3.4 Introduction

The primary etiological agent of human dental caries, *Streptococcus mutans*, lives in biofilms on the tooth surface. It has the ability to utilize a wide variety of sugars results in the production of acids which eventually dissolves the hard, crystalline structure of the teeth, resulting in carious lesions (Quivey *et al*., 2001; Burne, 1998). The critical factors responsible for the cariogenicity of this pathogen include its ability to adhere to and form biofilms on tooth surfaces, to catabolise carbohydrates and generate acids, and to survive low pH and other environmental stresses (Burne, 1998). *S. mutans* produces three glucosyltransferases (Gtf-B, -C, and -D) which are of central importance in dental plaque formation and development of caries (Ooshima *et al*., 2001, Tsumori and Kuramitsu, 1997). These Gtfs synthesize adhesive extracellular glucans from sucrose, especially the α (1, 3)-linked, water-insoluble forms, facilitate adherence of *S. mutans* to the tooth surface and modulate cell-cell interaction by serving as binding sites for Gtf proteins and glucan binding proteins which are a group of proteins that contribute to sucrose-dependent adherence and biofilm cohesiveness (Matsumura *et al*., 2003; Hazlett *et al*., 1999, Hazlett *et al*., 1998). As a major constituent of the biofilm matrix, glucans can further influence the structure of oral biofilms by serving as an extracellular energy source thereby modulating permeability to water and nutrients (Sutherland, 2001).

The quest for plants with medicinal properties continues to receive attention as scientists survey plants for a complete range of biological activities, which range from antibiotics to antitumor. Natural products from some plants continue to be used in pharmaceutical preparations either as pure compounds or as extracts. In light of the emergence of microbes which are resistant to multiple antimicrobial drugs posing a
challenge for the treatment of infections (Service, 1995), there is an urgency to discover non-toxic drug candidates against wide range of infections. Therefore, medicinal plants are the most potential source of antimicrobial agents (Cordell, 2000). The active constituents can be derived from different parts of the plant (Gordon and David, 2001).

*Trachyspermum ammi* (Ajowan), from the Apiaceae family, is an important commercial product for the food/flavoursing industry, and they accumulate up to 5% essential oil in compartments referred to as canals (Bhargava and Haksar, 1959). The earlier reports on the antimicrobial and antioxidant properties have been attributed to the extracts of ajowan seeds (Gersbach *et al*., 2001; Srivastava *et al*., 1999; Mehta *et al*., 1994). It is also reported to exhibit bronchodilatory effect on asthmatic airways and analgesic effect (Boskabady *et al*., 2007; Dashti-Rahmatabadi *et al*., 2007).

*Prosopis spicigera* (family: Leguminosae) is prickly tree or shrub and commonly found in dry and arid regions of north-western India, southern India, Pakistan, Afghanistan, Iran and Arabia (Kirtikar and Basu, 1984). Leaves and pods are extensively used as fodder for cattle, camels and goats. *Prosopis* species have also been extensively used in indigenous system of medicine as folk remedy for various ailments (Kirtikar and Basu, 1984; Duke 1985) like leprosy, dysentery, bronchitis, asthma, leucoderma, piles, muscular tremors and wandering of the mind. It is also known to possess anthelmintic, antibacterial, antifungal, antiviral, anticancer and several other pharmacological properties. Leaf paste of *P. spicigera* is applied on boils and blisters, including mouth ulcers in livestock and leaf infusion on open sores on the skin (Usmanghani *et al*., 1974; Chopra *et al*., 1958; Chopra *et al*., 1956; Nadkarni 1954). The smoke of the leaves is considered good for eye troubles. Earlier
studies have reported different phytochemicals in the leaves of *P. spicigera*, namely spicigerine; steroids namely campesterol, cholesterol, sitosterol, stigmasterol; alcohols namely octacosanol and triacontan-1-ol; and alkane hentriacontane (Jewers *et al*., 1976).

However, there are hardly any reports on the anticariogenic activity of these plants. Therefore, it was thought of interest to study the efficacy of *T. ammi* and *P. spicigera* in combating the virulence traits of the causative agent of caries, i.e., *Streptococcus mutans*.

### 3.2 Experimental procedure

The crude extracts from seeds of *T. ammi* and *P. spicigera* leaves were evaluated for its antimicrobial and anti-adherence effect (Section 2.2.4). They were further fractionated using solvents of increasing polarity as outlined in section 2.2.3. The best effect was observed in petroleum ether (PE) fraction of *T. ammi* and the ethanolic (ETH) fraction of *P. spicigera* and hence their effect on biofilm formation of *S. mutans* on polystyrene microtitre plates was also evaluated by methodology outlined in section 2.2.6. The mode of cariogenicity inhibition was explored by various assays mentioned in methodology (Section 2.2.9 and 2.2.11).

### 3.3 Results

#### 3.3.1 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was found to be 320 µg/ml and 40 µg/ml (crude-CR and petroleum ether-PE fractions of *Trachyspermum ammi*);
while 625 µg/ml and 78.13 µg/ml for crude-CR and ethanol-ETH fractions of *Prosopis spicigera*, respectively.

### 3.3.2 Adherence to smooth glass surfaces

The adherence assay was performed at concentrations below MIC to rule out the reduction in biofilm due to antimicrobial activity of the extracts tested (Figure 3.1A&B). It was found that at 20 µg/ml the PE fraction of *T. ammi* exhibited strong anti adherence effect with 84.25% reduction while its CR fraction at this concentration showed 11.57% reduction. The effective reduction by *T. ammi* (CR) was observed at 40 µg/ml. In case of *P. spicigera*, the ETH fraction as well as its CR fraction exhibited around 50% reduction in adherence at 9.76 µg/ml.

### 3.3.3 Biofilm formation assay

The inhibitory effect of the CR and the active fractions of *T. ammi* and *P. spicigera*, on biofilm formation are shown in Figure 3.2(A&B). Biofilm formation by *S. mutans* was significantly inhibited by CR and PE fractions of *T. ammi* at concentrations 80 µg/ml and 10 µg/ml compared to control (p < 0.01). The effective concentrations in case of *P. spicigera* were found to be 4.88 µg/ml and 19.53 µg/ml for CR and ETH fraction, respectively. The reduction in the ability to form biofilm was in a dose dependent manner. As these fractions did not affect the growth of *S. mutans*, as evaluated through determination of the minimum inhibitory concentration, the extracts affected the ability of *S. mutans* to form biofilm rather than its growth.
3.3.4 Effect on acid production

Both the plant extracts were efficient in reducing the production of acids (Table 3.1). The decrease of pH was significantly inhibited in the presence of CR (160 µg/ml) and PE (20 µg/ml) fractions of *T. ammi*, showing at the onset pH of 7.2 and 7.2 while after 24h of incubation pH 6.5 and 6.8, respectively. Similarly, the pH incase of *P. spicigera* (CR-312.5 µg/ml and ETH-39.06 µg/ml) were 7.1 and 7.2 at the start of the experiment, while after 24h it was found to be 6.4 and 6.1, respectively. On the other hand, untreated cells (control) showed pH 7.3 at the onset, and pH 4.8 after 24 h of incubation. This is significant, because of the fact, when the process of acidification of the medium between the bacterial plaque and the outer surface of the tooth is decreased, the possibility of demineralization of this surface, an essential factor in the formation of dental caries, is also decreased.

3.3.5 Inhibition of water insoluble glucan synthesis

Figure 3.3 (A&B) shows the inhibition of insoluble glucan synthesis (%) by crude GTF with increasing concentrations (2.5-312.5 µg/mL) of the extracts. The effectiveness in inhibition of insoluble glucan synthesis was found to be dependent on the extract concentration in the reaction mixture. The inhibitory effect of the active fraction in case of both the plants was stronger than their respective crude extracts.
Figure 3.1 (A): Effects of *Trachyspermum ammi* (crude-CR and petroleum ether-PE) fractions on the adhesion of *S. mutans* to glass surface (Data= mean ± SD). The % adherence means the relative amount (%) of adhered cells to the glass surface at a certain concentration of extract, as compared to the control. Each value is the average of triplicate assays and each bar indicates mean (Data= mean ± SD).
Figure 3.1 (B): Effects of Prosopis spicigera (crude-CR and ethanol-ETH) fractions on the adhesion of S. mutans to glass surface (Data= mean ± SD). The % adherence means the relative amount (%) of adhered cells to the glass surface at a certain concentration of extract, as compared to the control. Each value is the average of triplicate assays and each bar indicates mean (Data= mean ± SD).
Figure 3.2 (A): Effects of *Trachyspermum ammi* (crude-CR and petroleum ether-PE) on biofilm formation by *S. mutans* (Data = mean ± SD). The % biofilm formed means the relative amount (%) of biofilm formed in the presence of a certain concentration of extract, as compared to that formed in control. Each value is the average of triplicate assays and each bar indicates mean (Data = mean ± SD).
Figure 3.2 (B): Effects of *Prosopis spicigera* (crude-CR and ethanol-ETH) on biofilm formation by *S. mutans* (Data= mean ± SD). The % biofilm formed means the relative amount (%) of biofilm formed in the presence of a certain concentration of extract, as compared to that formed in control. Each value is the average of triplicate assays and each bar indicates mean (Data= mean ± SD).
Table 3.1: Effect of *T. ammi* (CR and PE) and *P. spicigera* (CR and ETH) on production of acids by *Streptococcus mutans*.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>pH ± SD (onset)</th>
<th>pH ± SD (after 24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.3 ± 1.23</td>
<td>4.8 ± 2.98</td>
</tr>
<tr>
<td><em>T. ammi</em> (CR)</td>
<td>7.2 ± 1.09</td>
<td>6.5 ± 1.23</td>
</tr>
<tr>
<td><em>T. ammi</em> (PE)</td>
<td>7.2 ± 3.12</td>
<td>6.8 ± 1.65</td>
</tr>
<tr>
<td><em>P. spicigera</em> (CR)</td>
<td>7.1 ± 2.11</td>
<td>6.4 ± 3.43</td>
</tr>
<tr>
<td><em>P. spicigera</em> (ETH)</td>
<td>7.2 ± 2.99</td>
<td>6.1 ± 2.19</td>
</tr>
</tbody>
</table>
Figure 3.3 (A): Inhibitory effects of *Trachyspermum ammi* (crude-CR and petroleum ether-PE) on insoluble glucan synthesis by crude GTF of *S. mutans*. The % glucan synthesis means the relative amount (%) of insoluble glucan produced at a certain concentration of extract, as compared to the amount produced in its absence. Each value is the average of triplicate assays and each bar indicates mean (Data= mean ± SD).
Figure 3.3 (B): Inhibitory effects of *Prosopis spicigera* (crude-CR and ethanol-ETH) on insoluble glucan synthesis by crude GTF of *S. mutans*. The % glucan synthesis means the relative amount (%) of insoluble glucan produced at a certain concentration of extract, as compared to the amount produced in its absence. Each value is the average of triplicate assays and each bar indicates mean (Data= mean ± SD).
3.4 Discussion

The formation of dental biofilm on teeth is the primary step leading to oral diseases. The sucrose-dependent mechanism (via synthesizes of glucans and fructans by GTF and FTF, respectively) is one of the most important means by which bacteria adhere to hard surfaces in the oral cavity. Gtf$s secreted by *S. mutans* not only binds to the tooth surface, but also on the bacterial surface in an active form (Vacca-Smith and Bowen, 1998), which are advantageous to the organisms for the persistent colonization of tooth surfaces (Schilling and Bowen, 1992). Biofilms containing lower amounts of insoluble glucans across the depth of the biofilms could influence the pathogenesis by disrupting physical integrity and stability (Cross et al., 2007), affecting the diffusion properties (Dibdin and Shellis, 1988), and reducing the binding sites for *Streptococcus mutans* (Vacca-Smith and Bowen, 1998; Schilling and Bowen, 1992). The altered exopolysachharide matrix containing less insoluble glucans may also be more susceptible to the influences of antimicrobials and other environmental assaults (Kreth et al., 2008). Higher content of insoluble glucans in the matrix is associated with increased cariogenicity of biofilms in humans (Paes et al., 2006). A recent study showed that Gtf$B$ levels in saliva correlated with presence of clinical caries in humans (Vacca-Smith et al., 2007). Aciduric bacteria such as the mutans streptococci can carry out glycolysis at low pH values within the biofilm's matrix even though glycolytic enzymes are not acid tolerant, because the bacteria maintains pH across the cell membrane with the interior more alkaline than the exterior. Clearly, the extracts affected acidurance (and acid production) of the biofilms as indicated by higher final pH values of the surrounding medium when compared to control group.
In this study, adherence to glass was tested in the presence of *T. ammi* (crude-CR and petroleum ether-PE fractions) and *P. spicigera* (crude-CR and ethanol-ETH fractions). The PE fraction of *T. ammi* and ETH fraction of *P. spicigera* were selected for further analysis as they showed the least MIC amongst the other solvent fractions. The crude extract of *T. ammi* caused about a 50% reduction in adherence of *S. mutans* at a concentration of 40 µg/mL while this reduction was obtained at 5 µg/mL in case of PE fraction. In case of *P. spicigera*, this 50% reduction in adherence was obtained at 9.76 µg/mL for the CR as well as the ETH fraction. In study concludes that the seeds of *Trachyspermum ammi* and leaves of *Prosopis spicigera* possessed good caries inhibitory activity against *Streptococcus mutans*. Their active fractions could be further used to identify single compound which may be synthesized and used as a putative drug candidate against dental caries.