et al. 1996, Kazmi et al. 1994, Souza et al. 2011, Cushnie and Lamb 2005). These phytochemicals like peptides, flavonoids, tannins, steroids, quinones are known to form pores by forming complex with the cell walls (Zhang and Lewis 1997, Cowan 1999). Stern et al. (1996) proposed that quinone derivatives have ability to induce free radical production in bacterial cells. The plant extracts in the present study, showed positive results for presence of various
phytochemicals, which have probably formed pores in the cell wall thus helping in penetration of the chemical constituents inside the cell, triggering different biochemical pathways which in turn produces excessive by products in bacterial cells and eventually caused cell death.

In order to study morphological changes of treated cells TEM analysis was done. From the TEM micrographs it was clear that extract treatment caused considerable changes in the cell morphology. Treatment of CHF extract of *M. pruriens* and *L. salicifolia* to *E. coli* resulted in the separation of inner membrane from the cell wall. Some cells showed disruption of the inner and outer membrane. The treated cells also showed abnormal swollen periplasmic space filled with cytoplasmic ingredients. Treated cells were reduced in size than the normal cells, which may be due to stress, induced by plant extracts.

In order to defend pathogen attack, plants are known to synthesize antimicrobial agents as a first line of defense mechanism and these antimicrobial agents are known to form complex with membrane lipids (Stotz *et al.* 2013). Disruption of the outer membrane of *E. coli* might have caused due to the interaction of the phytochemicals present in the extract with the outer membrane which in turn destabilized the outer surface eventually splitting the inner membrane. Probably membrane disruption caused accumulation of cytoplasmic material in the periplasmic space.

*S. aureus* cells treated with extract also showed damage in the cell wall. In some cells the cell wall was completely missing. The probable reason for this was explained by Cowan (1999) as the ability of some of the chemical constituents of plants to inhibit the bacterial cell wall formation resulting in cell wall disruption (Cowan 1999). Hu *et al.*
(2011) reported similar findings for *S. aureus* cells treated with *Magnolia officinalis* extract. Kamonwannasit *et al.* (2013) reported that the antibacterial action of *Aquilaria crassna* leaf extract against *Staphylococcus epidermidis* also involved cell wall disruption. Cao *et al.* (2012) reported that excessive oxidative stress due to ROS production could interfere with bacterial membrane transporters expression thus leading to membrane damage.

Comparative study of plasmid DNA of treated and control bacterial cells showed that there were considerable changes in the banding pattern of plasmid isolated from treated and control cells on agarose gel. There was an additional band in the *M. pruriens* and *L. salicifolia* treated plasmids on gel which was missing in case of control plasmid. In case of *E. coli* also, there was considerable differences in the banding pattern of treated and control cell DNA in the gel. Plasmid DNA isolated from treated *E. coli*, showed an extra band in the gel that was not visible in control plasmid. The possible reason for this could be due to the interaction of the plant constituents with the bacterial DNA or with the enzymes associated with bacterial DNA replication, interfering with DNA synthesis, resulting in formation of relaxed DNA. The wells loaded with treated DNA were filled with some amount of DNA. The probable reason for this phenomenon could be due to binding of the protein present in the extract, peptides or other phytochemicals to the DNA which in turn interfere with the movement of the DNA in the gel resulting in formation of faint bands. Flavonoids and alkaloids were known to induce antibacterial activity by binding to the bacterial DNA (Mori *et al.* 1987). Puroindoline protein isolated from wheat was reported to exhibit their antimicrobial action binding to DNA as proved by gel retardation assay.
(Alfred et al. 2013). Oxidative stress could be the cause or the consequence of structural and DNA damage eventually leading to cell death. Brudzynski et al. (2012) showed cause-effect relationship of DNA degradation of buckwheat honey treated bacterial strains and bacterial killing.

In the present work, time kill experiment was carried out with the PE, CHF, MT and AQ extracts of *L. salicifolia* leaves in order to study their bactericidal action *E. coli* and *S. aureus*. This is the first report on time kill kinetics of *L. salicifolia* leaf extract against *E. coli* and *S. aureus*. The extract exhibited bactericidal activity at 10 h of exposure to extract when the viable cell count decreased to 2.04 log10 cfu/ml from 5.17 cfu/ml of initial density. In case of *E. coli*, the CHF extract showed bactericidal activity after 6 h of exposure. PE, AQ and MT extract showed bacteriostatic activity to all the four bacteria up to 10 h of exposure. The data showed that the response of the *S. aureus* to the tested extract is both time and dose dependant, which may be explained as the ability of the antibacterial agents in the extract to interfere with the peptidoglycan layer of the bacterial cell wall indicating cell wall related mode of action (Mandal et al. 2011, Okemo et al. 2001, Mandal et al. 2007). The rate kill kinetics of *Azadirachta indica* was investigated against *S. aureus*. Time kill experiments for *Azadirachta indica* seed extract was studied against *Salmonella enterica* serovar Typhi by Mandal et al. (2007). Previous report on various classes of bactericidal antibiotics suggested bacterial killing by the mechanism of reactive oxygen species production (Kohanski et al. 2007), which supported our study of reactive oxygen species generation in the antibacterial activity *L. salicifolia* extract.
Among the four extracts of *M. pruriens*, CHF extract showed maximum antibacterial activity. Although the MIC value of the CHF extract was greater than 1 mg/ml which are not generally considered to be strong, sometimes purification of the crude extract can result in increase in bioactivity of the purified compound with MIC value less than 100µg/ml. This is because of antagonistic effect of different compounds in the crude extract or fractions lowering the activity of the active principle. Therefore, the crude extract was further analysed by bioactivity guided fractionation. Besides, analysis with TLC revealed that there are more components in CHF extract than PE, MT and AQ extracts. Therefore, it was logical to proceed with the CHF extract for further analysis. But the extracts with lower activities could still serve as source of useful compound after bioassay guided fractionation and purification (Akinpelu et al. 2008, Ndip et al. 2009). Further extracts showing very low or no activity might show similar properties with pro drug administered in inactive form (Njume 2012).

The CHF extract was further fractionated. The 6 fractions eluted from column chromatography were subjected to study antibacterial activity on *S. aureus, E. coli, P. aeruginosa* and *S. pneumoniae*. Maximum activity was exhibited by fraction 1 followed by Fraction 5> Fraction 6> Fraction 3> Fraction 4> Fraction 2. The antibacterial activity of fraction 1 was higher in *S. aureus* than in *E. coli*. However, *P. aeruginosa* and *S. pneumoniae* were resistant to all the fractions. However, the MIC value of all the 6 fractions was much higher than the crude extract. Therefore, further fractionation was not carried out. The possible reason of low activity of the fraction could be insufficient quantity of active components in the individual fractions. The chemical composition and proportion
of each component in the fraction as well as their mode of interactions with the test organism play an important role in the activity of the fractions (Saidana et al. 2008). This suggested that the phytochemicals acted synergistically on the bacterial cells resulting in growth inhibition. Partial purification of the crude extract into fraction could also result in decrease in activity because of the loss of the activity of the individual components.

The TLC profile showed that CHF of *L. salicifolia* seed extract contains some additional components that could be the possible cause for its higher activity than PE, MT and AQ extracts. Therefore, the CHF extract was further fractionated to obtain five fractions showing antibacterial activity in the order of Fraction 1 > Fraction 2 > Fraction 4 > Fraction 3 > Fraction 5 against *S. aureus*. The Fraction 1 was further purified by bioassay guided fractionation because Fraction 1 showed highest antibacterial activity with lowest MIC value of 0.08± 0.017 mg/ ml which is almost equal to the crude extract. Moreover, only the fraction 1 showed activity against both Gram positive and Gram negative bacteria. Five different fractions F 1a, F1b, F1c, F1d and F1e were eluted from Fraction 1 using preparative TLC method. Antibacterial assay by disc diffusion method showed that F1b is the most active among all the five extracts. MIC value determined by broth dilution method also showed lowest value for F1b.

Comparison of the data from FT-IR, NMR and mass spectra with literature for the purified compound of F1b suggested the compound to be β- sitosterol. Our data suggested that β- sitosterol may be one of the compounds responsible for antibacterial activity of the *L. salicifolia* extract. Comparison of MIC values of β- sitosterol, Fraction 1 and the crude extract indicated that antibacterial activity induced by β- sitosterol was significantly lower
than Fraction 1 and crude CHF extract. This is indicative of the fact that β-sitosterol is not the major active antibacterial compound present in Fraction 1 responsible for the antibacterial activity. The crude extracts and the fraction showed good antibacterial activity because of synergistic action of various other components together triggering the antibacterial activity.

Our results showed similarity with the reports of Kiprono et al. (2000) and Sen et al. (2012). They reported strong antibacterial activity of β-sitosterol against various bacterial and fungal species including *E. coli*, *P. aeruginosa* and *S. aureus*. There are previous published report on anticancer (Jourdain *et al*. 2006), anti-mutagenic (Villasenor *et al*. 2002), anti-inflammatory (Prieto *et al*. 2006), anti-diabetic (Jamaluddin *et al*. 1994) and antioxidant activities (Baskar *et al*. 2012) of β-sitosterol. The antibacterial mechanism of action β-sitosterol is not clearly understood.