5. DISCUSSION

5.1. *Streptococcus pneumoniae*

*S. pneumoniae* or pneumococcus is a species of the genus *Streptococcus*, which belongs to the family of *Streptococcaceae* (Lund & Henrichsen 1978). The pneumococcus was first isolated in 1881 independently by Sternberg in the USA and Pasteur in France, who both recovered diplococci from the blood of rabbits injected with human saliva. In 1886, the organism was referred to as *Pneumococcus*, but in 1920 it was renamed *Diplococcus pneumoniae*. It was not until 1974 that the pneumococcus received its present name, *S. pneumoniae*. (Austrian 1981, Watson et al. 1993).

*S. pneumoniae* (pneumococcus, Pnc) is a gram-positive, encapsulated, facultative anaerobic coccus, with a distinctive asymmetric "lance" shape. *S. pneumoniae* was formerly known as *Diplococcus*, because it usually appears in pairs and consequently was believed to belong to a genus separate from *Streptococcus*. These morphological characteristics help in identification of the bacteria. Three morphological layers can be distinguished in the surface of Pnc: plasma membrane, cell wall, and capsule. The peptidoglycan of the cell wall anchors the cell wall polysaccharide (CPS), and also proteins, in addition to the capsular polysaccharide. The *S. pneumoniae* bacteria have a thick polysaccharide capsule that covers the inner structure of the bacteria (Skov Sorensen et al., 1988). Some proteins, such as the pneumococcal surface protein A (PspA), however, are exposed beyond the capsule (Gray 1996). Moreover, in the present study, *S. pneumoniae* is typically small, slightly elongated cocci, with one end broad and other end is pointed, presenting a flame shaped appearance. They occurred in pairs (diplococci), with broad ends in apposition. They are capsulated enclosing each pair.

Biochemical test is the effective test for identify and differentiate the bacterial Species. Normally, *S. pneumoniae* showed gram positive, methyl red positive, Voges-proskur negative, Citrate negative and sugar production positive. *S. pneumoniae* differs from other streptococci chiefly in its morphology and bile solubility (Watson et al. 1993). Similarly, in the present study, *S. pneumoniae* showed same results such as gram positive, methyl red positive, Voges-proskur negative, Citrate negative sugar production positive and bile positive.
5.2. Screening of virulence factors in *S. pneumoniae*

The pathogenicity of pneumococci has been attributed to various structures, most of which are situated on its surface. The high morbidity and mortality caused by this microorganism are still poorly understood, and the list of virulence factors is probably far from complete. One group of factors, such as the capsule and a recently identified protein (Neelaman, C. et al., 1993), provides resistance to phagocytosis and thus promotes the escape of pneumococci from the host immune defense. Other factors, including cell wall components and the intracellular toxin autolysin. This autolysin produced enzyme like protease, hydrolase, amilase, and phospholipases which involved mainly in the inflammation caused by infection. The inflammation process fully develops only after lysis of bacteria by autolysin. Since inflammation is thought to induce most of the symptoms of pneumococcal disease (Johnston, R. 1991; Musher, D. M., 1992), this group of virulence factors directly responsible for the morbidity and mortality caused by pneumococci. Pneumococcal enzymes, such as autolysin, neuraminidase, hyaluronidase and IgA1 protease, have also been suggested to play a role in the pathogenesis of pneumococcal disease. (Paton et al., 1993, AlonsoDeVelasco et al., 1995, Brooks-Walter et al., 1999).

Extracellular phospholipases facilitate the ability of organisms to injure, invade the tissue of various host cells (Saffer et al., 1989; Silverman et al., 1992). In the present study, all 10 isolates of *S. pneumoniae* were responsible for invasive diseases and were able to grow on egg yolk agar plates (Table 3 and Fig 8). Among these isolates, six isolates of *S. pneumoniae* were negative and remaining four isolates were positive producing phospholipase production. Among the positive isolates, S2 and S10 were significantly scored more than S7 and S8.

Besides, in the present study it was found that the enhanced protease production by isolates of *S. pneumoniae* using skim milk agar medium is corresponding to the development of new multiple drug resistance activity. Protease production by *S. pneumoniae* on skim milk agar medium was observed. The higher protease (13 mm) production was found in S2 and S3 isolates and moderate protease (10 mm) was detected in S1, S6 and S9 isolates. However, the lowest protease productions was observed in S7 and S10. Biofilms are structured populations of microorganisms adhered to a surface (or interface) and embedded in an extracellular matrix consisting mainly of exopolysaccharides (sometimes bound together by proteins and
DNA) (Hall-Stoodley L and Stoodley P 2009) In nature, these communities can be mono or more frequently, multispecific (Ferrera I, et al., 2007; Souza-Egipsy JV et al., 2008) and show a modified phenotype in terms of their growth rate and different gene expression patterns. Biofilms have been likened in their level of organization to a eukaryotic organism, undermining the frontier between the biology of eukaryotes and prokaryotes (Costerton, JW et al., 1995). The polysaccharide surrounding the biofilm is frequently composed of one or more anionic uronic acids.

Over 60% of bacterial infections (and up to 80% of chronic infections) are currently considered to involve microbial growth in biofilms. This peculiar form of life poses an array of problems in human clinical practice, from infections associated with the implant of prosthetic devices and dental plaque formation to diseases such as cystic fibrosis, otitis media, and endocarditis (Bogaert D, et al., 2004). Biofilms are also produced by S. pneumoniae and this bacterium often colonizes the upper airways in humans as a normal commensal, yet it may spread to other areas of the body, causing otitis media, pneumonia, or invasive diseases such as bacteremia and meningitis. The capacity of S. pneumoniae to form biofilms had not been explored until recently. Several newly developed in vitro systems have allowed to test the capacity of S. pneumoniae to form biofilms, and to analyze the influence of several factors, including DNA and proteins which play a role in the virulence of this “supergerm” in the formation and development of biofilms (Miriam Moscoso et al., 2009).

In the present study, isolates of S. pneumoniae showed the different ranges of slime production on brain heart infusion agar medium supplemented with Congo red (Fig 10). Slime production was demonstrated in all of S. pneumoniae isolates, (Table 3). Strong slime production was found in two isolates such as S2 and S4 which appeared as thick black colour. White layer with black bubble formation exhibited in two isolates (S6 and S9) indicating as moderate and one isolates (S5) showed light brown colour representing as weak positive slime producer.

5.3. Genotypic analysis of S. pneumoniae using PCR analysis

S. pneumoniae infection is a life-threatening disease; however, it is proven that early diagnosis improves clinical outcomes (Tunkel et al., 2004). Recently developed molecular methods could allow an early and make it possible to achieve the accurate etiological diagnosis of pneumonial infection (Deutch et al., 2006; Dicuonzo et al., 1999; Margall et al.,
PCR tests are available as diagnostic procedures for specific organisms (such as \textit{S. pneumoniae}, \textit{N. meningitidis}, \textit{H. influenzae}) (Bossard \textit{et al.}, 2004; Kotilainen \textit{et al.}, 1998; Poppert \textit{et al.}, 2005). The use of broad-range bacterial PCR could help to improve our knowledge of the etiological spectrum of bacterial meningitis, allowing the detection of bacteria infrequently cultivated, or not yet recognized as causative agents of meningitis (Boudewijns \textit{et al.}, 2006; Claridge, 2004; Greisner \textit{et al.}, 1994; Poxton, 2005). Amplification using 16S rDNA primers has seen various applications. Recently it has been proposed as a strategy for the diagnosis of culture-negative bacterial meningitis and applied in daily microbiological practice (Deutsch \textit{et al.}, 2006; Dicciono \textit{et al.}, 1999; Margall \textit{et al.}, 2002; Meybeck \textit{et al.}, 2006; Pandit \textit{et al.}, 2005; Sacchi \textit{et al.}, 2002; Saravolatz \textit{et al.}, 2003).

Commercial tests are available for the identification of bacterial species based on the nucleic acid sequences of 16S rRNA (Schuchat \textit{et al.}, 1997). The MicroSeq 500 16S ribosomal DNA (rDNA) based bacterial identification system (Applied Biosystems Division) has been marketed for rapid and accurate identification of bacterial pathogens: the first 500-bp of the 16S rRNA gene of the bacterial strain are amplified, sequenced and analysed using the database provided by the system or, alternatively, public databases such as GenBank, NCBI and the organism was confirmed by phylogenetic tree (Fontana \textit{et al.}, 2005; Woo \textit{et al.}, 2003).

Similarly, in the present study, 16S r DNA sequencing of \textit{S. pneumoniae} done by universal bacterial primers, 16s-UP-F, R4 these universal bacterial primers were amplified with template DNA yielded bands at 1500 bp which were specific for the strain of \textit{S. pneumoniae}. In this study, DNA was isolated from the S2 isolates and its quality was checked by loading in 1% agarose gel with the Lambda DNA Hind III digest DNA marker which showed the intact DNA.

16S r DNA region of the S2 isolate was amplified through PCR which showed the molecular weight of 1500 kb corresponding to that of the 100 bp DNA ladder in 1% agarose gel (Fig.11) and this positive amplification of 1500 kb specific for the 16S r DNA regions of \textit{S. pneumoniae} genes. The amplified product was purified to remove the excess primer for sequencing and it was sequenced using the automated DNA sequencer. Sequence of the S2 isolate showed partial 16S r DNA sequences, consisting of 1441 nucleotides (Figs. 12),
which was submitted to the GenBank (National Center for Biotechnology Information, USA) and an Accession Number (JQ247720) was obtained.

Further, Phylogenetic tree was deduced from species of *S. pneumoniae* using Neighbour-joining method. The Phylogenetic tree was constructed based on 16S r DNA sequences of S2 isolate of *S. pneumoniae* in the present study and 16S r DNA sequences retrieved from the database of NCBI. The phylogenetic relationship between the various species of *S. pneumoniae* was obtained. The dendrogram placed the S2 isolate in a separate line of descent within the genus *S. pneumoniae* representing a distinct phylogenetic lineage. The S2 isolate of multiple resistant *S. pneumoniae* showed 100% similarity with the already established the species *S. pneumoniae*. From this study, S2 isolate was confirmed as the isolate of the species *S. pneumoniae*

5.4. Phytochemical analysis

It is emphasized that a rapid and accurate analytical technique is necessary to check wide difference in the plant samples and also in their quality. The quantitative GC/MS phytochemical analysis showed totally 40 compounds from *A. cepa*, 31 compounds from *A. sativum* and 18 compounds from *F. bengalensis*. These compounds belonged to various groups like alkaloids, flavonoids, carbohydrates, saponins, tannins, phenol and terpenes.

Flavonoids are reported to exhibit antioxidant activity (Ramanthan, *et al.*, 1989) and are effective scavengers of superoxide anions (Robak and Gryglewski, 1988). Alkaloids have been identified to have antibacterial activity; for example Allicin a compound of the alkaloid class isolated from *Allium spp.*. The mechanism of action here is attributed to the alkaloid ability to intercalate with DNA (Ghoshal *et al.*, 1996). Terpenoids and alkaloids have been proved to have antifungal activity. Presence of alkaloids and terpenoids in the leaf extracts of *A. sativum* exhibit antibacterial properties could serve as a basis for its traditional use as a medicinal plant. This agrees with what was reported by Ghoshal *et al.* (1996) that alkaloids, terpenoids and lactones are responsible for antibacterial activity. Terpenoids are synthesized from acetic units, and as such they share their origins with fatty acids. They however differ from fatty acids in that they contain extensive branching and are cyclized and have been found to inhibit bacterial growth.
Emergence of multi-drug resistance in human and animal pathogenic bacteria as well as undesirable side effects of certain antibiotics has triggered immense interest in the search for new antimicrobial drugs of plant origin (Ahmad and Beg, 2001). The antimicrobial activity of phenolic compounds found in Alliaceae and Moraceae have been studied mostly against various bacterial infections. In the current study, the tested phenolic compounds showed potential against S. pneumoniae, which are known to cause several oral infections. The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports (Komali et al., 1999; Moller et al., 1999).

Polyphenolic compounds have an important role in stabilising lipid oxidation and are associated with antioxidant activity (Gulcin et al., 2003; Yen et al., 1993). The phenolic compounds may contribute directly to antioxidative action. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when up to 10 g is ingested daily from a diet rich in fruits and vegetables (Tanaka et al., 1998). This present study is the first report on the antibacterial activities of A. cepa, A. sativum and F. bengalensis. The extracts of these three test plants can be used as natural healers against S. pneumoniae because of their rich phenolic content.

Saponins are secondary plant metabolites that occur in a wide range of plant species (Hostettmann and Marston, 1995). They are stored in plant cells as inactive precursors but are readily converted into biologically active antibiotics by plant enzymes in response to pathogen attack. The natural role of saponins in plants is thought to be protection against attack by pathogens and pests (Morrisey and Osbourn, 1999). These molecules also have considerable commercial value and are processed as drugs and medicines, foaming agents, sweeteners, taste modifiers and cosmetics.

According to our knowledge, saponins of A. cepa, A. sativum and F. bengalensis have never been studied for Streptococcal spp. On the other hand it is known that all of these species are also rich in highly bactericidal saponins. It was concluded from this study that the presence of these phytochemical in, A. cepa, A. sativum and F. bengalensis should be the reason for its antibacterial activity. The result of this experiment indicates that these medicinal plants have potentiality to treat multiple resistant S. pneumoniae causing pneumonial diseases and it could be utilized to create a healthy environment.
5.5. Anti-bacterial activity of medicinal plants

The search for newer sources of antibiotics is a global challenge preoccupying research institutions, pharmaceutical companies and academia, since many infectious agents are becoming resistant to synthetic drugs (Latha and Kannabiran, 2006). Infectious diseases are the world’s major threat to human health and account for almost 50,000 deaths every day (Ahmad and Beg, 2001). The situation has further been complicated with the rapid development of multiresistance to the microorganisms to the antimicrobial agents available. Plants have the major advantage of still being the most effective and cheaper alternative sources of drugs (Pretorius and Watt, 2001). The local use of natural plants as primary health remedies, due to their pharmacological properties, is quite common in Asia, Latin America and Africa (Bibitha et al., 2002). The antibacterial activity of onion juice can be attributed to the presence of flavonoids and polyphenols which has been reported to have broad spectrum of antibacterial activity (6). Polyphenols from plants have been reported to have antibacterial activity.

*Allium* is the largest and important representative genus of the *Alliaceae* family comprises 450 species. *Allium cepa* L., (Onion) which belongs to the family *Alliaceae* is also known as ‘garden onion’ or ‘bulb onion’. It is one of the oldest cultivated vegetables in history. It is thought that bulblets from the onion family have been utilized as a food source for millennia. Above ground, the onion shows only a single vertical shoot; the bulb grows underground, and is used for energy storage, leading to the possibility of confusion with a tuber which it is not. The leaves are bluish-green and hollow, the bulbs are large, fleshy and firm.

Three main varieties of onion are available viz: red, white and purple skinned (Ying M.C., et al., 1998). Onions are easily propagated, transported and stored. Onions are effective against common cold, heart disease, diabetes, osteoporosis, coughs and sore throat (Augusti K., 1996). They also act as bacteriostatic (Saulis A.S., et al., 2002). Certain chemical compounds believed to have anti-inflammatory, anti-cholesterol, anticancer and antioxidant properties such as quercetin are present in onions (Wilson E.A., 2007). They are high in flavonoids which is concentrated on the outer layer of the flesh (Nemeth K et al., 2007). Onions are also high in polyphenols than other *allium* vegetables (Wang L. et al., 2009).
*As* *at* *ivum* has been used in the history for both culinary and medicinal purposes. They exhibit different properties such as antibacterial, anti-fungal, anti-septic, anti-viral, expectorant, anti-histamine. It has been used as a food additive and also used in traditional remedies in flavonoids and a principle odour allicin (dialyl thiosulphinate). The extracts have been found to anti-histamine. It has been used as a food additive and also used in traditional remedies in flavonoids and a principle odour allicin (dialyl thiosulphinate). The extracts have been found to have a significant protective action against a fat induced increase in serum cholesterol and plasma fibrinogen, fibrinolytic activity and also possess pharmacodynamic properties. Allicin in its pure form, exhibit anti-bacterial activity against a wide range of Gram negative and Gram positive bacteria including multiple resistant strains of *E. coli*. The increased permeability of allicin through membranes may greatly enhance the intra cellular interaction with thiols was reported (Mirelman, D et al., 2000). Didry N et al., 1987 has tested antimicrobial activity against pathogenic aerobic and anaerobic bacteria from the crude extracts of garlic, onion and shallots.

The genus *Ficus* (Moraceae) includes some 750 species of woody plants occurring in most tropical and subtropical forests throughout the world (Berg C.C., 1989). The genus is remarkable for the large variation in the habits of its species (Jander E.A., et al., 2008). All plants containing active compounds are important. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, and phenol compounds, which are synthesized and deposited in specific parts or in all parts of the plant. Many *Ficus* species are commonly used in traditional medicine to cure various diseases. They have long been used in folk medicine as astringents carminatives, stomachics, vermifuges, hypotensives, anthelmintics and anti-dysentery drugs (Trivedi, P., et al., 1969).

Many species are cultivated for shade and ornament in gardens. Several species produce edible figs of varying palatability. Some species are producing latex. The fig is a very nourishing food and used in industrial products. Figs contained water, fats, high amounts of amino acids, such as leucine, lysine, valine and arginine, and minerals, such as potassium, calcium, magnesium, sodium, phosphorus and vitamins. Some of the most important species of *Ficus* are, *F. racemosa*, *F. religiosa* and *F. bengalensis*. It is propagated by seeds. *F. bengalensis* is commonly known as a Banyan tree. This tree is considered to be sacred tree in
India. The bark, leaves and fruits of this group are used as astringent, haemostatic, anti-septic, anti-inflammatory, antioxidant and anticancer agent and also in the treatment of diarrhoea, dysentery, and in the treatment of skin diseases, ulcers, vaginal disorders, leucorrhoea, deficient lactation (Khare, C.P., 2004).

Yusha’u Met et al., (2011) studied antibacterial activities of ethanolic extracts of *Annona squamosa* (L.) leaves were studied against clinical respiratory tract isolates of *Klebsiella pneumoniae*, *Proteus species*, *Pseudomonas species*, *Staphylococcus aureus*, *S. pneumoniae* and *α*-haemolytic *Streptococci* using disc diffusion and micro-broth dilution techniques. Sensitivity test results showed that water fraction of the plant was active on *S. aureus* and *S. pneumoniae* (10 mm) at 50μg/disc concentration while ethanolic extract of the plant was active against *K. pneumoniae*, *S. pneumoniae* and *Proteus species* at 200μg/disc concentration with zone diameter formed by *K. pneumoniae* (11 mm) being wider than that formed in response to standard Augmentin disc (6 mm).

Shiv Shanker Gautam et al., (2012) studied the antibacterial potential of various extracts (petroleum ether, acetone, methanol and aqueous) of *Neepa ciliaris* against three gram-positive (*S. aureus*, *S. pneumoniae* and *S. pyogenes*) and one gram-negative (*Pseudomonas aeruginosa*) bacterial pathogens. The agar well diffusion method was adopted to examine antibacterial and minimum inhibitory concentration (MIC) values of most effective extracts against the susceptible bacteria. Erythromycin was used as positive control to determine the sensitivity of the strains. Out of the four bacterial species tested, *S. pneumoniae* was the most susceptible. The *N. ciliaris* is potentially a good source of antimicrobial agents.

In the present study from the medicinal plants such as *A. cepa*, *A. sativum* and *F. bengalensis* fractions showed antibacterial activity against *S. pneumoniae*. The individual fractions (water, ethanol, methanol, acetone, hexane and butanol) of *A. cepa*, *A. sativum* and *F. bengalensis* showed moderate antibacterial activity. The average zone of inhibition of 2.5, 3.2, 1 and 0.5 mm was observed in *A. cepa* and to 2, 7, 3, 2, 2, 0.5 mm was noted in *A. sativum* and 3, 6, 4, 0.5, 1, 2 mm was analyzed from *F. bengalensis* in disc diffusion method (Table 8).

Among all the fractions, only ethanol and methanol showed maximum activity against *S. pneumoniae*. Hence these two fractions were once again treated with *S. pneumoniae* strain at
various combinations, which showed effective results (Table 9). The significant anti-bacterial activity of 4 mm, 8 mm and 9mm zone of inhibition was observed with ethanol and methanol fraction of *Acepa* (1:82), *A. sativum* (12:18) and *F. benghalensis* (2:18) against *S. pneumoniae* strain. The MIC of *F. benghalensis* effectively inhibited the growth of *S. pneumoniae* at concentration of 11.5 mg/ml (Table 10), which was higher than others. However, the MIC for *Acepa* and *A. sativum* values were found to be 10.8 mg/ml and 10.6 mg/ml respectively against *S. pneumoniae*.

5.6. Molecular docking Nan A of *S. pneumoniae*

The treatment of patients with pneumonial diseases involving only antibacterial medications is difficult to achieve or it may not be possible in patients with streptococcal infection. Successful treatment of streptococcal infection could be hampered when *S. pneumoniae* get well established in the oral cavity. *S. pneumoniae* in the oral cavity exhibits significantly higher tolerance to traditional antifungal agents. As a consequence, alternative strategies like elucidation of bioactive compounds from medicinal plants to treat the oral streptococcal infection. This process involves screening of various bioactive compounds from medicinal plants through GC-MS analysis.

The protein-ligands interaction plays a significant role in determining the suitable drugs for the treatment. A variety of computational methods to identify the suitable drugs are available. One such method is docking of drug molecules with receptors. The energy value obtained through docking is used as a criterion for the selection of drugs. Based on the energy values, lead molecules are identified. This infers that the lead molecules are one with maximum interaction having high negative e-value (Virupakshaiah et al., 2007). Thus the concept of protein-ligand docking helps in finding the suitable drugs for pneumonial diseases.

Among the variety of computational methods, Molecular docking using Hex is an effective method and it is an interactive molecular graphics program for calculating and displaying feasible docking modes of pairs of Protein and DNA molecules. The docking results are ordered by negative energy values. The high negative energy value having compound is the seed member for drug designing. (Ritchie, 2003).

In molecular docking bioactive compound act as ligand and virulence factor of *S. pneumoniae* act as receptor. *S. pneumoniae* produce several virulence extra cellular hydrolytic enzymes such as autolysin, neuraminidase, hyaluronidase and IgA1protease
(AlonsoDeVelasco et al., 1995, Brooks-Walter et al., 1999). Among the virulent factors secreted neuraminidase are considered to be the most important virulence factor in Nan A enzyme (Paton et al., 1993) and it helpful to adhere and invade the host during the infection.

In the present study, the structure of Nan A were retrieved from PDB data bank and the structure of bioactive compounds from drug bank. All the ligand molecules were made to dock against the active sites of the target antigen using Hex software. Nan A enzymes were acting as receptors and the bioactive compounds act as ligands. Nan A when docked with various ligands (bioactive compounds), some bioactive compound showed high negative energy values which was selected as suitable lead compound to treat pneumonial diseases. The docking results were represented in the form of e-negative values. In the docking studies, higher negative e-values represent high binding affinity between the receptor and ligand molecules, indicating the higher efficiency of the bioactive compounds.

Nan A of *S. pneumoniae*, on docking with bioactive compounds namely 2-Furanearboxaldehyde, 5-(hydroxymethyl), 4H-Pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl, Maltol, Propanetriol, 1-acetate of *A. cepa* produced energy values such as -141.82, -140.48, -137.34, and -104.48 respectively. Similarly, the bioactive compounds, Urs-12-en-3-ol, acetate, 12-Oleanen-3-yl acetate, (3â€‘) Sucrose of *A. sativum* produced energy values such as -199.02, -197.05 and -156.06 respectively. Docking of Nan A with bioactive compounds, including Oxalic acid, 2-ethylhexyl ethyl ester, 1,2-Benzene diol, Dodecanic acid of *F. bengalensis* produced energy values -139.61, -111.99 and -120.79 respectively. The results showed that all the bioactive compounds with target antigens produced high negative e-value. Thus, it is clear that the bioactive compounds were able to interact with any of the available binding sites of the Nan A enzymes effectively. The above study clearly indicates that the bioactive compounds were able to inhibit the activity of the Nan A enzyme.

Among the bioactive compounds studied, Urs-12-en-3-ol, acetate, (3â€‘) from *A. sativum* showed a higher negative energy value of -199.02 than that of other compounds indicating as effective antibacterial activity over other compounds.