5.1 Introduction

Dental caries is a transmissible infectious disease that occurs at sites with a pre-existing natural and diverse microflora in the human oral cavity (Ramos-Gomez et al. 2002). Among the oral bacterial flora in human, *Streptococcus mutans* has been confirmed to be a highly cariogenic pathogen (Hamada and Slade 1980). The essential virulence factors of *S. mutans* are acidogenicity and aciduricity together with its ability to form extracellular glucans from sucrose catalyzed by glucosyltransferases (GTFs). Three distinct GTF isoforms, GTF B, C, and D were shown to participate in the sucrose-dependent adherence process (Koo et al. 2000). The synthesis of water-insoluble glucans from sucrose is a major cause of plaque formation. These glucans promote the adherence and accumulation of mutans streptococci and other oral bacteria on the tooth surface resulting in the formation of cariogenic dental biofilms (De Stoppelaar et al. 1969). The production of acid during the metabolism of dietary carbohydrates and the ability of the bacteria to withstand acidic pH in the dental plaque favours its survival under low pH conditions (Nascimento et al. 2004). Despite several antiplaque agents being available, the search for an effective agent still continues. An important strategy for dental caries prevention is the interference with *S. mutans*’ ability to colonize teeth. In addition to being a vaccination target (Chia et al. 1993), inhibition of GTFs and sucrose-dependent *S. mutans* colonization has been a subject of many *in vitro* studies in which different agents, including, plant extracts (Saito et al. 2001; Islam et al. 2009) and natural substances were shown to possess such inhibitory properties (Islam et al. 2008; Shouji et al. 2000). Among active components present in plant extracts are tannins and other polyphenols (Yanagida et al. 2000).
In recent years, there has been a revival of interest in the use of medicinal plants in developed as well as developing countries, because of the fact that compounds obtained from medicinal plants have been shown to be an effective source of chemotherapeutic agents, without undesirable side effects (Rahman and Lowe 2006; Khan et al. 2009).

*Trachyspermum ammi,* Ajowan caraway belongs to the family Apiaceae. Also known as, bishop’s weed, it is an aromatic spice closely resembling thyme in flavour. It is native of Egypt and is distributed in Mediterranean region and South-West Asia. It has long being used as the principal source of thymol. *T. ammi* seeds are employed as an antiseptic, aromatic, carminative and antioxidant source. Its oil is used in preparation of lotions and ointments in cosmetics industries and as spice in many food preparations. It is reported to possess strong insecticidal activity, bronchodilatory effect on asthmatic airways and analgesic effect (Boskabady et al. 2007; Dashti-Rahmatabadi et al. 2007).

Despite the traditional use of *T. ammi* seeds in a number of ailments, its use against dental caries, remains unknown. Therefore, in view of its medicinal significance, we have initiated our study to isolate and characterize a novel compound with anti-biofilm and anti-adherence activities against *S. mutans*.

### 5.2 Experimental procedure

Purification of the active compound from the seeds was performed by silica gel chromatography and spectroscopic methods (FTIR, NMR and MS) were employed for its identification and structure determination as mentioned in section 2.2.13 and 2.2.14. Antibiofilm and antiadherence activities of the active compound
against *S. mutans* were analysed (Sections 2.2.4 to 2.2.6). Confocal microscopy was performed to visualize the effect of compound on biofilm structure of *S. mutans* as described in methodology section 2.2.7.

**5.3 Results**

**5.3.1 Identification of the compound**

The purified compound (112 mg) was obtained as white amorphous solid. Its melting point was measured as 82°C and was uncorrected. The compound was completely soluble in ethanol while insoluble in water. The positive ion FAB MS spectrum showed peaks at m/z 236, 193, 181, 166, 152, 150, 138, 98, 84, 70, 55, 43 and 42. The molecular ion peak at m/z 236 was compatible with molecular formula \( \text{C}_{16}\text{H}_{28}\text{O} \). The base peak was observed at m/z 55. The FTIR spectrum exhibited bands \( \nu (\text{cm}^{-1}) \) at 2920, 2840, 1700, 1440, 1400, 1310, 880 and 600. The band at 1700 cm\(^{-1}\) is characteristic for carboxylic function. \(^1\)H NMR spectrum displayed signals at \( \delta 0.89 \) (t, J= 7.2 H\(_3\)-3’ and H\(_3\)- 3’’), \( \delta 1.26 \) (brs, methylene protons), 1.64 (m, J= 7.6 H-5 and H-4a) and 2.36 (t, J= 7.6 H\(_2\)-2). The \(^{13}\)C NMR gave signals for sixteen carbons. The peaks appeared at \( \delta c \) 210.16 (C-1), 40.25 (C-2), 22.69 (C-3), 24.75 (C-4), 35.36 (C-5), 31.93 (C-6), 21.70 (C-7), 33.65 (C-8), 52.60 (C-8a), 46.45 (C-4a), 35.09 (C-1’), 19.26 (C-2’ and C-2’’), 14.12 (C-3’ and C- 3’’), 14.12 (C-3’ and C- 3’’), 35.43 (C-1’’). The compound was identified as \((4\text{aS, 5R, 8aS}) 5, 8\text{-di-1-propyl-octahydro-naphthalen-1-(2H)-one}\). The structure is shown in figure 5.5.
Figure 5.1: Mass spectra of the compound purified from seeds of *Trachyspermum ammi*. The peaks are representative of the relative intensity of the fraction with corresponding m/z (amu).
Figure 5.2: FTIR spectra of the isolated compound from the seed of *Trachyspermum ammi.*
Figure 5.3 (A): $^1$H NMR of the isolated and purified compound from the seed of $Trachyspermum ammi$. 


Figure 5.3 (B): $^1$H NMR of the isolated and purified compound from the seed of Trachyspermum ammi.
Figure 5.4: $^{13}$C NMR spectra of the novel compound isolated from the seeds of *Trachyspermum ammi.*
**Figure 5.5:** Chemical Structure of (4aS, 5R, 8aS) 5, 8a-di-1-propyl-octahydonaphthalen-1-(2H)-one isolated from *Trachyspermum ammi.*
5.3.2 Determination of MIC and MBC

The MIC of the compound against *S. mutans* was found to be 156.25 µg/ml. Inoculums from wells with no visible growth were subcultured on BHI agar. The maximum concentration that showed no growth on the plate, that is, the MBC was found to be 312.50 µg/ml.

5.3.3 Inhibitory effect on bacterial adherence

The effect on the adherence of *S. mutans* by different concentrations of the compound are given in Figure 5.6A. The reduction in the adherence was found to be in a dose dependent manner. The least concentration to give atleast 50% inhibition was 39.06 µg/ml. Even though the MIC is not very effective, that is, 156.25 µg/ml, the adherence properties were effected at a concentration as low as 9.77 µg/ml with 30% reduction.

5.3.4 Effect on *Streptococcus mutans* biofilm formation

Figure 5.6B shows the influence of the compound on biofilm of *S. mutans*. The biofilm formation was assessed using increasing concentration of the compound. At 78.13 µg/ml, the compound inhibited the biofilm formation of *S. mutans*. As observed in the adherence, the inhibitory effect was also found to be in a dose dependent manner. As expected, the compound completely inhibited the biofilm formation at 156.25 µg/ml (MIC) due to its antimicrobial effect.
5.3.5 Confocal microscopy

The biofilm of *S. mutans* was analysed under the confocal laser scanning microscope to observe the changes in its morphology shown in figure 5.7. In the absence of the compound, the cells showed clumps and aggregate that was absent when treated with sub inhibitory concentration of the compound. The cells in the treated sample were more spread out and dispersed. Each panel of the image is a representative view of 141.14 µm by 141.14 µm along the xy axis. The xy analyses provide the biofilm surface coverage, while the z-section analyses establish the thickness of the biofilm. The control cells have biofilms that are clumped and less spread along the xy lane, show a thickness of 19.74 ± 0.5 µm. In the presence of 78.13 µg/ml of the compound, the cells show a complete absence of clumped cells and the thickness is 12.8 ± 0.7 µm. The cells were individually scattered over the surface rather than in any arrangement.

5.3.6 Inhibition of acid production

The pH of the culture medium was recorded after 24h of treatment in order to determine the effect of this compound on acid production. The reductions in pH drop at various concentrations of the compound are reported in Table 5.1. At a concentration of 78.13 µg/ml, there is maximum reduction in acid production as is evident by an increase in pH from 4.7 (control) to 7.5. This suggests that 78.13 µg/ml compound effectively inhibits acid production in *S. mutans*. 
Figure 5.6: Inhibitory effects of the purified compound on A) Adherence; B) Biofilm formed; C) Hydrophobicity and D) Water-insoluble glucan formed. The percentage indicates the relative amount (%) formed or produced in the presence of different concentrations as compared to the amount formed or produced in the absence of the compound. Each value is an average of triplicate assays and each bar indicates ± standard deviation (n=3)
Figure 5.7: CLSM images of *Streptococcus mutans* biofilm formed in the presence or absence of the compound after 24 h incubation. The assays were performed in triplicates, and similar results were obtained.
Table 5.1: Effect of the compound on the pH of *Streptococcus mutans*. Different concentration of the compound was added to 1.5 X $10^6$ CFU per ml *S mutans* cells. pH was recorded after 24 h incubation at 37°C. Each concentration was taken in triplicates calculated (n=3).

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>pH ± SD (onset)</th>
<th>pH ± SD (after 24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.44 ± 1.09</td>
<td>4.71 ± 3.41</td>
</tr>
<tr>
<td>2.44</td>
<td>7.28 ± 0.09</td>
<td>4.77 ± 1.69</td>
</tr>
<tr>
<td>4.88</td>
<td>7.24 ± 1.33</td>
<td>4.84 ± 1.20</td>
</tr>
<tr>
<td>9.76</td>
<td>7.33 ± 1.11</td>
<td>5.49 ± 1.05</td>
</tr>
<tr>
<td>19.53</td>
<td>7.38 ± 1.91</td>
<td>5.62 ± 1.96</td>
</tr>
<tr>
<td>39.01</td>
<td>7.43 ± 1.13</td>
<td>5.68 ± 6.78</td>
</tr>
<tr>
<td>78.13</td>
<td>7.43 ± 0.15</td>
<td>6.48 ± 5.97</td>
</tr>
<tr>
<td>156.25</td>
<td>7.38 ± 1.29</td>
<td>7.55 ± 2.65</td>
</tr>
</tbody>
</table>
5.3.7 Effect of the compound on the hydrophobicity of *Streptococcus mutans*

It was shown that the cell surface hydrophobicity of *S. mutans* is affected following treatment with the compound obtained from *T. ammi* as shown in Figure 5.6C. Exposing the bacteria to 9.76 µg/ml of the compound drastically reduced the cell surface hydrophobicity to more than half when compared to untreated condition.

5.3.8 Inhibition of water insoluble glucan synthesis

The influence of different concentrations of the compound was assessed for the synthesis of insoluble glucan. The synthesis was found to be suppressed by the compound in a dose dependent manner. There was a 25% reduction in the synthesis of insoluble glucan at a concentration as low as 4.88µg/ml as shown in figure 5.6D.
5.4 Discussion

Authors have described various attributes of *T. ammi* previously (Kaur and Arora 2009; Kaur *et al.* 2009). However, to the best of our knowledge there is no literature dwelling upon its influence on dental caries to date. This is the first study to report anti biofilm and anti cariogenic properties of a novel compound isolated from the seeds of *T. ammi*. The characterization of the compound was done by FABMS, FTIR, $^1$H NMR and $^{13}$C NMR spectroscopic techniques.

Functional group analysis was done by FTIR spectrum. It exhibited characteristic absorption band for carboxyl group at 1700 cm$^{-1}$. The other bands at 2920, 2840, 1440, 1310, 880 and 600 cm$^{-1}$ in fingerprint region represents CH$_2$ and CH groups. The FAB mass displayed molecular ion peak at m/z 236, corresponded to molecular formula C$_{16}$H$_{28}$O, which indicated three double bond equivalents, two of them adjusted in bicyclic carbon framework of the molecule and remaining one in carboxyl group. The $^1$H NMR spectrum displayed the presence of H$_3$-3' and H$_3$-3″ terminal methyl groups were indicated by six proton triplet at $\delta$ 0.89(J=7.2 Hz). A two proton multiplet at $\delta$ 1.64 was ascribed to H-5 and H-4a methine protons with coupling constant of 7.6 Hz suggesting trans diaxial coupling. A two proton triplet at $\delta$ 2.36 (J= 7.6 Hz) was accounted for C-2 methylene protons. The slightly downfield signal for C-2 protons was explained by the presence of neighbouring carboxyl group. A broad singlet at $\delta$ 1.26 was associated with other methylene groups of cyclic system and side chain. According to the above $^1$H NMR data trans diaxial coupling between H-5 and H-4a proton is possible when the compound rings are trans fused. Also, trans fused conformation place C-5 bulky propyl group at equatorial position, which further stabilizes the molecule.
Futher information of the compound was obtained from $^{13}$C NMR spectrum which showed signal attributable to the expected structure. The important signals appeared for carboxyl carbon at $\delta_c$ 210.16 (C-1), C-2 carbon at $\delta_c$ 40.25, quaternary carbon (C-8a) at $\delta_c$ 52.60 and tertiary carbons (C-5; C-4a) at $\delta_c$ 35.36 and 46.45 respectively. The peak for terminal methyl groups is indicated by $\delta_c$ 14.12. The signals appeared in the range $\delta_c$ 19.26-35.09 represents other methylene carbons.

On account of these spectral evidences the compound was characterized as (4aS, 5R, 8aS) 5, 8a-di-1-propyl-octahydronaphthalen-1-(2H)-one. This compound has been reported for the first time from any plant or synthetic source. It is a derivative of naphthalene. Compounds of naphthalene have been reported to show a variety of biological activities including antimicrobial activities (Eid et al. 2004; Goksu et al. 2005), HIV-1 integrase inhibitory effect (Burke et al. 1995), anti-inflammatory activity (Huang et al. 2003), inhibition of protein tyrosine kinases and inactivation of enveloped viruses (Burke et al. 1993). Naphthalene glycosides and naphthoquinones are other naphthalene derivatives to act as antibacterial agents against microbes present in the oral cavity (Cai et al. 2000).

*S. mutans* plays an essential role in the pathogenesis of dental caries and consequently becomes the prime target for prevention of caries. The antimicrobial activity of the compound against *S. mutans* was evaluated. The MIC and MBC were found to be 156.25 and 312.5$\mu$g/ml, respectively. Amongst the important steps to form dental plaque is the adherence of *S. mutans* to the tooth surface, its inhibition could be one of the therapeutic approaches to reduce its virulence (Matsumoto et al. 1999). Thus we investigated the inhibitory effect the compound on adhesion at various concentrations (2.44, 4.88, 9.77, 19.53, 39.06 and 78.13 $\mu$g/ml). Figure 5.6A
shows that the compound significantly reduced the adherence of *S. mutans* compared to the control.

Glucan is believed to be the major factor contributing to the ability of *S. mutans* to adhere to the tooth surface and for aggregation of the bacterial cells within a biofilm (Munro *et al.* 1995). Bacterial aggregates in a biofilm are generally enveloped in large amounts of exopolymeric matrix and are interspersed by less cell dense regions of the matrix (Nivens *et al.* 1995). The sticking of cells on a polystyrene surface forms a model for the *in vitro* biofilm formation of bacteria (Loo *et al.* 2000). This approach was used to study the biofilm of *S. mutans* in the presence of the compound at various concentrations. The dye released by the bound cells is an indirect revelation of the bacterial monolayer formed as well as due to reduced in the number of cells adhering to the surface in the presence of the compound. The OD at 600 nm decreases in its presence, as the case may be (Figure 5.6B) in a concentration-dependent manner.

CLSM images display the architecture of cells in a biofilm. The cells in control are embedded in a polysaccharide matrix that stimulates cell clustering (Wimpenny *et al.* 2000). Figure 5.7 shows representative CLSM images of biofilms containing *S. mutans* alone and with the compound at sub-MIC concentration. Calculations based on z-series showed that the thickness of biofilms formed in control was 19.74 ± 0.5 µm and that in the presence of the compound was 12.8 ± 0.7µm .The action of natural anticaries compounds could occur either by inhibition of the bacterial adhesion without killing the bacteria (Islam *et al.* 2008) or by destroying the integrity of the cell wall (Kim *et al.* 2008). Images indicated latter mechanism being followed, wherein *S. mutans* exposed to sub-inhibitory concentration of the compound, show
biofilm cells scattered individually along the polysaccharide matrix. The classic biofilm architecture of mushroom cap and stalk seems to absent and there is complete loss of aggregates.

*S. mutans* is an acidogenic bacteria that grows in plaque, and releases various organic acids such as lactic acid, formic acid, butyric acid and propionic acid during metabolizing carbohydrates. The organic acids demineralize tooth surfaces and initiate the dental caries (ten Cate 2006). In our investigation, there was a reduction observed in acid production of *S. mutans*. Moreover, *S. mutans* adheres to the enamel surface by hydrophobic bond interaction. Therapeutic agents, that help prevent hydrophobic bond formation, would reduce the incidence of caries. Addition of increasing concentrations of the compound decreased the hydrophobicity of this bacterium. These results clearly showed that our compound decrease hydrophobicity, one of the most important initial factors for the oral pathogenic bacteria to adhere to the tooth surface (Weiss et al. 1982). Cell-surface hydrophobicity of *S. mutans* is mainly associated with cell-surface proteins (Wen et al. 2005) and it is possible that the active compound binds to cell-surface proteins reducing the overall cell hydrophobicity.

Mutans streptococci can colonize the tooth surface and initiate plaque formation by their ability to synthesize extracellular polysaccharides from sucrose, mainly water-insoluble glucan, using glucosyltransferase (Jacquelin et al. 1995). De novo synthesis of water-insoluble glucan is essential for the adherence of *S. mutans* and other oral microorganisms to the tooth surface, forming a barrier that prevents the diffusion of acids produced by the bacteria. Glucosyltransferases (GTFs) synthesize extracellular polysaccharides (glucans) that promote adhesion and colonization of
cariogenic organisms and mediate protection against antimicrobial agents and resistance to toxic compounds. The compound might have exerted its influence either by affecting the ability of *S. mutans* to produce extracellular glucans which resulted in a decrease in extracellular matrix component or by increasing the diffusion coefficient of the biofilm, making it more permeable and thus more prone to reduced microbial adhesion (Hillman *et al.* 2007). The water insoluble glucans are of selectively more importance than water-soluble glucan in adhesive interactions by *S. mutans*, so we tested the influence of the compound on the water insoluble glucan and found it to be effective with a 50% reduction in its synthesis at 19.53μg/ml.

In summary, (4aS, 5R, 8aS) 5, 8a-di-1-propyl-octahydronaphthalen-1-(2H)-one, a novel compound reported for the first time from the seeds of *T. ammi* was examined for its activity against cariogenic properties of *S. mutans*. It significantly reduced its adherence as well as biofilm formation, insoluble glucan synthesis by glucosyltransferase and hydrophobicity. These results indicate that this compound possesses powerful anticariogenic potential.