3. MATERIALS AND METHODS

3.1 Collection and transport of specimen

A total of one hundred clinical dental caries infected samples from patients attending the outpatient ward of Government Headquarters Hospital; Erode (Tamilnadu, India) was collected during the period between Sep 2009 and Sep 2010. The source materials used for caries pathogen isolation were the carious dentine removed from the carious tooth during endodontic restorative procedures. The samples were received after obtaining the informed consent from the patients. A proforma containing the demographic details viz. the age, sex, occupation, marital status, risk factors such as drinking, smoking, tobacco chewing, food habits viz., vegetarian/non-vegetarian, economical status and detailed clinical examination were recorded for each study case for further analysis. After the removal of superficial plaque and debris overlying the lesion, the carious zone of decalcified and partially decalcified dentine was washed with sterile saline carious dentine was excavated and used as explained (Smiline et al., 2012). Properly sterilized cotton swabs were also used for the collection of the specimens. The specimen was collected from the anterior and molar teeth of both jaws from patients on the portion of the teeth lesions and caries affected areas. Care was taken to swab only the teeth infected areas. The collected swabs were immediately dipped into the screw-cap tube containing 1ml of 1 X phosphate buffered saline (PBS). Then the screw-cap tubes were wrapped nicely in thermo cool box and transported to the laboratory and processed within 2 h.

3.2 Screening and identification of causative organisms

Samples were dispersed, inoculated onto selective media, after incubation the isolates were identified by colony morphology; Gram’s staining and was characterized biochemically following standard methods (Cappuccino and Sherman, 1996; Monica Chesbrough, 1998; Senthilkumar et al., 2013)

3.2.1 Cultural characteristics of the isolates

Each collected specimens were inoculated immediately into common medium of nutrient broth (Himedia, India) and allowed incubation for 24 h at 37°C. A loopful of culture was taken from the common medium, and quadrant streaking was done on the selective, and differential media’s including Lactic bacteria differential agar, Mitis salivarius bacitracin agar, Yeast nutrient gelatin medium, Mannitol salt agar, Eosin
methylene blue agar and Cetrimide agar for selective isolation of organisms like *Lactobacillus* sp., *Streptococcus* sp., *Kurthia* sp., *Staphylococcus* sp., *Klebsiella* sp., *Escherichia coli*, *Pseudomonas* sp., etc. Further, inoculated culture plates were incubated for 24 h at 37ºC. The colony morphological characteristics and the growth pattern of the bacterial isolates were noted. The bacterial isolates were preserved by routine subculture techniques and subjected to further studies.

3.2.2 Morphological & biochemical characteristics

**Gram’s staining**

This test was performed to find out whether the bacteria are Gram positive or Gram negative. It is the ability of Gram positive bacteria to take up crystal violet (CV) after mordant with iodine (I) to retain crystal violet iodine (CVI) complex. Following the extraction with alcohol, if bacteria loose CVI complex after alcohol treatment, they are Gram negative. Saffranin was used as counter stain. Gram positive will appear as violet color and Gram negative as purple colour. Isolated unknown colonies from the selective media were smeared in a clean glass slide and the Gram’s staining procedure was performed. The test bacterial cultures were smeared on a clean glass slide and heat fixed. The smear was flooded with crystal violet for a min and the stain was rinsed with water. The smear was flooded with Gram’s iodine for a min, rinsed with water, decolorized with 95% alcohol, rinsed out and the smear was counter stained with saffranin for a minute and rinsed with water. The slide was dried and examined under a microscope in oil immersion. Gram-positive bacteria were appeared purple or violet and Gram-negative bacteria were pink in color.

**Motility test**

It determines the motile nature of the bacterial isolates. The 14-18 h old young bacterial culture was placed on a clean cover slip and very small amount of vaseline was applied on each corner of the cover slide. Then the cavity slides were placed on the top of the cover slide and quickly inverted so that the culture hangs inside the cavity. The preparations were observed under the low power of light microscope.

**Catalase test**

This test determines the aerobic nature of the isolate. The test bacterial cultures were placed on a clean glass slide, and 3% of hydrogen peroxide (H₂O₂) solution was added. A positive test is indicated by evolution of bubbles.
**Oxidase test**

Whatman No. 1 filter paper was placed on the growth of test bacterial cultures, and a drop of N-tetra methyl paraphenylenediamine hydrochloride reagent was added. The color development was observed after 10 sec, the appearance of blue color indicates a positive result (production of oxidase enzyme).

**Carbohydrate fermentation test**

The test bacterial cultures were inoculated into sterile phenol red carbohydrate broth with NaCl. The broth tubes were added previously with different carbon sources (glucose, lactose, maltose, sucrose, mannitol and fructose) and inverted Durham’s tube. The tubes observed for the medium color change and accumulation of gas in Durham’s tube after incubation at 37°C for 24 h.

**Indole test**

The test bacterial cultures were inoculated into sterile tryptone broth and incubated at 37°C for 24 h. After incubation, the production of indole was assessed by the addition of Kovac’s reagent and the development of cherry red ring at the top of the medium.

**Methyl red test**

This test was performed to find out the ability of the bacteria to oxidize glucose with the production and stabilization of high concentration of acid end products. The test bacterial cultures were inoculated into sterile MR-VP broth and they were incubated at 37°C for 24 h. After incubation, the methyl red indicator was added. The color change of broth to red indicated the positive result.

**Voges- proskauer test**

The test bacterial cultures were inoculated into sterile MR-VP broth, and they were incubated at 37°C for 24 h. After incubation, Barrit’s reagent A (alpha-naphthol) and B (40% potassium hydroxide) were added, gently mixed, allowed to stand for 15 min. The formation of pink color indicated the positive result.

**Citrate utilization test**

The test bacterial cultures were inoculated into sterile Simmons citrate agar slants and incubated at 37°C for 24 h. After incubation, the color change of the medium from green to blue indicating a positive result was observed.
**Triple sugar iron agar test (TSI)**

It differentiates the isolates on the basis of their dextrose, lactose and sucrose fermentation and hydrogen sulfide production. The test bacterial cultures were both stabbed and streaked on TSI agar slants and incubated at 37°C for 24 h. After incubation, the tubes were observed for acid, gas and H₂S production.

**Nitrate reduction test**

The test bacterial cultures were inoculated into sterile nitrate broth and incubated at 37°C for 24 h. After incubation, nitrate reagents A&B (containing sulphaniline and α-napthalamine) were added, and the development of red color indicating a positive result (the organism’s ability to reduce nitrates to nitrites) was noted.

**Starch hydrolysis**

The test bacterial cultures were streaked on the sterile starch agar medium and incubated at 37°C for 48 h. After incubation, iodine solution was poured onto the growth of organisms in the plates. Development of clear zone around colony area indicates a positive result.

**Casein hydrolysis**

The test bacterial cultures were streaked on the sterile nutrient agar plates supplemented with 0.2g of casein per plates and incubated at 37°C for 24 h. After incubation, a transparent zone observed surrounding colonies indicates a positive result and the obtained results were recorded.

**Urea hydrolysis**

The test bacterial cultures were inoculated into sterile urea broth and incubated at 37°C for 48 h. After incubation, the broth color changes to pink color indicating a positive result were observed.

**Gelatin hydrolysis**

The test bacterial cultures were stabbed into the gelatin agar tubes and incubated at 37°C for 24-48 h. After incubation, the tubes were placed into the refrigerator at 4°C for 15 min. The contents of tubes in a state of liquefaction due to the production of gelatinase indicated positive test, whereas tubes remained in solid demonstrated negative reaction.
3.3 Determination of biofilm forming ability

The biofilm formation, slime and extracellular polysaccharide (EPS) production and their characterization of all the bacterial isolates were evaluated by the following three different methods.

3.3.1 Tube adherence test (Christensen et al. 1982)

A loopful of the bacterial culture (each isolates) from the agar plate was inoculated into sterile glass test tube containing 5ml of trypticase soy broth (TSB) and incubated at 37°C for 48 h. Each tube was decanted, stained with 0.25% of crystal violet, gently rotated to ensure uniform staining, and then the contents were gently decanted. The tubes were then placed upside-down to drain. The color of the inner surfaces of the tubes was observed. An adherent film on the surface of the glass tube was taken as an evidence of slime formation. The absence of a film represented as a negative result (-). Based on slime production, the positive results were recorded as strong (+++), moderate (++), weak (+) (Murugan et al., 2010). Each test was interpreted by two different observers.

3.3.2 Congo red agar (CRA) method (Freeman et al., 1989).

The slime production by the each bacterial isolates was determined by CRA method as described by Freeman et al. (1989). Congo red agar plates were streaked with each bacterial isolates and incubated aerobically for 24 h at 37°C. The appearance of dark-black colonies with a rough, dry, and crystalline consistency was considered as indicative of slime production. Non slime isolates produced pinkish red smooth colonies with a darkening at the centre.

3.3.3 Micro titer plate assay (Pitts et al., 2003; Motegi et al., 2006; Sohaibani and Murugan, 2012)

Micro titer plate assay was performed for the biofilm assay of different isolates as accordingly as described (Pitts et al., 2003; Motegi et al., 2006; Sohaibani and Murugan, 2012) with certain modifications. All the bacterial isolates were individually grown overnight in trypticase soya broth (TSB) at 37°C and diluted (1:40) with the same broth supplemented with 0.25% glucose. All the bacterial strains were assayed for biofilm formation by mixing 40µl of cell suspension (8 x 10^-6 colony-forming units CFU) and 160µl of dextrose-free TSB supplemented with 0.25% sucrose in the wells of sterile 96-well U-bottom polystyrene tissue culture plates (Tarsons, Mumbai, India) that had
previously been coated with whole saliva and incubated for 24 h at 37°C without agitation. After incubation, the culture medium was then decanted, and the plates were gently washed twice with 200µl of sterile phosphate buffer saline (pH 7.2) to remove planktonic and loosely bound cells. The adherent bacteria were stained with 250µl of 0.1% crystal violet for 15 min. After rinsing twice with 250µl of sterile phosphate buffer saline and drying 5 min at room temperature, light absorbance was measured at 490nm using micro-titer reader (model 680, Bio-Rad, Hercules and CA). Uninoculated wells containing TSB with glucose served as blanks. Blank corrected absorbance values of strains were used for assessing biofilm production.

A three grade scale was used to evaluate the slime producing ability of the strains which included 1. Negative ODs < 0.500, 2. Positive ODs 0.500 < 700, 3. Strong 700 < ODs as scored by Alcaraz et al. (2003) with certain modifications. Each strain was tested for biofilm production in duplicate, and the assay was repeated two times.

### 3.3.4 Exopolysaccharide (EPS) analysis

This technique was performed for each confirmatory isolates by the method of Majumdar et al. (1999) with certain modifications. The selected abundant biofilm forming bacterial cultures were grown in a basal salt solution (BSS) having (g/l) glucose 10.0g and trace metal solution (1ml). Carbon and nitrogen sources and the concentrations of phosphate were varied as required. The pH of the medium was adjusted to 7.5 with 1N of NaOH. The medium was sterilized by autoclaving and 24 h old cultures were grown in the same medium at room temperature on a rotary shaker at 100rpm.

After incubation, the bacterial cultures were grown in the BSS medium wherein glucose was replaced by trisodium citrate (5.0 g/l) in conical flask in a rotary shaker (100rpm) at 37°C for 48 h after which the cultures were grown in an incubator for 24 h at 37°C. Cells were removed by centrifugation at 4000rpm for 10min at 4°C, and the pellets were discarded. Then the supernatant (100ml) was precipitated by adding 5ml isopropanol and 150ml cold ethanol and incubating the mixture at 4°C for 3 h. The precipitated (gel like, white colored) EPS was pipetted out, collected in eppendorf tubes then dried at room temperature in a sterile condition. The matrix was analyzed through HPLC Agilent 1200 series.
3.3.4a HPLC - instruments details (SOP / R / 11.)

Model no : 1200 series  
Make : Agilent  
Detector : Refractive index  
Refractive index range : 1.00-1.75 RIU  
Measurement range : +/- 600x 10-6 RIU  
Column used : Meta-Crap 87C  
Column length : 300mm  
Column ID : 7.8mm  
Packaging materials : 7.8 mm x 30 cm bed packed with cation – exchange resin in the Ca++ ionic form  
Flow rate : 0.6 ml / min  
Operating temperature range : 10 – 80°C  
Mobile phase : De – ionized water  
Sample cell volume : 8µl  
Pump type : Quart pump  
Pump control : Multi Channel Gradient Valve  
Operating pump pressure : 0 - 400 bar  
Line filter : 20 µm

3.3.4b Estimation of Exopolysaccharide (Aljane et al., 2007)

The standard sugars were used at the concentration of 25mg/50ml with 500ppm. The isolated EPS was weighted and diluted in 200 µl of distilled water. From this 50 µl sample was injected into the system. The flow rate was adjusted to 0.6ml / min and 80°C was maintained. Sugars were identified based on the peak value. Concentration of sugar was calculated by the following formula.

\[
\text{Concentration of sugar} = \frac{\text{Amount of sugar in 200ml of sample}}{\text{Weight of the sample}}.
\]

3.3.5 Epi-fluorescence microscope biofilm study (Dhandapani et al., 2012)

A loopful of the strain (each isolates) from the stock culture was inoculated into sterile glass test tube containing 5ml of trypticase soya broth. The test tube contains half immersed sterile SS plate (Stainless steel - 316L grade with 0.2 mm thickness) and
balanced with the help of threads. This plate is commonly used for tooth filling purpose in commercial. The tubes were incubated at 37°C for 48 h with 100rpm agitation. After the incubation, the biofilm grown SS plates was carefully taken out from the test tube and gently rinsed with sterile 0.1M phosphate buffer (pH 7.2) to remove unattached platonic bacterial species. Each plate was stained with fluorescein isothiocyanate (FITC; Hi-Media, India). FITC can stain both the live cells and biofilm. It can penetrate into the cell membrane and illumination of green fluorescence indicated the presence of viable bacterial cells and yellow patches. The biofilm was studied under the epi-fluorescence microscope (E200 Coolpix -Nikon, Tokyo, Japan). Each test was interpreted by two different observers.

3.4 Kirby-Bauer antibiotic sensitivity test (CLSI, 2006)

The antibiotic resistance or sensitivity profile of the isolates against commonly used antibiotics to treat or prevent infections was assessed in vitro by the disc diffusion method according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2006). Inoculums for each confirmatory isolates was prepared and swabbed onto Mueller Hinton agar plates (HiMedia, Mumbai, India). The commercial antibiotic discs (HiMedia, Mumbai, India) such as amoxicillin (10mcg), ampicillin (10mcg), erythromycin (15mcg), gentamicin (10mcg), methicillin (5mcg), nalidixic acid (30mcg), penicillin G (10 units), rifampicin (5mcg), streptomycin (10mcg), tetracycline (30mcg) was placed in petridish maintaining equal distance. Following incubation at 37°C for 18-24 h, the inhibition zone of bacterial growth around the disc was analyzed and compared with standard chart.

3.5 Ethno-botanical studies

The documentation of the available traditional knowledge with the Malayalies ethnic group and their ethno medicinal practices for oral health and other diseases who are living in Kolli hills, Namakkal District, Tamilnadu, and Southern India.

3.5.1 Description of the study area

Tamilnadu is situated in the Southern end of India, east of Kerala and south of Andhra Pradesh and Karnataka states. Namakkal district is located in Tamilnadu, in between the geocoordinates 11° 00’ 00” and 11° .34’ 8” N and longitude 77° 40’ 15” and 78° 29’ 30” E. It is bounded by Salem on the North, Karur in the South, in the East by Tiruchirappalli and in the West by Erode district. The study undertaken area Kolli hills
(Kollimalai in Tamil), Namakkal district is an ideal religious place famous for diverse flora of ethno-medicinal significance. It is located in the extreme eastern part of Namakkal district (Fig 3.1). From Namakkal to Kolli hills the distance is 55 km. The area falls within the latitudes 11°55′05″ to 11°21′10″N and 78°17′05″ to 78°27′45″ E. Minimum and maximum temperature ranges from 10°C to 20°C and 20°C to 30°C respectively (Kumaran et al., 1998). It stretches 29 km from north to south and 19 km from east to west and is bounded on the north by Namagiripet block, east by Uppiliapuram block (Trichirapalli District), West by Senthamangalam and Erumaipatty blocks and South by Erumaipatty block. Various villages of Kolli hills include Nachiyarkovil, Nattukulpatti, Solakkadu, Semedu, Kuzhivalavu shoal, Arapallieswararkovil and Sengari shoal. The Semmedu is head of Kolli hills is located about 60 km away from the district headquarters. There are 14 village panchayats (Alathurnadu, Ariyurnadu, Bailnadu, Cithoornadu, Devanurnadu, Edapulinadu, Gundurnadu, Kunduninadu, Perakarinadu, Selurnadu, Thinnanurnadu, Thirupulinadu, Valappurnadu, and Valavanthinadu) and 275 hamlets and 70 hair-pin dead ends top to bottom. The total geographical area accounts for 36,397.06 ha (Harikrishnan, 1977).

3.5.2 Malayali people

Since ancient times, Kolli hill is known for medicinal plants growing there and is believed that the Sithars (ancient medicine and meditation men) were lived in the hill, and they used to meditate and practice on various medicinal plants for many ailments, as well as the regular health care of human beings. Moreover, there are a number of sacred groves in Kolli hills. Stories abound of people chancing upon the stone mortars used by the sithars to prepare their medicines and concoctions. Kolli hills constitute about 68.34% of the total tribal population in the district. The population predominantly consists of scheduled tribes called Hindu Malayali whose spoken language is only Tamil, which constitutes about 98.8% (Thurston, 1907). The resident Malayalis (literally meaning people of the hills) are a friendly, sturdy and hardworking people, who generally keep to themselves. Many workers were reported the worthiness of plants for the treatment of several diseases of Malayali tribal community (Franchis Xavier et al., 2011; Kuru Suresh et al., 2011; Rajendran and Manian, 2011).
Fig. 3.1 Regional map showing the location of Kolli hills (Namakkal, Tamilnadu, India).
3.5.3 Sacred groves of Kolli hill as medicinal plants

The strong linkages of the Malaiyalis with forest and trees can be seen in the context of Samisolai or sacred groves. Certain beliefs and faiths have helped to protect particular patches of land with good vegetation cover. It is a general feature in Kolli hills that one finds a group of villages worshipping a particular deity who usually is found in the interior of the forests or on the periphery of it. Some of these areas fall under reserve forests. Rituals on a big scale are conducted periodically to these gods. The groves very often harbour remnant of the past vegetation of landscape. Some of the forest patches are considered as the abode of four or five goddesses and gods which are housed amidst trees like Syzigium cumini, Ficus racemosa, F. bengalensis, Artocarpus integrifolia, Mangifera indica, Michelia campaca, Persia micrantha, Meliadubia, Terminalia chebula, T. bellerica, and several other medicinal flora.

3.5.4 Ethnobotanical survey

Field visits were undertaken between January 2011 and March 2011. This study was conducted among the Malayali tribal practitioners living in the interior area such as Semmedu, Keel Solakadu, Vasular patty, Villaram, Parriyur, Arikkal, Valavanthinadu, Valluppurnadu, Arriyurnadu and around Arrappulliseswerkovil (People like family heads, healers and old experienced knowledgeable informants). Information was collected about the medicinal plants used in oral health care, the method of medicinal preparation and its administration (i.e., chewing sticks, powder, paste, decoction and juice) form of usage either fresh or dried and method of application. The other ethnobotanical data regarding the medicinal plants which have been used traditionally for health, hygiene and other ailments were gathered from them, through personal interviews according to the methodology suggested (Jain, 1963; 1964; 1967; 1989).

3.5.5 Interviews with tribal practitioners

The results presented in this study, are the outcome of a series of interviews conducted among traditional healers who were trained by traditional ways, but not through government run institutions, were identified by a preliminary survey conducted by us with the help of Dharma Herbal Research Foundation & Herbal Gardens Ariyurnadu in Kolli hills. The first two to three visits were used to establish a good contact with the healers. The purpose and nature of the investigation were described to
them in a simple language, to get prior informant consent. By this way, 13 traditional healers who were willing to share their knowledge were selected for further interviews. A total of 13 (between the ages of 37 to 68) informants (2 ritualists, 3 traditional healers, 4 folk practitioners and 4 elderly people) were interviewed for the purpose of documentation of various herbal plants used in oral health.

The ethnomedicinal investigation was done systematically using a specific questionnaire (Data sheet, Appendix). Interview was carried out in local language (Tamil). One questionnaire was used for recording single use of a single species by a single respondent. The plant specimens were collected with the help of traditional healers and medicine men by knowing their local names as per suggestion. In addition, general details such as routine teeth cleaning, tooth decay, gum diseases, oral mucosal diseases and other medicinal uses were also collected. The plants documented are arranged alphabetically of their scientific names along with family followed by local name, parts used, mode of preparation and administration. Moreover, voucher specimens were prepared and identified with the help of suitable literature Dhar et al. (1968) and Nadkarni (1976). The collected plants were photographed; its voucher specimens in duplicates were submitted to herbarium, Entomology research institute, Loyola College, Chennai and were identified by the taxonomist Dr. P. Pandikumar, Scientist of the same institute.

3.5.6 Plant extracts preparation

The collected plant’s bioactive compounds were extracted through sequential extraction by following the cold percolation method as explained (Saxena and Yadav, 1983) for determining their anticaries activity. Different parts of the ethnobotanical survey identified 15 medicinal plants were collected from Kolli hills. The collected plant material was cut into small pieces, and shade dried at room temperature. The dried plant material was coarsely powdered in a hammer mill. About 200 grams of each plant powder was extracted (soaked) twice with hexane (1:3, w/v) in a cold (48 h). The extract was filtered through Whatman No. 1 filter paper under suction. The filtrate was evaporated to dryness under reduced pressure using rotary evaporator at 60°C. The mark left after extraction with hexane was successively extracted (soaked) with chloroform, ethyl acetate
and methanol in the cold (48 h) in a similar manner using cold percolation method. The extracts were preserved in air tight containers and stored at 5°C until further use.

3.6 Anticaries activity screening

Hexane, chloroform, ethyl acetate and methanol extracts (successive) of the above 15 plants were screened their anticaries activity against a total of five caries forming bacterial isolates viz. *Lactobacillus casei, Streptococcus mutans, Kurthia gibsonii, Staphylococcus aureus* and *Klebsiella pneumoniae*.

3.6.1. Inoculum preparation

Active cultures were prepared by transferring a loopful of isolated caries forming bacterial strains from the stock cultures maintained at 4°C to test tubes containing Mueller-Hinton broth (MHB). They were incubated with agitation for 18 h at 37°C.

3.6.2 Anticaries susceptibility test (Bauer et al., 1966; Murray et al., 1995)

*In vitro* anticaries activity of the plant extract was screened by disc diffusion assay using Mueller Hinton agar (MHA). The MHA plates were prepared by pouring 20 ml of media into sterile petriplates. The plates were allowed to solidify for 10 min. The test cultures were swabbed uniformly on the top of the solidified media and allowed to dry for 10 min. Sterile discs (Hi-media, Mumbai, India) of 6mm diameter were loaded with 10mg/disc of each extract. The loaded disc was placed on the surface of the medium and allowed for 5 min for the compound to diffuse and incubated at 37°C for 24 h. Respective vehicles and nalidixic acid (30 µg/disc) were used as negative and positive controls respectively. The crude extracts were dissolved in DMSO (Dimethyl sulphoxide) and were used as the vehicle for hexane, chloroform, ethyl acetate and methanol extracts. At the end of incubation, inhibition zones formed around the disc were measured with transparent ruler in millimeter and the studies were repeated twice.

Based on the experimental results five plants (leaves of *L. innermis, M. elengi, P. gaujava* and twigs (sticks) of *A. indica A* and *T. purpurea*) were short listed for further study. The anticaries activities were determined at a concentration level of 5 mg/disc. The hexane and chloroform extracts were found less active when compared to others, so these two extracts were short out of the next stage.
3.6.3 Bulk extraction of *Mimusops elengi* L. (Saxena and Yadav, 1983).

The plant material (*M. elengi* leaves) was collected from Kolli hills, the identity of the plant and its voucher specimen was certified and authenticated by the Scientist, Botanical Survey of India, Ministry of environment and forests, Government of India, Coimbatore (Certificate No. BSI/SRC/5/23/2012-13/Tech.1478, Appendix)

The 6 kg of fresh leaves were carefully selected excluding young, old, insect-damaged and fungus infected leaves. The collected plant leaves were washed with distilled water twice and were cut into small pieces and spread out for drying in shade. The material was coarsely powdered in a hammer mill from which 2kg of the dried material was finally obtained.

The above obtained 2 kg leaf powder was taken in an aspirator bottle, and cold extracted (soaked) twice with chloroform for 48 h with intermittent shaking. Since chloroform extract and also hexane extract did not show activity during preliminary screening, so only the chloroform extraction was carried out. Hexane soluble material would also be removed while extracting with chloroform. The extract was filtered through a vacuum sucker (Buchner funnel) with Whatman No. 1 filter paper. The filtrate was evaporated to dryness under reduced pressure using rotary evaporator at 60°C. The mark (remains) left behind was extracted with ethyl acetate (sequentially) in a similar manner using cold percolation method and the ethyl acetate extractions were performed thrice which completely removes ethyl acetate soluble compounds. The obtained 65g dried extract yield was stored in a dry and clean glass container at 5°C for further studies.

3.6.3a Preparation of admixture

The 65g of active crude ethyl acetate extract was dissolved in hexane in a china dish and warmed with a help of water bath. To this enough amount of (acme’s 100–200 mesh size) silica gel was added and the same was made into a slurry form. This mixture was air dried at room temperature and then in a vacuum in desiccators. The finally obtained slurry was a light green fine powder.

3.6.3b Column chromatography (CC)

Chromatography, the new method of separation for a mixture of substances when they are available in small amounts, have almost the same physical and chemical
properties and hence cannot be separated by other usual methods of separations is used here to separate the phytochemicals.

A chromatography column (60×3cm) fitted with a teflon stopper (2mm bore) was packed with silica gel (Acme’s 100–200 mesh) in low polar solvent, hexane and the admixture was added to the column. Column was initially eluted with hexane, followed by the combination of hexane in order of gradual increase (hexane, chloroform, ethyl acetate) in the polarity mixtures of solvents (Table 3.1). Finally, the column was eluted by adding methanol which elutes the compounds that could not move with the other low polar solvents. Every 150ml eluent were collected in dry and clean conical flasks, and the active fractions needed further purification if any, were subjected to sub-chromatogram. The *M. elengi* compound isolation sequence followed was also depicted (Fig 3.2).

**Table 3.1 Stepwise elution of compounds from ethyl acetate extracts using column chromatography.**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Elutent</th>
<th>Fraction no (collections)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexane</td>
<td>100%</td>
</tr>
<tr>
<td>2</td>
<td>Hex: CHCl₃</td>
<td>80:20</td>
</tr>
<tr>
<td>3</td>
<td>Hex: CHCl₃</td>
<td>60:40</td>
</tr>
<tr>
<td>4</td>
<td>Hex: CHCl₃</td>
<td>40:60</td>
</tr>
<tr>
<td>5</td>
<td>Hex: CHCl₃</td>
<td>80:20</td>
</tr>
<tr>
<td>6</td>
<td>Chloroform</td>
<td>100%</td>
</tr>
<tr>
<td>7</td>
<td>CHCl₃: EtOAc</td>
<td>80:20</td>
</tr>
<tr>
<td>8</td>
<td>CHCl₃: EtOAc</td>
<td>60:40</td>
</tr>
<tr>
<td>9</td>
<td>CHCl₃: EtOAc</td>
<td>40:60</td>
</tr>
<tr>
<td>10</td>
<td>CHCl₃: EtOAc</td>
<td>20:80</td>
</tr>
<tr>
<td>11</td>
<td>Ethyl acetate</td>
<td>100%</td>
</tr>
<tr>
<td>12</td>
<td>EtOAc: Methanol</td>
<td>90:10</td>
</tr>
<tr>
<td>13</td>
<td>Methanol</td>
<td>100%</td>
</tr>
</tbody>
</table>

**3.6.3.3 Thin layer chromatography** (TLC) (Marston *et al.*, 1997)

Simple, quick, and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture, the TLC is used for ascertaining the
compound purity. 0.25mm silica gel 60 F/254, percolated aluminum plates (Merck) were used. The TLC plates after development with suitable solvents system were dipped in vanillin sulphuric acid reagent, heated at 105-110°C until the clear appearance of spots. The phenolic compounds were identified by visualization with alcoholic ferric chloride solution and by observing the color developments after iodine vapor exposure.

(Retention factor or Rf = distance travelled by the compound /distance travelled by the solvent front).

3.7 Instrumentation studies

(Fleming and Williams, 1967; Williamkemp, 1987; Beckett and Stenlake, 1988; Sharma, 1999; Silverstein et al., 2005)

All melting points were uncorrected and were determined by open capillary method on a heating block apparatus. UV – VIS spectra were recorded in the range 200-500 nm on Thermo Fisher grading spectrophotometer using spectroscopic grade methanol. FT-IR spectra were recorded on a Shimadzu FT-IR grating spectrophotometer in KBr disc in a range, 4000-500 cm⁻¹. ¹H-NMR and ¹³C-NMR spectra were recorded on Bruker instrument, (400 and 100 MHz respectively), FT-NMR spectrometer and recorded in CDCl₃ or DMSO-d6 were used as the solvents, and TMS was used as the internal standard. Chemical shifts values are given in δ scale. The GC-MS spectra were taken on Agilent instrument, and the values were compared with NIST-11 Library. Optical rotations were measured using a Jasco digital polarimeter in chloroform. All the spectral methods Viz. IR, UV, NMR and GC-MS were used in the quantification analysis.

3.7.1 Fourier Transform Infra-Red Spectroscopy. (FT-IR)

The IR region of the spectrum used in organic chemistry is 4000-500cm⁻¹ this far infra-red region. Absorption in infrared region corresponds to vibration and corresponding rotational energies in the molecule. The vibration modes include sym-stretching, unsym-stretching and sketatal vibrations. Sketatal vibrations include bending, rocking, wagging etc. The Infra red spectrum is useful in detecting functional groups with characteristics vibrational frequencies. e.g. the hydroxyl groups in a compound absorbs at 3600-3200 cm⁻¹ due to OH stretching. The carbonyl group of ketones would give a strong
band at 1710cm⁻¹ due to stretching of the CO band. COOMe would give a band around 1720 cm⁻¹ due to C=O stretching and a band around 1230 cm⁻¹ due to COC bending. Structural changes like α, β unsaturation hydrogen bonding, etc, modify the absorption frequencies. Hydrogen bonding α, β unsaturation cause decrease in absorption frequencies.

3.7.2 Ultra-Violet and Visible Spectroscopy. (UV-Vis)

The technique of UV-Vis spectrophotometry is one of the most frequently employed methods it involves the measurement of the amount of ultraviolet (190-380) or visible (380-800 nm) radiation absorbed by a substance in solution. Absorption of light in both the ultra-violet and visible regions of the electromagnetic spectrum occurs when the energy of the light matches that required to induce (an electronic transition) in the molecule and its associated rotational and vibrational transitions. The various electronic energy levels involved are σ (bonding), π (bonding), n (nonbonding), π *(anticoding), and σ *(anticoding), in the order of increasing energy. The energy required for various transitions obey the following order:

\[ \Sigma \rightarrow \sigma^* > n \rightarrow \sigma^* > \pi - \pi^* > n \rightarrow \pi^*. \]

UV-Vis spectroscopy is useful particularly in the study of conjugated systems e.g. diene, dienones, aromatic systems, enols etc. The chromophore is defined as any isolated covalently bonded group that shows a characteristic absorption in the Ultra-Violet or the visible region. e.g. ethylenic, acetylenie, carbonyls, acids, esters, nitrile group etc.

An auxochrome can be defined as a group which in combination with the chromophore leads to a pathochromic shift. Auxochromic groups do not show characteristic absorption above 200nm. E.g. –OH, -OR, -NH₂, -NHR, -NR₂, -SH etc. UV-Vis spectra are used in natural product chemistry in the structural elucidation of compounds like flavonoids, xanthones, coumarins, alkaloïdes, and steroids etc containing conjugated systems.

Use of shift reagents in the structural elucidation of flavonoids compounds. Flavones generally show two absorption bonds corresponding to the benzoyl and cinnamoyl moieties. Bond I of longer wave length (320-380nm) represents the cinnamoyl moiety, while bond II of shorter wavelength (240-270nm) represents the benzoyl moiety.
Flavones usually contain phenolic hydroxyl groups at C-3, C-5, C-7, C-3\(^1\) and C-4\(^1\) and C-5\(^1\) addition of sodium acetate causes shift of band II by about 8 to 20 nm if 7-OH is present. Addition of boric acid causes a further shift of about 15-30 nm if ortho-dihydroxy system is present. Addition of aluminum chloride helps in the detection of the chelated hydroxyl groups at C-3 and C-5. These can be differentiated by addition of one or two drops of concentrated hydro-choleric acid. Addition of aluminum chloride causes a bathochromic shift of about 60 nm band I if both 5-OH and 3 are present. A shift of about 45 nm of band I with aluminum chloride with hydrocholeric acid (AlCl\(_3\)+Hcl) indicates 3 glycosylation or absence of 3-OH. Substitution at C-6 either hydroxyl or Alkali group will cause little or no shift on addition of AlCl\(_3\). Similarly this substitution hinders shift with sodium acetate. Addition of sodium methoxide causes a shift of band I by about 50-60 nm without decrease in intensity if 4\(^1\)-OH is present. Alkaline sensitivity of compounds having 5, 3, 3\(^1\), 4\(^1\), 5\(^1\) hydroxyl groups in succession causes degradation and decrease in intensity of the longer wave length band. (Leonard Jurd and Geissman, 1962).

3.7.3 Fourier Transform Nuclear Magnetic Resonance Spectroscopy (FT-NMR)

The nuclei’s of an atom as positive charge which is a multiple of the charge of hydrogen nucleus may be compared to a spring magnetic and thus has a angular momentum characterized by spin quantum number I.(I=0,\(\frac{1}{2}\),1,\(\frac{3}{2}\),…etc). Magnetic moment \(\mu\) is given by \(\mu = \gamma \cdot p = \gamma \cdot (h \cdot I / 2\pi)\).

(P is the spin of the nucleus; \(\gamma\) is a constant describing the type of the nucleus (gyro magnetic ratio) \(h\) is Planck’s constant).

The nuclei with even mass and atomic number such as \(^{12}\)C, \(^{16}\)O and \(^{32}\)S have no magnetic moment and so do not produce NMR signals. Other nuclei having (I\(\neq\)O) when placed an external uniform magnetic field can have 2I +1 discrete orient nous levels. The nuclei which are most common in organic chemistry as \(^1\)H, \(^{13}\)C, \(^{15}\)N, \(^{19}\)F, \(^{31}\)P etc, have spin I = \(\frac{1}{2}\) . In an external magnetic field (Ho). Two orientations are possible - parallel. (m= \(\frac{1}{2}\)) and antiparallel (m=\(-\frac{1}{2}\)). m= magnetic quantum number. The distance between the two levels.

\(\Delta E\) corresponds to the radio frequency reign by \(\gamma = V / 2\pi \cdot X \cdot Ho\)
Different protons in molecules will see the applied magnetic field in different amounts depending upon the shielding or deshielding due to chemical environment. The chemical shift $\delta$ is given in ppm, with a reference standard TMS (Tetra Methyl Silane) which is given $\delta = 0$. Since parallel and antiparallel spins state are involved splitting of NMR signals takes place and characteristic splitting constants ($J$ in Hz) have been evolved. The magnitude of the splitting constant mainly depends upon the dihedral angle. The values of chemical shift for $\delta$ varies mostly from 0-10 for protons ($^1H$) and 0-220 for $^{13}C$, chemical shift are also quoted in $\tau$ scale. ($\tau = \text{tou}$). $\tau = 10 - \delta$

Both $^1H$ and $^{13}C$ NMR are used in the structural elucidation of natural products, the field strength for $^{13}C$ NMR is usually $\frac{1}{4}$ of the field strength for $^1H$ NMR in the same instruments. Thus when $^1H$ NMR is taken at 400 MHz, the field strength for $^{13}C$ NMR in the same instrument is 100 MHz’s.

3.7.4 Gas chromatographic - Mass spectrometry (GC-MS)

Agilent GC-MS – 5975C fitted with a DB - 5 MS Agilent ultra inert 30.0m x 0.25mm, film thickness 0.25 µm capillary GC column. Helium was the carrier gas at a flow rate of 1.51 ml/min. The injector port temperature was 240°C, the detector temperature was 240°C and the oven temperature was maintained at 70°C. Oven Temperature Program: 70-300°C; hold time 2 min. One microliter of the sample was injected by Split injection mode.

**MS condition**

Agilent mass spectrometer with dual FID, a split ratio 1:30. Ion source temp: 200°C, Interface temp: 240°C, Scan range: 40 – 1000 m/z, Solvent cut time: 5mins; MS start time: 5(min), MS end time: 35 (min), Ionization: EI (-70ev), Scan speed: 2000, the ionization voltage was 70 eV. MS Library: NIST- 11.

3.7.5 Spectroscopic structural interpretation studies

The obtained compounds structures were interpreted by consulting with Dr. K. Balakrishna, Senior Chemist (Retd. Captain Srinivasa Murthi Drug Research Institute of Ayurdha, Arumbakam Chennai, India). The structure of the compounds was derived and the drawn structures were given.
Mimusops elengi (leaves)

↓

Ethyl acetate extracts (65g)

↓

Column chromatography (100-200 mesh)

↓

142 collections

↓

Combined based on TLC

↓

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

↓

Active fractions

↓

Re-chromatographed

↓

Washed with methanol washed with Chloroform: Ethyl acetate (25:75)

↓

Single spot on TLC

↓

Subjected to IR, UV, NMR, and GC-MS

↓

Compound 1

(Chondrillasterol)

Compound 2

(Myrecitin-3-0-α-L-Rhamnopyronoside)

Fig. 3. 2 Flow chart detailing the sequence of compound isolation from M. elengi extract
3.8 Anticaries and antibiofilm activity of the isolated phytoconstituents

3.8.1 Determination of minimum inhibitory concentration (MIC) (CLSI, 2006)

Anticaries activity and MIC value of the two isolated compounds (chondrillasterol and myrecitin-3-O-α-L-rhamnopyronoside) were determined against oral bacteria by micro dilution technique using Clinical and Laboratory Standards Institute guidelines method (CLSI, 2006). The required concentrations of the compounds were dissolved in 2% DMSO aqueous solution. The initial concentration (800µg/ml) of the compounds were serially diluted in two-fold and were prepared in a volume of 100µl per well, in 96 well micro titer plates. The concentration of compounds were in a range of 800µg/ml, 400, 200, 100, 50, 25, 12.5, and 6.25µg/ml. Mueller Hinton broth with 20% DMSO as solvent control, broth without antibacterial agent was used as a negative control and chlorhexidine (25µl/ml) as a positive control. Five caries isolates used in this study were grown to Mueller Hinton broth at stationary phase for 18 h at 37°C. After addition of 100µl of bacterial cell suspension (1x 10^8 CFU/ml bacteria) into each well, the plate was vibrated slightly for 1min and then the plates were incubated 24 h at 37°C. After incubation, 5µl of each tested organism (from the wells) were placed on the sterile MHA plates and incubated at respective temperature. The MIC for each bacterium was determined as the lowest concentration of the compound inhibiting the visual growth of the test culture on the agar plate. The experiment was repeated twice.

3.8.2 Biofilm inhibition assay

3.8.2a Modified gradient plate technique (Murugan et al., 2013)

The action of the *M. elengi*, leafs isolated compounds (chondrillasterol and myrecitin-3-O-α-L-rhamnopyronoside) on caries bacterial exopolysaccharides (EPS) production was determined by a modified gradient plate technique as described (Murugan et al., 2013). Continuous gradient of plant extract on the CRA medium was prepared as follows: Initially, an arrow was drawn in the center of the plate indicating concentration gradient, placed on pencil at an angle; 10ml of the molten CRA was poured and allowed to harden. Plate was then placed flat, and 10ml of the plant compounds (25mg/50ml) incorporated CRA was again poured and allowed to solidify. The selected biofilm forming isolates were inoculated into the center of the plate as a single streak. The selected biofilm forming isolates were zigzag streaked by crossing each time the original streak and observed for the color of the colonies along the line of streak after incubation.
3.8.2b Microtitre plate analysis of anti-biofilm activity (Pitts et al., 2003)

Antibiofilm activity of the isolated compounds, chondrilasterol and myricetin 3 rhamnoside on biofilm forming caries isolates was determined by microtiter plate analysis (Pitts et al., 2003). 10ml of 18 h old each isolated strains was added with 90ml of freshly prepared trypticase soy broth and mixed thoroughly. From this 200µl of each strain was transferred to every well and incubated at 37°C for 48 h. At regular intervals of eight hours, utilized broth was replaced with freshly prepared one. After incubation, wells were emptied, and 200µl of fresh medium were added that contains the respective compounds. Respective concentration of the plant compounds chondrilasterol (L. casei, S. mutans, S. aureus (25µg/ml); K. gibsonii and K. pneumoniae (50µg/ml) and myricetin 3 rhamnoside (L. casei and S. mutans, 12.5µg/ml; K. gibsonii, S. aureus, K. pneumoniae 25µg/ml) were added according to their already determined susceptibility values. Equal amount of trypticase soy broth and chlorhexidine at a concentration of 25µl/ml were added as negative and positive controls respectively. Treated wells were incubated for 24 h and were stained with 0.1% crystal violet stain for 5 min at room temperature. By rinsing all the wells twice with sterile distilled water, the spent media, plant extracts, and free floating cells were removed. The absorbance of the wells was determined at 490nm using a micro plate reader (model 680, Bio-Rad, Hercules and CA). The OD values were used to calculate the standard deviation and coefficient of variation to determine the stability of the organism against antibiofilm activity of chondrilasterol and myricetin 3 rhamnoside, and the percentage of inhibitions were calculated (Sandasi et al., 2010; Sohaibani and Murugan, 2012).

3.8.2c Epi-fluorescence microscopic determination of anti-biofilm activity (Teixeira et al., 2006; Dhandapani et al., 2012)

The antibiofilm activity of isolated compounds chondrilasterol and myricetin 3 rhamnoside on biofilm forming caries isolates cultures was determined by epi-fluorescence microscope following the methods of Teixeira et al. (2006) and Dhandapani et al. (2012) with some modifications. Briefly, 1ml of 18 h old cultures of each isolates were added with 9ml of freshly prepared trypticase soy broth and mixed thoroughly. From this 200µl of each culture was transferred to 96-well microtitre plate. Sterile SS coupons (Stainless steel - 316L grade with 0.2 mm thickness) were placed and incubated static condition at 37°C for 48 h. After incubation, wells were emptied and 200µl of fresh
medium preparation was containing respective compounds were added. Varying concentration of chondrilasterol (L. casei, S. mutans, S. aureus (25µg/ml); K. gibsonii and K. pneumoniae (50µg/ml) and myricetin 3 rhamnoside (L. casei and S. mutans, 12.5µg/ml; K. gibsonii, S. aureus, K. pneumoniae 25µg/ml) were tested for the antibiofilm activity. Equal amount of trypticase soy broth and chlorhexidine (25µl/ml) were used as negative and positive controls respectively. Treated wells were incubated for 24 h. After 24 h the SS coupons were taken out of the wells and rinsed carefully with sterile distilled water two times to remove the spent media, plant compounds, and the free floating cells. Then it was stained with the dual stains of fluorescein isothiocyanate (FITC; Hi-Media, India) and propidium iodide (PI; Hi-Media, India) in ratio (FITC: PI; 1:1). The adherence of bacteria was observed by epi-fluorescence microscope (E200 Coolpix -Nikon, Tokyo, Japan).

3.9 In silico molecular interaction study

The three-dimensional crystal structures Gram-positive infectious bacteria (Streptococcus pneumoniae) aspartate-beta-semialdehyde dehydrogenase (Pubmed ID: 2GYY), Gram-positive bacteria Rap phosphatase quorum-sensing receptors (Pubmed ID: 4I1A), Streptococcus mutans glycosyltransferase (Pubmed ID: 3AIC), glycosyltransferases involved in LPS biosynthesis from family GT4 (Pubmed ID: 2IW1), Streptococcus mutans cell surface-localized salivary component and extracellular matrix molecule adhering antigen I/II (AgI/II) (Pubmed ID: 3QE5), Enterococcus faecalis peptide sex pheromone receptor (Pubmed ID: 2AXU), signal transduction protein TRAP (Pubmed ID: 4AE5) and human membrane-associated Torpedo acetylcholine (ACh) receptor (Pubmed ID: 2BG9) were obtained from protein data bank and used for in silico molecular docking studies. AutoDock 4.0 was used for performing docking simulation. Kollman united atom charges and polar hydrogens were added to the protein PDB using Autodock tools. All rotatable bonds in the ligands were kept as free to allow for flexible docking (Sohaibani and Murugan, 2012). Best conformers were searched using Lamarckian genetic algorithm and 10o independent docking runs were carried out for each ligand. Molinspiration (Ertl et al., 2000) was used to calculate the molecular descriptors and Lipinski’s rule was used to calculate the number of violations for all analyzed ligands (Ertl, 2012).