CHAPTER 5: DOWNREGULATION OF ComDE PATHWAY OF
Streptococcus mutans BY AROMATIC 1,3-DI-M-TOLYLUREA AND ITS
CARIOSTATIC EFFECT WITH FLUORIDE IN WISTAR RATS

5.1 Introduction

We have previously reported ComA as a potential target for drug development. In silico findings showed 1,3-disubstituted ureas as potential ligands followed by synthesis and in vitro validation of five derivative ligands along with parent ligand (ComAI). To explore the possibility of reducing fluoride concentration as discussed in previous chapter, we had included various concentrations of fluoride in our in vitro assays and investigated its synergistic activity along with our synthesized compounds. Results of in vitro validation indicated that ComAI and could act as a potent biofilm inhibitor alone as well as along with lower concentration of fluoride (31.25 ppm) among all the synthesized compounds. Thus, to further explore the target specific activity of ComAI, the objectives of this specific aim were (i) to elucidate the target specific mechanism of ComAI on various quorum regulated genes and (ii) to test and validate the activity of ComAI at preclinical stages using Wistar rat model for dental caries.

5.2 Materials and methods

5.2.1 Cell culture:

Liver hepatocellular carcinoma (Hep G2) cells were received from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in Dulbecco’s modified Eagle’s medium (HiMedia) supplemented with 10% fetal bovine serum (FBS) and 1% pen/strep. The cells were incubated and maintained at 37°C in a saturated humidified incubator with 5% CO₂. MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) for cell proliferation assay was purchased from HiMedia, Mumbai, India.
5.2.2 Bacterial strains and growth medium:

*Streptococcus mutans* MTCC 497 was received from Microbial Type Culture Collection (MTCC), Chandigarh, India and was used as a standard strain in the study. A clinical isolate of *S. mutans*, 4SM (Multidrug resistant) was received from JSS Medical College, Mysore, India. Both the strains were grown at 37°C in brain heart infusion broth/agar (HiMedia) supplemented with 2% sucrose.

5.2.3 Test compounds:

The synthesis of aromatic 1, 3-disubstituted ureas was carried out by a simple one pot reaction of aryl isocyanates with the selective amines has been reported in the previous chapter 4 (Section 4.2.1). Further, the derivatives of the lead compound were synthesized and screened *in vitro* for their activity. Amongst all the synthesized compounds, ComAI (1, 3-di-<i>m</i>-tolylurea) was found to be the most effective based on their biofilm inhibiting activity against *S. mutans*.

5.2.4 Quantification of gene expression using RT-PCR

*S. mutans* (MTCC 497 and SM4) were grown in BHIB in the presence and absence of ComAI (3.75μM) and fluoride (31.35 ppm) till 24 h at 37°C. Cells were harvested by centrifugation from grown cultures (0.5 ml) at early log phase (3-5 h), mid log phase (8-10 h) and stationary phase (24 h) and immediately stored at -80°C. RNA was isolated using a Qiagen RNeasy mini Kit in accordance with the manufacturer’s instructions. RNA concentrations were determined by OD<sub>260</sub> measurements in a NanoDrop (Thermo Scientific, USA). cDNA synthesis was carried out using the iScript<sup>TM</sup>cDNA Synthesis Kit according to the manufacturer’s instructions. Briefly, the reaction mixture was incubated for annealing at 25°C for 5 min, extension at 42°C for 30 min and inactivation of samples at 85°C for 5 min.
qRT-PCR was used to assess the transcription levels of biofilm and virulence related genes (*comA*, *nlmC*, *immA*, *immB*, *bsmI*, *bsmH*, *comDE*, *comX*, *comB*). Sequences for the primers used in this study are furnished in Table 5.1. Reaction mixture in a total volume of 20 μl, consisted 10 μl 2X SYBR Green PCR Master Mix, forward and reverse primers (1μl each), 4 μl of nuclease free water and 4 μl of 20X diluted cDNA. PCR conditions included an initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation (95°C for 15 sec), annealing (55-57°C for 15 sec), and extension (72°C for 20 sec). To ensure the samples were free from contamination, negative controls containing nuclease-free water instead of cDNA were run in parallel. The relative gene expression was analysed using the $2^{-\Delta\Delta CT}$ method with 16S r-RNA as reference gene.

### Table 5.1: Primer sequence for qRT-PCR analysis of various genes in *S. mutans*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence (5’-3’)</th>
<th>Reverse Primer Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>comDE</td>
<td>ACAATTCCCTTGAGTTCCATCCAAG</td>
<td>TGGTCCTGCTGCTTGTTGC</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>CCTACGGGAGGCAGCAGTGA</td>
<td>CAACAGAGCTTTACGATCCCCAAA</td>
</tr>
<tr>
<td>nlmC</td>
<td>TTGTGCAGCAGGATTGCTTC</td>
<td>AAGAGCTCTCCGATTTCCCT</td>
</tr>
<tr>
<td>immA</td>
<td>TCTCCCTGCTTGGCATG</td>
<td>GCTGGCAAATTTGCTTTACTT</td>
</tr>
<tr>
<td>immB</td>
<td>GCTAGAGAGGGCAATGGCA</td>
<td>CAGCAGCAGCTGAGAAGATG</td>
</tr>
<tr>
<td>bsmI</td>
<td>GAAACAATGGATACAGAGAGC</td>
<td>GGAACAAATAGAGGATTGG</td>
</tr>
<tr>
<td>bsmH</td>
<td>AGACATGTAGCCTGTGAAG</td>
<td>AAGCGCTGTCCAATCCTA</td>
</tr>
<tr>
<td>comX</td>
<td>CTGTGCTCAAGTGGCGGTA</td>
<td>GCATACTTGGCTCTCCCACA</td>
</tr>
<tr>
<td>comA</td>
<td>ACGAGCCTAAACAAGGGGATT</td>
<td>CCCTGAGGCAATTGTTCAAT</td>
</tr>
<tr>
<td>comB</td>
<td>CCAGTCCAAACCAGTCAACT</td>
<td>GCTGCTTCTCTTGTCTTTCG</td>
</tr>
</tbody>
</table>
5.2.5 MTT cell proliferation assay:

For toxicity analysis, Liver hepatocellular carcinoma (Hep G2) cells were cultured in the presence of ComAI and ComAI\(^1\). After trypsinization and counting the cells with hemocytometer, 10,000 cells were seeded in 96 well plate along with 1, 3-disubstituted ureas. The cells were allowed to proliferate for 24 hours at 37°C and at the endpoint, MTT reagent was added and further subjected to 4 hours of incubation [131]. The formazan crystals formed were solubilized using DMSO (100 μL) and the absorbance was measured at 570 nm in a microtitre plate reader (iMark, BIORAD, Japan).

5.2.6 Animal study

5.2.6.1 Acute Oral Toxicity (AOT) analysis:

Healthy female Wistar rats aged 8 weeks used for the AOT analysis were bred and reared in Central Animal Facility, SASTRA University, Thanjavur, Tamil Nadu, India. The animals were acclimatized to animal house conditions for one week prior to the treatment with ComA. The animals were housed and maintained in polypropylene cages consisting of clean paddy husk bedding with stainless steel grill lids at a temperature of 25 ± 2 °C under 12:12 hour light dark cycle. The rats were fed with pelleted feed (M/S ATNT Laboratories, Mumbai, India) and filtered tap water *ad libitum* throughout the experiment.

The acute oral toxicity test of ComAI was evaluated in rats using the up and down procedure in accordance with OECD 425 guidelines [132]. Briefly, the rats were divided into 5 groups with first group receiving a limited dose of 175mg/kg orally using a suitable intubation canula. The animal was observed for toxic symptoms continuously for the first 3 hours after dosing. The animal was further observed for 48 hours and based on survival of the first group rat, the second group was dosed with 550mg/kg
orally. Similar observations were carried out for the second group and subsequently based on the survival of rat, the dosing was increased to 2000 mg/kg for next three groups. All these animals were then maintained for 14 days further with feed intake observations made on daily basis and weight observations on weekly basis. At the 14th day, the animals were sacrificed and vital organs were observed macroscopically by a calibrated professional histopathologist for any lesions.

5.2.6.2. Efficacy studies:

The animal experiments were reviewed and approved by Institutional Animal Ethics Committee (IAEC) with approval number 382/SASTRA/IAEC/RPP of SASTRA University, Thanjavur, Tamil Nadu, India and was performed according to the methods described previously [129]. To determine the effects of ComAI on caries establishment, a total of 42 SPF female Wistar rats aged 21 days were purchased from Central Animal Facility, SASTRA University, Thanjavur, India. After acclimatization for 5 days, the 30 animals were infected with clinical isolate of *S. mutans*, SM4 (10⁵ cfu/mL) and randomly divided into five groups (n= 6 per group): a diseased control, a ComAI treated group (3.75μM), a fluoride treated group (500ppm), a synergy group (3.75 μM ComAI and 31.25ppm fluoride), a 10 X ComAI group (37.5 μM) to determine the long term effects of high dose of ComAI). The swab was obtained and plated on Mitis Salivarius Agar with 0.2 U/mL bacitracin to confirm the colonization of *S. mutans* on dentine. Each group was fed with diet 2000 (contains 56% sucrose) and 5% sucrose water *ad libitum*. In addition to these five groups, two other groups were maintained as controls (n= 6 per group): a control group without sucrose diet and another control group with diet 2000 and 5% sucrose water. From this point, the molars of animals were given topical treatments with their corresponding concentrations once daily by using camel hair brush. The animals were noted for their body weight weekly and physical
appearance was noted daily. The treatment was carried out for 7 weeks, and at the end of the experimental period, animals were euthanized by CO₂ asphyxiation. The lower jawline was dissected aseptically and suspended in 10 ml of sterile phosphate buffer saline and subjected to sonication (20s pulses at 10s intervals for two times) to recover the maximum adhered viable counts. The solution was further serially diluted and plated on Mitis Salivarius Agar with 0.2 U/mL bacitracin to estimate the S. mutans population. The determination of severity of caries developed on molars of the animals were scored according to a Larson’s modification of the Keyes system [133] and was performed by expert examiner in caries.

5.2.6.3. Histopathological evaluation:

For histopathological evaluation, the liver tissues and decalcified dentine was collected and post-fixed in 4% PFA for 24 hours at 4°C, embedded in paraffin (Leica EG1150H, Leica Microsystems, Heerbrugg, Switzerland), and sectioned into ~5 μm thick sections (Leica RM2125 RTS, Leica Microsystems, Heerbrugg, Switzerland). The sections were further stained with hematoxylin and eosin using an automated tissue processing and staining system (Leica TP 1020; Leica FG1150; Leica RM 2125 RTS and Leica ST4040) and scored blindly by a veterinary pathologist to be examined under a binocular microscope (Nikon Eclipse Ci-Ds-Fi2)[134].

5.2.6.4. Inflammatory parameters evaluation:

The inflammatory makers were assessed using qRT-PCR method. Blood samples (5ml each) from all the rats were collected in EDTA-treated collection tubes, just before the necropsy was carried out. The blood samples were further centrifuged at 2000 rpm for 10 minutes at 4°C for plasma collection [135]. The plasma samples were immediately stored at -20°C. RNA was isolated using a Qiagen RNeasy mini Kit were assessed using
the same procedure as described in section. Similarly, cDNA synthesis was carried as described previously in section 5.2.4

qRT-PCR analysis was carried out in 96 well plate (Thermofisher) using Realplex 2 (Eppendorf) to assess the transcription levels of genes related to inflammatory markers (IL-1, IL-6, C- Reactive protein, TNF-α) (Table 5.2). Reaction mixture in a total volume of 20 μl, consisted 10 μl 2X SYBR Green PCR Master Mix, forward and reverse primers (1μl each), 4 μl of nuclease free water and 4 μl of 20X diluted cDNA. PCR conditions included an initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation (95°C for 15 sec), annealing (52.9°C for 15 sec), extension (72°C for 20 sec). To ensure the samples were free from contamination, negative controls containing nuclease-free water instead of cDNA were run in parallel. The relative gene expression was analysed using the $2^{-\Delta\Delta CT}$ method with Gapdh as internal control.

**Table 5.2: Primer sequence used for qRT-PCR analysis of inflammatory markers in rat liver and blood**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence (5’-3’)</th>
<th>Reverse Primer Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>IL1</td>
<td>GGAAGGGGAGAAATCCAAG</td>
<td>TGGTTTTTTCACCCCCCTGAC</td>
</tr>
<tr>
<td>IL6</td>
<td>CCGGAGAGGAGACTTCACAG</td>
<td>ACAGTGCATCAGCTGTTTC</td>
</tr>
<tr>
<td>CRP</td>
<td>AACCTGGGAGAGGGTCAGAT</td>
<td>GACTCTGCTCCAGGGACAC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>AGTCCGGGCGGACGTCTACTTTT</td>
<td>GGCCACTCTTCAGCGTCTC</td>
</tr>
<tr>
<td>Gapdh</td>
<td>CATGGTCTACATGTTCCAGT</td>
<td>GGCTAAGCAGTTGGTGTTG</td>
</tr>
</tbody>
</table>
5.2.7 Statistical analysis

For qRT-PCR, one way ANOVA and multiple comparisons were carried out. The data from *in vivo* study were analysed by unpaired Student’s *t* test. The minimum level of significance was set at *p* ≤ 0.05. All the assays were carried out in triplicates and the results were expressed as mean ± SD. Graph pad prism software (version 6.01) was used for statistical analysis for all the experiments.

5.3 Results

5.3.1 Gene expression profiling using RT-PCR:

Gene expression study using RT-PCR in MTCC 497 revealed that the genes which were located downstream to ComA were downregulated at mid log phase and stationary phase except *immA* and *immB* genes which were found to be upregulated. The genes were found to have a basal level expression at early phase ([Figure 5.1 A and B](#)). In contrast, fluoride did not show any significant effect on the expression of quorum sensing genes at mid log phase as well as stationary phase. Similar results were obtained in SM4 strain treated with ComAI at the tested concentration ([Figure 5.2 A and B](#)).
Figure 5.1: qRT-PCR analysis of various genes involved directly and indirectly in quorum sensing circuit of MTCC 497 (S. mutans). The results are represented as ratios corresponding to the fold change of genes treated with ComAI (3.75 μM) and fluoride (31.25 ppm) as well as control (without treatment) at mid log phase (A) and stationary growth phase (B). 16S rRNA gene was used as an internal control for data normalization. * indicates significantly different (p < 0.05) compared to untreated genes. # indicates p> 0.05.
Figure 5.1 (Continued)
Figure 5.2: qRT-PCR analysis of various genes involved directly and indirectly in quorum sensing circuit of clinical isolate SM4. The results are represented as ratios corresponding to the fold change of genes treated with ComAI (3.75 μM) and fluoride (31.25 ppm) as well as control (without treatment) at mid log phase (A) and stationary growth phase (B). 16S rRNA gene was used as an internal control for data normalization. * indicates significantly different (p < 0.05) compared to untreated genes. # indicates p > 0.05.
5.3.2 Cytotoxicity analysis:

In the present study, MTT assay revealed that ComAI and ComAI\(^1\) does not have any quantitative cytotoxic effect on morphology and proliferation of Hep G2 cell lines when compared with the respective control as shown in Figure 5.3. The cells were found to have about ninety percent confluence after 24 hours of incubation. The results of cytotoxicity assay suggested that compounds can be further used in rodent animals for efficacy studies for validation of compounds.
Figures 5.3: Viability of HepG2 cells after 24 hour treatment: The cells did not exhibit any significant cytotoxicity with cells treated with ComAI at two as well as four times of the effective concentration (X= 3.75 μM).

5.3.3 Acute oral toxicity studies

In acute oral toxicity study, the rats did not show toxic signs or death during the 14 day observation period. External examination of the rats did not show any signs of disease development and uptake of feed was normal without significant differences in the average weight gains among the experimental groups (data not shown). The skin and natural orifices of all experimental animals revealed no morphologic alterations. The animals did not show any variation in their general physical appearance and behaviour and also, no signs of anorexia, depression, lethargy, jaundice, dermatitis throughout the study. Macroscopic observation of organs such as heart, lung, pancreas, spleen, liver, stomach, intestine, kidney, ovary, brain, eyes and tongue revealed indifference among all the rats without any detectable pathological symptoms.
5.3.4 ComAI reduce the incidence of dental caries \textit{in vivo}

The present study has revealed that ComAI acts as a potential cariostatic agent and thus hinder the occurrence of dental caries \textit{in vivo}. Macroscopic observations showed the development of brown and black lesions on the crown of diseased rats whereas, reduction in development of lesions was found in ComAI treated group (\textbf{Figure 5.4}). Moreover, in disease group, the tissue around the molar root was inflamed as compared to the normal control group. The group treated with fluoride (250ppm F, clinically proven anticaries agent) alone showed comparatively reduced lesions but not as significant as that of ComAI treated group. In this study, it is shown that ComAI (3.75μM) in combination with lower concentrations of fluoride (31.25ppm F) was considerably effective in reducing the occurrence of lesions and adherence of biofilm producing cells as compared to the fluoride alone. The colony count of SM4 showed significant reduction in the adherent cells in case of ComAI and combinatorial study group when compared with disease control (\textbf{Figure 5.5}). The total viable counts and the SM4 viable counts recovered from the rodents’ plaque were not significantly affected by treatments with ComAI when compared to the normal control (p > 0.05).
Figure 5.4: Incidence of dental caries: The image represents the occurrence of dental caries in various groups. (A) Normal control group without sucrose diet; (B) Normal control with sucrose diet; (C) Diseased control group, solid arrow represents the occurrence of black lesions on molar crown indicating development of caries and dotted arrow represents the inflamed gum tissue due to infection by \textit{S. mutans}; (D) ComAI (3.75 μM) treated group; (E) Fluoride (250 ppm) treated group; (F) ComAI (3.75 μM) along with fluoride (31.25 ppm) treated group.
Figure 5.5: Anti-adherent activity of ComAI in treated groups in comparison to untreated groups was evaluated by colony forming unit (CFU) assay and the results were plotted on a logarithmic scale. The columns in the graph represent the mean of six animals per group. Error bars represent standard deviations with *p < 0.05.

5.3.5 Histopathology studies

The liver of control as well as ComAI (10 X dose; X=3.75 μM) administered rats showed normal hepatic structure, characterized by polygonal-shape hepatocytes with well-defined boundaries, large centrally located nucleus with light stained acidophilic cytoplasm along with dispersed chromatin radially disposed in hepatic lobules (Figure 5.6). The incidence of dental lesions is summarized in Figure 5.7. Decalcified longitudinal sections of teeth of normal group showed healthy dentine, odontoblast and pulp, whereas in diseased group, the carious dentine lesions were moderate to severe
transcending through odontoblast into the pulp and completely decayed enamel crown. Almost no carious lesions were detected in ComAI treated as well combinatorial treated group whereas moderate carious lesions were recorded in fluoride treated group.

**Figure 5.6:** Hematoxylin and eosin staining of liver tissue from (A) normal control group and (B) the group treated with 10 X dose of ComAI (X= 3.75 μM). Normal as well as treated groups showed normal liver tissue histology without any pathological signs.
Figure 5.7: Hematoxylin and eosin staining of dental tissue from (A) Normal control group; (B) Normal control with sucrose; (C) Diseased group, solid arrow represents the lesions developed on the dentine and penetrated up to the dental pulp tissue; (D) ComAI (3.75 μM) treated group; (E) Fluoride (250 ppm) treated group; (F) ComAI (3.75 μM) along with fluoride (31.25 ppm) treated group.

5.3.6 Reduction in inflammatory markers

The inflammatory markers such as IL-1, IL-6, TNF-α, CRP showed varying expression levels in diseased as well as treated groups. In case of liver samples (Figure 5.8), levels of proinflammatory cytokines TNF-α, CRP, IL-1 and IL-6 were found significantly elevated in diseased group as compared to the control group (p < 0.05). Treatment with ComAI and ComAI along with fluoride significantly (p < 0.05) decreased the expression of TNF-α, CRP, IL-1. However, IL-6 levels were not affected by the ComAI treatments but ComAI along with fluoride was able to reduce the expression significantly. Furthermore in plasma (Figure 5.9), except IL-6, other inflammatory markers used in this study, i.e., IL-1, CRP and TNF-α showed significant reduction in
expression levels in treated groups (ComAI alone and Combinatorial group) as compared to the diseased group.

**Figure 5.8:** qRT-PCR analysis of inflammatory markers in liver tissues of Wistar rats. (A) IL-1; (B) TNF-α; (C) C- Reactive Protein (CRP); (D) IL-6. The results are represented as ratios corresponding to the fold change in various treatment groups when compared with normal control group (without treatment). Gapdh gene was used as an internal control for data normalization. * indicates significantly different (p < 0.05) compared to untreated tissues.
Figure 5.9: qRT-PCR analysis of inflammatory markers in samples collected from blood plasma of Wistar rats. (A) IL-1; (B) TNF-α; (C) C-Reactive Protein (CRP); (D) IL-6. The results are represented as ratios corresponding to the fold change in various treatment groups when compared with normal control group (without treatment). *Gapdh* gene was used as an internal control for data normalization. * indicates significantly different (p < 0.05) compared to untreated tissues.

5.4 Discussion

Oral cavity is one amongst the dynamic microbial community niche consisting of more than 700 species in equilibrium. Most of the species are commensal and helps in maintaining the normal balance and thus avoiding pathogenic interference by opportunistic pathogens. Emergence of multidrug resistant strains has raised the concern and need for the development of better anti-virulent drugs. In this context, our
present study focussed on the *in vitro* and *in vivo* validation of target specific anti-virulent drugs which were previously reported by us to have better binding to ComAI [136].

Our study examined the effects of ComAI and fluoride at mid-logarithmic growth phase and stationary growth phase. The genes considered in this study are reported to be directly and indirectly involved in quorum sensing circuit of *S. mutans*. At mid log phase, the expression of *immA* and *immB* (bacteriocin-immunity genes) were found to be upregulated. Similar results were observed previously by Wang *et al.*, [137] where the group reported upregulation of *immA* and *immB* genes upon treatment with chlorhexidine in *comC* mutant. Additionally, they also reported the enhanced sensitivity of *comC* mutant towards antimicrobials indicating the indirect involvement of quorum sensing in resistance towards various antimicrobials. In a previous study by Helena *et al.*, [138] the effect of *luxS* mutant on the expression of bacteriocin genes was explored and was in-line context with our present data except the *bsmH* gene which was found to be upregulated in their study. This can be attributed to the fact *luxS* might be regulating the expression of *bsmH* in an alternative way and not through the two component ComDE TCTS system. Similar reports by Banu *et al.*, [139] showed the downregulation of *bsmH* as well as other bacteriocin related genes in *pknB* mutant strains. They have speculated that *pknB* modulates the activity of ComDE TCTS system. On the other hand, as expected, treatment of MTCC 497 and SM4 strains with ComAI, resulted in downregulation of the genes involved in competence development and bacteriocin production through ComDE quorum sensing pathway. The effect of ComAI further transcended till stationary phase indicating that the effect is not temporary and has effect at later stages of growth in *S. mutans*. The *comA* gene was found to be downregulated as *S. mutans* enters from early to mid and then stationary
phase as a result of positive feed-back loop present in ComDE QS pathway. Interestingly, on exposure with fluoride alone, the relative expression of the genes was found to be at basal level when compared with the control samples. This signifies that fluoride does not have any effect on ComDE pathway and it might be affecting alternative pathway involved in the EPS production as well as sucrose metabolism.

MTT assay was carried out to evaluate the potential toxicity of ComAI in cell lines before proceeding for *in vivo* acute oral toxicity in Wistar rats. The *in vitro* cytotoxic results revealed that ComAI was not found to have any toxic effect on mammalian cell lines making it suitable for validating the drug *in vivo*. In acute oral toxicity studies, the rats were found to be healthy till the highest dose used (2000mg/kg/PO) as per OECD 425 guidelines. This shows that ComAI does not have short term as well as long term toxic effects.

The efficacy study was carried out to evaluate whether the antibiofilm and cariostatic activity of ComAI would be similar to that of *in vitro* study, with widely used Wistar rat model for dental caries study *in vivo* [140, 141]. The topical application of ComAI significantly reduced the formation of biofilm and effectively decreased the incidence of dental caries when compared with disease control (p <0.05) confirming previous *in vitro* findings. The anti-caries property of ComAI was observed even at its brief exposure of efficacious concentration in the presence of sucrose-rich diet when ingested by the animals as compared to the disease control (p<0.05). The ability of topically applied ComAI to have persistent anti-caries effect is a desirable characteristic of a novel chemotherapeutic agent targeting biofilm oriented dental diseases such as dental caries [142]. It is noteworthy that colony counts of total microflora of oral cavity and the SM4 *S. mutans* were not affected which approves well with its lack of antibacterial activity against biofilm results of our previous findings. Furthermore,
these observations indicate that the caries preventive mechanisms of ComAI may be related to its effects on ComA in quorum sensing circuit resulting in downregulation of several virulence attributes of *S. mutans*, such as biofilm formation and bacteriocin production. Combinatorial study group in rodent model showed that ComAI in combination with fluoride enhance the anti-cariogenic effect of fluoride thus, signifies its potential clinical application to reduce the prevalence of dental caries at lower concentrations without increasing the concentration of fluoride exposure. As mentioned earlier, fluoride does not alter the expression of genes involved in quorum sensing of *S. mutans*. In the previous reports, fluoride levels found in plaque, affects the glycolytic activity and production of Gtfs via., disrupting the proton permeability of the cell membrane in *S. mutans* [141, 143]. The intracellular polysaccharides (IPS) are metabolized by oral pathogens when external sources of fermentable carbon have been depleted in the oral cavity. Thus, IPS promotes the occurrence of carious lesions by enhanced exposure of tooth surfaces to lower pH in the biofilm niche. IPS is synthesized as result of ATP pools in cells of biofilm matrix. Fluoride significantly reduces the ATP pools and results in substantial reduction in IPS synthesis and as a result reduces the incidence of lower pH in oral cavity. In addition, fluoride also enhances the remineralization process and cause reduction in the demineralization process at the tooth-biofilm interface. The present data not only corroborate previous *in vitro* findings but also support the hypothesis that interfering with the quorum sensing circuit possibly results in the reduction of caries through downregulation of virulence attributes such as biofilm phenotype.

Liver is one of the major organs involved in the detoxification of body. It is necessary to evaluate the long term effects of higher doses of ComAI on hepatocytes to eliminate any toxicity pattern involved with administration of ComAI. In the present
study, liver sections did not show any degenerative signs thus, proving the administration of ComAI (upto 10 X doses for long term might not be toxic to the recipients at clinical stages. Histopathological examination of teeth and absence of lesions in ComAI treated group provides evidence that topical applications of ComAI and in combination with fluoride have cariostatic effect. In disease group, the caries had penetrated enamel (decalcified), dentine and reached the depth of teeth i.e., pulp (soft connective tissue) [Figure 5.7]. Entrance of S. mutans in pulp can lead to the invasion of bacteria to the blood stream causing systemic pro-inflammatory response in the body [144]. A review study by Esser et al. [145], has reported the potential link between low grade chronic inflammation and systemic diseases such as obesity and Type 2 Diabetes. High level expression of inflammatory response may lead to the development of systemic diseases such as cardio-vascular disease, rheumatoid arthritis, type 2 Diabetes, premature birth of babies and so on [146]. To investigate the above relationship, the expression levels of inflammatory markers such as TNF-α, IL-6, IL-1, and CRP were analysed in real-time using qRT-PCR in both plasma as well as liver tissues. The liver has a remarkable capacity to adapt to the injury stress through tissue repair as compared to any other solid organ in the body. Complex interactions of the immune cell subsets regulate tissue repair process. Levels of pro-inflammatory cytokines TNF-α, CRP, IL-1 were significantly downregulated in case of plasma as compared to liver, attributing to the fact that, cytokines are more readily diffused. The present data was in concordance with the earlier studies, where the ComAI mediated biofilm inhibition may prevent the formation of carious lesions and further inhibit the invasion of S. mutans in the pulp.
5.5 Conclusion

Collectively, the data in our present study shows that ComAI reduces the incidence of caries development by targeting the ComA in quorum sensing pathway of *S. mutans* thus, further affects the major virulence factors such as biofilm formation, bacteriocin production without causing mortality of bacteria. ComAI along with lower concentrations of fluoride could be used as a potential cariostatic measure to reduce the incidence of caries without affecting the remineralization property of fluoride. The combination of ComAI with fluoride at lower concentrations may provide a potential substitute to the current chemotherapeutic approaches to prevent the incidence of dental caries. In addition, the prevention of caries also results in the reduction of inflammatory markers as shown in this study at pre-clinical stages.