3. MATERIALS AND METHODS

3.1. Phenotypic analysis of *S. pneumoniae*

3.1.1. Sample collection and clinical examination

Oral samples were collected from different age patients who were belongs to various clinics in and around Thanjavur and Chennai, Tamil Nadu, India. Oral sample collection involved oral swab and rinsing the mouth with 10 ml of sterile phosphate-buffered saline (PBS; 0.1 M; pH 7.2) and it was collected through sterile container. Each rinse was centrifuged at 2,000 ×g (10 min), the supernatant was removed, and deposit was dissolved in 1 ml of PBS. A portion (50 µl) of the concentrate was inoculated in to the nutrient agar medium containing petridishes and incubated at 37°C for 24 h.

3.1.2. Biochemical study of *S. pneumoniae*

3.1.2.1. Gram staining

A thin smear of streptococcal isolates were separately made on a clean glass slide and heat fixed. Then the smear was stained using crystal violet for 1 min and then washed with water followed by flooding with Gram’s iodine. After 1 min, the slide was washed again in tap water and decolorized. Then the smear was counterstained with safranin for 1 min. Finally the slide was washed, air dried and observed under microscope.

3.1.2.2. Motility test

Petroleum jelly was applied around the cavity slide. A loopful of streptococcal isolates culture as placed in the center of a clean coverslip. The petroleum jelly applied slide was placed with the concave surface facing down over the coverslip. Then the slide was quickly turned upside down and it was observed under the microscope. The motility of the Streptococcal isolates was recorded.

3.1.2.3. Spore staining (Mc Kinney, 1953).

Bacterial smear was prepared, heat fixed and stained with 1 per cent alcian blue in 95 per cent ethanol for 1 min. The excess stain was washed with sterile water and allowed to air dry. Ziehl-Neelsen carbol fuchsin was used as counter stain for 1 min. The slide was washed, air dried and finally observed under microscope.
3.1.2.4. Indole test

Tryptone broth medium (Appendix 5) was prepared and poured into the test tubes. The streptococcal isolates were inoculated separately to the broth and incubated at 30 °C for 6 days. The uninoculated broth was maintained as control. After the period of incubation, 1 ml of Kovac’s reagent was added to each tube, including control. The tubes were gently shaken at an interval of 10 to 16 min, and allowed to stand until the reagent reached the bottom. The formation of red colour ring is indicative of positive results, whereas yellow colour ring indicates negative result.

3.1.2.5. Methyl red test

The MR-VP broth (Appendix 6) was prepared and 5 ml of the broth was poured into sterile test tubes. The streptococcal isolates were inoculated separately into the tubes and incubated at 30 °C for 6 days. After the incubation period, 5 ml of methyl red indicator was added to each tube. Red colouration of the broth indicates the positive reaction while turning of methyl red to yellow indicates a negative reaction.

3.1.2.6. Voges-Proskauer test

The MR-VP broth was prepared. 5 ml of the broth was poured into sterile test tubes. The streptococcal isolates were inoculated separately into the tubes and incubated at 30 °C for 6 days. After the incubation period, 5 ml of Barrett’s reagent A and B mixed (Appendix 4) was added. Development of red colour indicates positive results, while yellow colour indicates negative results.

3.1.2.7. Citrate utilization test

Simmon’s citrate agar medium (Appendix 8) was prepared and poured into sterile test tubes. The streptococcal isolates were inoculated separately into the test tube and incubated at 30 ± 2°C for 6 days. After the incubation period, if the green colour of Simmon’s citrate agar medium turns blue, it indicates positive result.

3.1.2.8. Catalase test

A clean glass slide was taken and a drop of streptococcal isolates suspension was placed. Few drops of hydrogen peroxide were added to the culture. The evolution of air bubbles from the suspension is indicative of positive result.
3.1.2.9. Carbon utilization test

Fermentation medium (broth) was prepared with carbon sources like lactose, mannitol, sucrose, glucose separately. After sterilization, streptococcal isolates were inoculated into the broth and in control no inoculation was made. The tubes were incubated at 30 °C for 6 days. After incubation, the reactions developed in the fermentation media were observed by comparing with the unoinoculated (control) tubes i.e., change in colour (due to production of acid) or change in colour and appearance of bubbles (due to production of acid and gas).

3.1.2.10. Bile salt solution test

*S. pneumoniae* produce a self-lysing enzyme to depress the growth of old colonies. The presence of bile salt accelerate this process. For this study, 10 ml of broth culture was taken and 1 ml of bile salt solution was added. It was incubated at 37°C for 15 minutes.

3.1.3. Screening of *S. pneumoniae* in selective medium

Blood agar medium is a selective medium to indicate the number of *S. pneumoniae* species present in the given sample and it showed the specific colour to every individual *pneumoniae* species. It can be used to distinguish the *S. pneumoniae* with other streptococcal species and species of non-streptococcal species.

For this study, tentatively identified streptococcal species from oral swab samples were inoculated on a blood agar medium and incubated at 28°C for 72 hours. After incubation, culture of pneumoniae species showed specific colour. Based on their specific colony colour, name of the pneumoniae species were identified.

3.1.4. Morphological study of *S. pneumoniae*

Oral swab samples smeared on the slides were stained with crystal violet and allowed to dry for about one minutes. Then the smear was flooded with grams iodine and kept for 1 minute. The 95% ethanol was added drop wise to decolourise the dye. Then the counter stain safranin was added, dried slides were examined under oil immersion compound microscope and high power (40X) of the compound microscope to identify *S. pneumoniae*. 

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3.2. SCREENING OF VIRULENCE FACTORS IN S. PNEUMONIAE

3.2.1. Phospholipase production by S. Pneumoniae

Price et al. (1982) described plate method for the detection of phospholipase activity in S. Pneumoniae which circumvents disadvantages. Egg yolk contains large amount of phospholipids, predominantly phosphatidylethanolamines and phosphatidylethanolamine, it was incorporated into a nutrient agar medium. This method is the traditional screening method for phospholipase activity for Streptococcal species.

When isolates of S. Pneumoniae grown on this medium indicating as phospholipase-positive and it forms distinct, well-defined, dense white zone of precipitation around the colony. This white zone is probably due to the formation of calcium complex with the fatty acids released by the action of phospholipase on the phospholipids present in the egg yolk. In this assay, phospholipase activity is defined as the ratio of colony diameter to the diameter of the dense white zone of precipitation around phospholipase positive colonies.

3.2.2. Protease production by S. Pneumoniae

Protease production of Streptococcal species was checked on the skim milk agar medium as per method described by Gokce et al. 2007. Skim milk agar medium possesses peptone from casein 5.0; yeast extract 2.5; skim milk powder (no inhibitors) 1.0; glucose 1.0; agar-agar 10.5; and distilled water 1000 ml maintained at pH 5.0. Ten µl samples (suspension) were introduced on a sterile paper disk placed on the surface of skim milk agar medium. The inoculated plates were incubated at 37°C for two days and diameters of zones of inhibition around the disks were measured for determination of protease activity.

3.2.3. Slime production by S. Pneumoniae

A method of screening slime production was described by Freeman et al. (1989). The composition of brain heart infusion medium (BHIM) contains 37 g/l, glucose 80 g/l, agar 10 g/l, and Congo red 0.8 g/l. The Congo red stain was prepared in aqueous solution which was autoclaved separately at 121°C for 15 min and it was added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically at 35°C for 48 h.

Slime production was evaluated according to the “Congo red phenomenon”. Isolates that produced dark red colonies showed as strong slime positive, whereas those showing pink or white colonies indicating as moderate and light brown colour representing as weak positive slime producer.
3.3. GENOTYPIC ANALYSIS OF S. PNEUMONIAE USING PCR ANALYSIS

3.3.1. 16S rDNA gene sequencing analysis

3.3.1.1. Bacterial Genomic DNA Isolation

Genomic DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen) following manufacturer’s instructions.

The cells are harvested in a microcentrifuge tube by centrifuging for 10 minutes at 5000 rpm. The supernatant is discarded and the pellet is resuspended in 180 μl of ATL buffer. Twenty micro litres of proteinase K was added and incubated at 56 °C in a water bath. After lysis, 5 μl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. 200 μl of AL buffer and 200 μl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm for 1 minute. The DNeasy mini spin column was transferred to a new 2 ml tube and washed with 500 μl of AW1 buffer. Wash step was repeated using AW2 buffer. After washing the DNeasy mini spin column was placed in a clean 1.5 ml tube and DNA was eluted out using 100 μl of AE buffer.

3.3.1.2. Quality and Quantity analysis for DNA by Agarose Gel Electrophoresis

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1μl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5μl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 μg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genci) and the image was captured under UV light using Gel documentation system (Bio-Rad).

DNA quantification was done by comparing the fluorescent intensity of the samples with a standard (100ng DNA). DNA was diluted to approximately 10ng/μl and stored at 4°C as working solution while the stock DNA (undiluted) was stored at -20°C.
3.3.1.3. Primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Name</th>
<th>Direction</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rDNA</td>
<td>16S-UP-F</td>
<td>Forward</td>
<td>CGAATTCGTCGACACAGAGTTGCATTGCTCAG</td>
</tr>
<tr>
<td></td>
<td>16S-UP-R</td>
<td>Reverse</td>
<td>CCCGGGATCCAGGTTACGGTACCTTGCAGACTT</td>
</tr>
</tbody>
</table>

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

3.3.1.4. PCR amplification profile

16S rDNA

\[
\begin{align*}
95 \, ^\circ C & \quad - \quad 5.00 \, \text{min} \\
95 \, ^\circ C & \quad - \quad 0.30 \, \text{min}
\end{align*}
\]

\[
54 \, ^\circ C \quad - \quad 0.40 \, \text{min}
\]

\[
72 \, ^\circ C \quad - \quad 1.00 \, \text{min}
\]

\[
72 \, ^\circ C \quad - \quad 7.00 \, \text{min}
\]

\[
4 \, ^\circ C \quad - \quad \infty
\]

\[
\begin{align*}
35 \text{ cycles}
\end{align*}
\]

3.3.1.5. Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 μg/ml ethidium bromide. 1 μl of 6X loading dye was mixed with 5 μl of PCR product was loaded on electrophoresis and it was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. 100 bp DNA ladder (USB) used as a molecular standard. The gel was visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.3.1.6. ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five micro litres of PCR product is mixed with 2 μl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes.
3.3.1.7. Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycle (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components:

- PCR Product (ExoSAP treated) - 10-20 ng
- Primer - 3.2 pM (either Forward or Reverse)
- Sequencing Mix - 0.28 μl
- Reaction buffer - 1.86 μl
- Sterile distilled water - make up to 10μl

The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes.

3.3.1.8. Post Sequencing PCR Clean up

Master mix I of 10μl milli Q 2μl 125mM EDTA per reaction, master mix II of 2 μl of 3M sodium acetate (pH 4.6) and 50 μl of ethanol were prepared. 12μl of master mix I was added to each reaction containing 10μl of reaction contents and was properly mixed. After that 52 μl of master mix II was added to each reaction and Contents were mixed by inverting and incubated at room temperature for 30 minutes. Then Spun at 14,000 rpm for 30 minutes and decanted the supernatant and added 100 μl of 70% ethanol. Again Spun at 14,000 rpm for 20 minutes and decanted the supernatant and repeated 70% ethanol wash. Decanted supernatant and air dried the pellet. The cleaned up air dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems).

3.3.1.9. Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment required editing of the obtained sequences were carried out using Geneious Pro v5 (Drummond et al., 2010).

3.3.1.10. Phylogenetic analysis

The 16s rDNA sequences of S2 isolates aligned with the sequences of pneumoniae species which were retrieved from the GenBank database. The sequences were converted into
FASTA format. The retrieved sequences were fed with ClustalW for multiple sequence alignment to observe sequence homology. Evolutionary tree was inferred by using the neighbour-joining method (Thompson et al., 1997). Dendrogram was constructed by neighbour-joining method using PHYLIP software package.

3.4. BIOLOGY OF PLANT MATERIALS

3.4.1. Taxonomy of *A. cepa* L.

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Division</td>
<td>Angiosperm</td>
</tr>
<tr>
<td>Class</td>
<td>Monocots</td>
</tr>
<tr>
<td>Order</td>
<td>Asparagales</td>
</tr>
<tr>
<td>Family</td>
<td>Amaryllidaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Allium</td>
</tr>
<tr>
<td>Species</td>
<td><em>A. cepa</em> L.</td>
</tr>
</tbody>
</table>

Tamil: vengayam. English: onion. It is found in India, Republic of Korea, Russia, Brazil, Pakistan, Turkey, Iran, Egypt, United States and China. It is a biennial plant, growing from a subterranean bulb. It can grow up to 70 cm in height. It has an erect stem and an umbel of soft, white to pink flowers on its top. Its underground bulb carries small, shallow roots. (Fig. 1).

**Bio active components:**

Calcium, iron, phosphorus, vitamin C, riboflavin, niacin, thiamine, carotene.

**Medicinal uses:**

Onion has a great variety of medicinal uses. It is considered to have anthelmintic, antioxidant, antiseptic carminative, diuretic, expectorant, febrifuge and vulnerary properties. It is very helpful to cure the common cold, heart disease and diabetes.
3.4.2. Taxonomy of *A. sativum* L.

- **Kingdom**: Plantae
- **Division**: Angiosperms
- **Class**: Monocots
- **Order**: Asparagales
- **Family**: Amaryllidaceae
- **Genus**: *Allium*
- **Species**: *A. sativum* L.

**Tamil**: Poondai, **English**: Garlic. It is generally found in India, China and Russia. It is a dioecious plant, smooth climbing vine reaching a height of 2 to 4 meters. Leaves are usually oblong-elliptic, oblong-ovate or ovate, 6 to 17.5 cm long, 3.5 to 10 cm wide. Fruit is coalescing, fully embedded in the pulp and concrecent with the rachis. Seeds are smooth, oblong to globose-obovoid, 2.25 to 2.6 mm long. (Fig. 2).

**Phytochemical constituents**

Butanimidamide, Pyrazinold, Piperazine, Dimethyl trisulphide, Butanal, Heptanoic acid, alliin, ajoene, diallyl polysulphides, vinylthiins, and S-allylcysteine.

**Medicinal Properties**

Garlic reported for various pharmacological activities such as antimicrobial, antioxidant, antimitagenic, and antiinflammatory. It also acts as a medicine for heart disease and also prevent certain types of cancer, including stomach and colon cancers. It has a efficiency to reduce platelet aggregation and hyperlipidemia.

3.4.3. Taxonomy of *Ficus benghalensis* L.

- **Kingdom**: Plantae
- **Phylum**: Angiosperms
- **Class**: Eudicots
- **Order**: Rosales
- **Family**: Moraceae
- **Genus**: *Ficus*
- **Species**: *F. benghalensis* L.
Tamil: Aalamedu. English: Banyan. The banyan is a large and extensive growing tree of the Indian subcontinent. It produces propagating roots which grow downwards as aerial roots. Once these roots reach the ground, they grow into woody trunks that can become indistinguishable from the main trunk. (Fig. 3).

**Phytochemical constituents**

Phenol, Piperazine, 1,2-Benzenediol, Oxalic acid and Dodecanoic acid.

**Medicinal Properties**

*F. bengalensis* decreased the blood sugar, anti-tumor activity, anthelmintic activity it has, Anesthetic, Antibacterial, Antihemorrhoidal, Antihydrocele, Anti-incontinence, Antivaginogynphotic, Antioxidant and Antiprostatitic.

3.5. **Collection of plant materials**

*A. cepa* and *A. sativum* and *F. bengalensis* were collected from various areas in Kolli Hills, Namakkal, Tamil Nadu, India. The plant materials were authenticated and voucher specimens were deposited in herbarium (Rajinath Herbarium) of St. Joseph’s College, Trichy, Tamil Nadu, India.

3.6. **Extraction of plant materials (Harborne, 1984)**

The bulbs of *A. cepa*, *A. sativum* and prop roots of *F. bengalensis* were washed with dechlorinated water, dried in shade and powdered with the help of an electric blender. The test materials (1.0 kg) were extracted with different organic solvents viz., acetone, hexane, petroleum ether, chloroform and ethanol in a soxhlet apparatus for 8 h and the extract was concentrated in a rotary vacuum evaporator to yield crude extract (Fig. 4 a and b).

3.7. **Column chromatography analysis of plant materials (Annamalai et al., 2007)**

The extracts of test plant materials obtained by soxhlet apparatus were subjected in to the silica gel column to collect the fractionation. For this study, 250 g of activated silica gel was loaded to a column and the extracts of test plant materials such as *A. cepa* and *A. sativum* and *F. bengalensis* was loaded on to each column. The compounds were eluted with high and low polar solvents such as water, ethanol, methanol, acetone, hexane and butanol (HPLC grade) solvents respectively (Fig. 4 c).
3.8. PHYTOCHEMICAL ANALYSIS

3.8.1. Qualitative Analysis

Phytochemical analysis of the all plant extracts was undertaken using standard qualitative methods as described by various authors (Kapoor et al., 1969; Odebiyi and Sofowora, 1990). The plant extracts were screened for the presence of biologically active compounds such as alkaloids, flavonoids, carbohydrates, phytosterols, proteins, phenolics, tannins and saponins.

3.8.1.1. ALKALOIDS (Salehi Surmaghi et al., 1992)

I. Dragendorff’s test (Kraut reagent – Potassium bismuth iodide)

8 g of Bi (NO₃)₃, 5 H₂O was dissolved in 20 ml of HNO₃ and 2.72 g of potassium iodide in 50 ml of distilled water separately. They were mixed and allowed to stand till KNO₃ got crystallized. The supernatant was decanted and made up to 100 ml with distilled water. The alkaloids were regenerated from the precipitate by treating with Na₂CO₃ followed by extraction of the liberated base with ether. To 0.5 ml of plant extract 2 ml of HCl was added. Then 1 ml of reagent was added to this acidic medium. An orange red precipitate was produced immediately, which indicated the presence of alkaloids.

II. Wagner’s reagent (Iodine-Potassium iodide solution)

1.2 g of iodine and 2.0 g of potassium iodide were dissolved in 5 ml of H₂SO₄ and the solution was diluted to 100 ml. 10 ml of plant extract was acidified by adding 1.5% v/v HCl and a few drops of Wagner’s reagent. The formation of a yellowish brown precipitate confirmed the presence of alkaloids.

III. Meyer’s reagent (Potassium mercuric iodide)

1.36 g of mercuric chloride was dissolved in 60 ml of distilled water and 5 g of potassium iodide in 10 ml of distilled water separately. The two solutions were mixed and diluted to 100 ml with distilled water. A few drops of the reagent were added to 1 ml of the plant extract. The formation of a pale precipitate showed the presence of alkaloids.

3.8.1.2. FLAVONOIDS (Somolenski et al., 1972)

In a test tube containing 0.5 ml of plant extract, 5-10 drops of diluted HCl and a pinch amount of zinc or magnesium were added, and the solution was boiled for a few minutes. In the presence of flavonoids, a reddish pink or dirty brown colour was produced.
3. 8.1.3. CARBOHYDRATES

I. Fehling’s test (Kokate, 1994)

Solution A:

34.65 g of copper sulphate was dissolved and made upto 500 ml with distilled water.

Solution B:

125 g of potassium hydroxide and 173 g of Rochelle’s salt (sodium potassium
 tartarate) were dissolved in water and made upto 500 ml.

The solutions “A” and “B” were added. The contents were boiled for a few minutes. The
formation of a red or brick red precipitate indicated the presence of carbohydrates.

II. Benedict’s test

173 g of sodium citrate and 100 g of sodium carbonate were dissolved in 500 ml of
 distilled water. 17.3 g of copper sulphate dissolved in 100 ml of distilled water was added to
the above solution. To 0.5 ml of plant extract, 5 ml of Benedict’s reagent was added and
boiled for 5 min. The formation of a bluish green colour showed the presence of
 carbohydrates.

3. 8.1.4. PROTEINS

I. Millon’s test (Walsh and Farrel, 1961)

One part of mercury was digested with 2 parts of concentrated HNO₃ and the resulting
solution was diluted with 2 volumes of water. To a small quantity of plant extract, 5-6 drops
of Millon’s reagent was added. A white precipitate which turned red on heat indicated the
presence of proteins.

3. 8.1.5. PHENOLS (Malick and Singh, 1980)

I. Ferric chloride test

One ml of plant extract, 2 ml of distilled water followed by a few drops of 10 per cent
aqueous FeCl₃ solution were added. Formation of a blue or green precipitate indicated the
presence of phenols.

II. Lead acetate test

1 ml of plant extract was diluted to 5 ml with distilled water and then a few drops of 1
% aqueous solution of lead acetate was added. Appearance of yellow precipitate indicated the
presence of phenols.
III. Libermann’s test

A small amount of plant extract was dissolved in 0.5 ml of 20% sulphuric acid solution followed by the addition of a few drops of aqueous sodium nitrate solution. A red colour was obtained on dilution and it turned blue when made alkaline with aqueous sodium hydroxide Solution, which indicated the presence of phenol.

3.8.1.6. Saponins (Malick and Singh, 1980)

In a test tube containing about 5 ml of plant extract, a drop of sodium bicarbonate solution was added. The mixture was shaken vigorously and kept for 3 min. Formation of a honeycomb-like froth showed the presence of saponins.

3.8.1.7. Tannins (Segelman et al., 1969)

1. Ferric chloride test

Two ml of plant extract, a few drops of 5% aqueous FeCl₃ solution was added. A bluish black colour was formed, which then disappeared and addition of few ml of dilute H₂SO₄. This was followed by the formation of yellowish brown precipitate.

2. Lead acetate test

In a test tube containing about 500 ml of plant extract, a few drops of 1 per cent solution of lead acetate was added. Formation of yellow or red precipitate indicated the presence of tannins.

3.8.1.8. Phytosterols (Malick and Singh, 1980)

About 0.5 ml of test solution was mixed with minimum quantity of chloroform and the 3-4 drops of acetic acid and one drop of concentrated H₂SO₄ were added. Formation of a deep blue or green colour showed the presence of steroids.

3.8.1.9. Terpenoids

5 ml of each extract was mixed in 2 ml of chloroform. 3 ml of concentrated H₂SO₄ was then added to form a layer. A reddish brown precipitate colouration at the interface formed indicated the presence of terpenoids.

3.8.1.10. Phlobatannins:

Deposition of a red precipitate when an aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid was taken as evidence for the phlobatannins.
3.9. Gas Chromatography - Mass Spectroscopy (GC-MS) analysis (Ivanova et al., 2002)

The powdered sample (20 g) were soaked and dissolved in 75 ml of methanol for 24 h. Then the filtrates were collected by evaporated under liquid nitrogen. The GC-MS analysis was carried out using a Clarus 500 Perkin-Elmer (Auto System XL) Gas Chromatograph equipped and coupled to a mass detector Turbo mass gold – Perking Elmer Turbomass 5.2 spectrometer with an Elite-1 (100% Dimethyl poly siloxane), 300 m x 0.25 mm x 1 mm df capillary column. The instrument was set to an initial temperature of 110°C, and maintained at this temperature for 2 min. At the end of this period, the oven temperature was raised up to 280°C, at the rate of an increase of 5°C/min, and maintained for 9 min. Injection port temperature was ensured as 250°C and Helium flow rate as 1 ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass Spectral scan range was set at 45-450 (m/z). The chemical constituents were identified by GC-MS.

The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using National Institute of Standards and Technology Mass Spectral database (NIST-MS). The percentage of each component was calculated from relative peak area of each component in the chromatogram.

3.10. ANTIBACTERIAL ACTIVITY OF PLANT MATERIALS


Various solvent fractions of test plant materials such as A. cepe and A. sativum and F. bengalensis were checked for anti pneumoniae activity using disc-diffusion method. Multiple resistant S. pneumoniae (S2 isolate) was swabbed on the surface of the nutrient agar plates. The disc (Whatman No.1 filter paper with 9 mm diameter) was impregnated with the 50 μl of each test plant sample and it was placed on the surface of nutrient agar plates.

To compare the antibacterial activities, nystatin (20 μg/disc) used as standard antibiotic and blank disc impregnated with water act as negative control. The plates (triplicates) were incubated 28°C for 72 h. The antimicrobial potency of the test samples was measured by determining the diameter of the zones of inhibition in millimeter.
3.10.2. Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MICs) of the test plant materials such as *A. cepa* and *A. sativum* and *F. bengalensis* against Multiple resistant *S. pneumoniae* (S2 isolate) which was determined by serial dilution technique (Noble et al., 1977; Reiner, 1982) in the presence of standard nystatin.

3.11. Molecular Docking

The structure of Nan A in *S. pneumoniae* were retrieved from PDB data bank and the structure of bioactive compounds from drug bank.

3.11.1. DATABASES

I. PDB (http://www.rcsb.org/)

The Protein Data Bank (PDB) is a depository for the 3-D structural data of large biological molecules, such as proteins and nucleic acids. (See also crystallographic database). The data, typically obtained by X-ray crystallography or NMR spectroscopy and submitted by biologists and biochemists from around the world, are freely accessible on the Internet via the websites of its member organisations (PDBe, PDBj, and RCSB). The PDB is overseen by an organization called the Worldwide Protein Data Bank, wwPDB.

II. Drug databank (http://www.redpoll.pharmacy.ualberta.ca/drugbank/).

The Drug bank database is a unique bioinformatics and cheminformatics resource that combines detailed drug (i.e. chemical, pharmacological and pharmaceutical) data with comprehensive drug target (i.e. sequence, structure, and pathway) information. The database contains 6828 drug entries including 1436 FDA-approved small molecule drugs, 134 FDA-approved biotech (protein/peptide) drugs, 83 nutraceuticals and 5208 experimental drugs. Additionally, 4439 non-redundant protein (i.e. drug target/enzyme/transporter/carrier) sequences are linked to these drug entries. Each Drug Card entry contains more than 150 data fields with half of the information being devoted to drug/chemical data and the other half devoted to drug target or protein data.

3.11.2. TOOLS USED

Hex is an effective tool for molecular docking among the variety of computational methods. In the present study, bioactive compound act as ligand and Nan A of *S. pneumoniae* act as receptor which made to dock with help of Hex software.
3.11.2.1. Hex (hex.loria.fr/dist50/)

Hex is an interactive protein docking and molecular superposition program, written by Dave Ritchie. Hex understands protein and DNA structures in PDB format, and it can also read small-molecule SDF files. Docking protein-protein and protein-ligand docking. Docking of receptor and ligand were carried out using Hex software (Ritchie and Venkatraman, 2010).