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Efficacy of ascorbic acid against biofilm formation by Streptococcus mutans - an in vitro study

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Abstract:
Background*: Dental caries still contributes a huge share of the burden of oral diseases in India despite the existence of several preventive strategies. Being the second highly populous country, Vitamin C, if found effective, would be the most cost effective, easily accessible and socially acceptable alternative to the existing preventive measures of dental plaque associated caries infection. The objectives of the study were to determine Biofilm Prevention Concentration(BPC) and Biofilm Eradication Concentration(BEC) of ascorbic acid against Streptococcus mutans.*
Materials and Methods*: Diluted solutions of L-ascorbic acid($C_6H_8O_6$) and S. mutans MTCC890 strain were used for the study. Micro titration plates were prepared and incubated for 24 hrs at 35 ^{0} C, with concentrations of ascorbic acid at 15-30 mg/ml. MIC and MBC were determined using MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Exopolysaccharide(EPS) analysis and crystal violet biofilm assay were then performed to assess biofilm formation at different concentrations of ascorbic acid.*
Results*: The increasing diameter of zone of inhibition of Streptococcus mutans were found to be proportional to the increasing concentrations of ascorbic acid. MIC and MBC were observed at 19 mg/ml. In the EPS analysis at 25mg/ml, the biofilm eradication was observed to be 91.5%. In the crystal violet biofilm assay, at 19 mg/ml, ascorbic acid was found to have effective biofilm prevention properties.*
Conclusion: *It reflects the prospective role of ascorbic acid as a potential therapeutic agent against biofilm formation by S.mutans. Further studies are needed to establish vitamin C as a promising preventive agent for dental caries.*
Key Word*: Biofilm, Streptococcus mutans, ascorbic acid, Crystal violet assay, EPS analysis.*

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I. Introduction

Dental caries and periodontitis, the most common oral infections, affect all population, irrespective of gender, age or socioeconomic status. This situation is more distressing in developing countries due to lack of access to oral health care services and preventive measures like fluorides[1].Globally, oral health is very gradually improving, despite the development of increasingly sophisticated technologies in the prevention and treatment of these diseases[2]. The costs associated with conventional treatment of caries and periodontal diseases are also enormous[3].

The infectious diseases caused by biofilm forming microbes are the most difficult to eradicate in humans. In oral cavity, the first stage to develop dental caries or periodontal disease is to obtain an oral biofilm that is called "dental plaque"[4]. When organisms form a biofilm, they are able to adapt to environmental change by altering their gene expression patterns. The biofilm structure and corresponding change in gene expression can protect the microbes from antimicrobials[5].

Ascorbic acid, also known as ascorbate or vitamin C is a water-soluble ketolactone with two ionizable hydroxyl groups[6]. It is used to prevent and treat scurvy and also has implications in cancer and chronic disease prevention attributed to its antioxidant effects[7].However the antibacterial and anti biofilm effects of ascorbic acid has largely remained underexplored.

In the study done by Isela S R et al (2013)[6], at 20 mg/ml, ascorbate was found to inhibit the oral biofilm formation by *Streptococcus mutans*, *Staphylococcus aureus*, *Porphyromonas gingivalis*, *Candida albicans* and *Enterococcus faecalis* added at the inoculation time. In the study done by Pandit S et al(2017)[8], quantitative proteomics analysis revealed that specific concentrations of vitamin C inhibit bacterial quorum sensing and other regulatory mechanisms underpinning biofilm development of Bacillus subtilis. As a result, the extracellular polymeric substance biosynthesis is reduced beyond a critical point and bacterial cells get fully

exposed to the medium. At this stage, the cells are more susceptible to killing, either by vitamin C-induced oxidative stress, or by other antimicrobials or treatments.

Since Streptococcus mutans is the key causative organism associated with oral biofilm formation, the current study aimed to assess the efficacy of ascorbic acid against the biofilm formation by Streptococcus mutans.

II. Materials And Method

It was an invitro study conducted at Dextrose Technologies Private Limited, Bangalore urban, Karnataka. L-ascorbic acid($C_6H_8O_6$ – powder form) was purchased from Bangalore fine chemicals, Bengaluru and the solution was prepared at Dextrose technologies Pvt.Ltd Laboratory.

Materials used:

1. Streptococcus mutans strain MTCC890
2. Diluted concentrations of ascorbic acid
3. Brain Heart Infusion broth
4. Agar plates
5. Vernier calipers
6. Trichloroacetic acid
7. Ethanol
8. Concentrated Sulphuric acid
9. Crystal violet dye
10. TSB medium
11. 33% acetic acid
12. Spectrophotometer

Methodology:

Sample preparation: *S. mutans* MTCC890 was stored and preserved at Dextrose technologies Pvt.Ltd Laboratory, Bangalore. The sample was sub-cultured in BHI broth and stored at 37℃ incubation until further use.

Zone of inhibition Test: Anti-bacterial activity was evaluated by well diffusion technique. After, autoclaving the plates were allowed to dry and 6mm wells were punctured on the surface of the agar plate. The agar plates were seeded with 100µl of the inoculums and spread evenly over the plate with a sterile glass spreader. Each sample (10mg, 15mg, 20mg, 25 mg, 35mg, 40mg) were added to separate wells in the culture plates and incubated at 30℃ for 24hrs. After 24 hrs of incubation diameter of the zone of inhibition was measured to nearest millimeter (mm) using a vernier caliper.

Biofilm Assay:

EPS extraction and estimation:

The protein content of the EPS was separated by precipitating it with 25% (w⁄v) trichloroacetic acid on ice for 2h. The reprecipitation of EPS was carried out by addition of two volumes of ice cold ethanol. The precipitate was dried overnight at 70°C. This precipitate was dissolved in hot D/W and further used for the estimation of exopolysaccharide quantity[9].

Exopolysaccharides were estimated as total carbohydrates by phenol-sulphuric acid method[10]. To 1 ml of sample, 1 ml 5% (w/v) phenol was added followed by 5 ml concentrated sulphuric acid. The sample tubes were kept in ice while adding sulphuric acid. The mixture was incubated at room temperature for 20 min and the absorbance was read at 490 nm. Glucose was used as the standard in the range of 20-100 µg concentration. A standard graph was plotted with absorbance at 490 nm against concentration of glucose.

Crystal Violet Assay:

S. mutans biofilm was formed on glass test tubes. Briefly, A 100 µl of cell 10 suspension having 0.5 O.D600 nm was inoculated in 20mL TSB medium in each test tube. Then the test tubes were incubated for 16 h at 37^0 C. After aspiration of planktonic cells biofilms were fixed with 99% methanol. Plates were washed twice with phosphate buffer saline or sterile saline water and air-dried. Then, 200 µl of crystal violet solution (0.2%) was added to all wells. After 5 min, the excess crystal violet was removed and plates were washed twice and air dried. Finally, the cell bound crystal violet was dissolved in 33% acetic acid[11]. Biofilm growth was monitored in terms of O.D 530 nm using spectrophotometer (Multiskan, Thermo Lab systems).

MIC: Microbial Susceptibility was also tested by a spectrophotometric method using the dye MTT. Micro titration plates were prepared and incubated for 24 h at 35^0 C, with the concentrations 15-30mg/ml. Then, 25 µl

of MTT was added at the above-mentioned concentrations to each well. After 30 min of incubation at room temperature and gentle agitation, the OD was measured spectrophotometrically at 660 nm.

MBC: The same sample was also plated on to TSA agar by adding 100µL sample and incubated at 37C for 24 hours. After incubation the total viable colonies were counted and noted.

Statistical analysis: Descriptive statistics was used to analyze the data. Data analysis was done using SPSS software Version 25.0.

III. Results

Biofilms are dense micro-communities that grow on surfaces and encapsulate themselves with secreted polymers [12]. Bacteria attached in a biofilm manufacture extracellular polysaccharides and proteins not found in free floating cells creating extracellular polymeric substances. They play a major role in maintaining the integrity of the biofilm and as well as preventing desiccation and attack by harmful agents [13]. The resultant biofilm develops resistance to antimicrobials and can pose a serious public health issue.

The current preventive strategies and programmes have not produced the desired outcome in our country. Provision of professional dental services and fluoride resources has always remained major challenges in a highly populous country as India. Most of the available services have also remained largely underutilized. Anti microbial resistance of pathogenic microorganisms is also one of main problems in modern medicine today.

Vitamin C, if proven effective, could be the most cost effective, easily accessible and socially acceptable alternative to the contemporary preventive strategies in a highly populous country as India.

Among the microorganisms involved in oral biofilm formation, Streptococcus mutans, which is a renowned member of the Streptococci group and naturally found in the oral cavity, is broadly accepted to be a major dental caries pathogen[14].

The present study aimed to assess the Minimum Inhibitory Concentration(MIC), Minimum Bactericidal Concentration(MBC), biofilm prevention concentration(BPC) and biofilm eradication concentration(BEC) of ascorbic acid against Streptococcus mutans.

Table 1 shows the Zone of inhibition diameter in mm at different concentrations of ascorbic acid ranging from 15-40 mg/ml. Zone of inhibition was observed at 15 mg/ml of ascorbic acid and the diameter was approximately 14.5 mm. As the concentration of ascorbic acid increased, the zone of inhibition also exhibited an associated increase of diameter. At 25 mg/ml, the zone of inhibition diameter was 19 mm.

Table 1: Zone of inhibition diameter in mm at different concentrations of ascorbic acid ranging from 15-40 mg/ml

Concentration (mg/ml)	Zone of Inhibition diameter (mm)
5	-
10	-
15	14.5
20	16
25	19
30	21.5
35	22
40	23

Table 2 exhibits the minimum inhibitory concentration(MIC) of ascorbic acid at different concentrations ranging from 15-30 mg/ml, spectrophotometrically quantified at 660 nm. The corresponding biofilm prevention percentage and minimum bactericidal concentration(MBC) have also been depicted. Minimum bactericidal concentration has been determined considering the colony forming units per ml. The corresponding values of negative and positive control samples have also been given in the table. At 19 mg/ml of ascorbic acid, the MIC was observed to be 0.020 with a biofilm prevention percentage of 97.9% and MBC at 4 CFU/ml. At 25 mg/ml, MIC stood at 0.012 with a biofilm prevention percentage of 98.7% and MBC at 2 CFU/ml.

Table 2: Minimum Inhibitory Concentration, Minimum Bactericidal Concentration and biofilm prevention percentage at different concentrations of ascorbic acid ranging from 15-30 mg/ml

Concentration (mg/ml)	**MIC at 660 nm**	**Biofilm prevention %**	**MBC CFU/mL**
-Ve Control (Only medium)	0.00	-	-
+Ve Control (Chlorhexidine)	0.967	0	$9.34x10^3$
15	0.643	33.5	$0.61x10^3$

16	0.241	75.0	$1.22x10^2$
17	0.134	86.1	$1.1x10^2$
18	0.115	88.1	$1.3x10^2$
19	0.020	97.9	4
20	0.020	97.9	4
21	0.018	98.1	3
22	0.011	98.8	2
23	0.011	98.8	2
24	0.011	98.8	2
25	0.012	98.7	2
26	0.004	99.5	-
27	0.002	99.7	-
28	0.003	99.6	-
29	0.003	99.6	-
30	0.003	99.6	-

Graph 1: Bar chart depicting biofilm prevention percentage at different concentrations of ascorbic acid ranging from 15-30 mg/ml

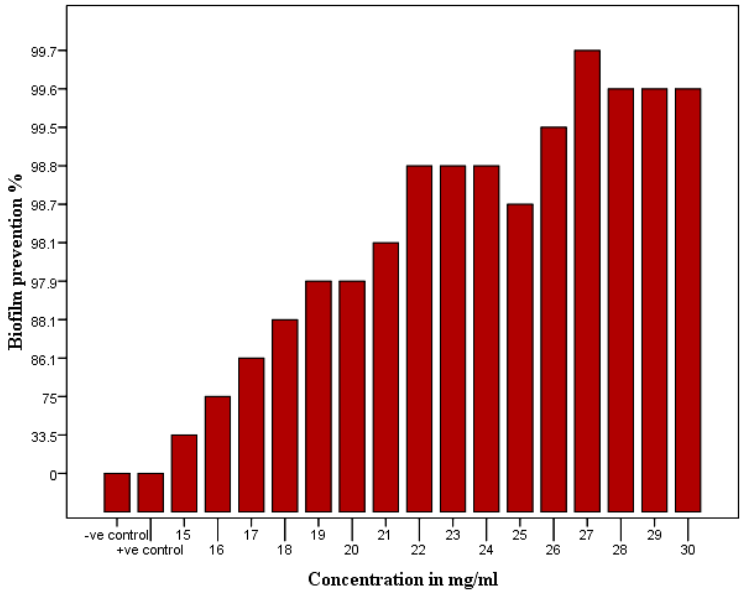
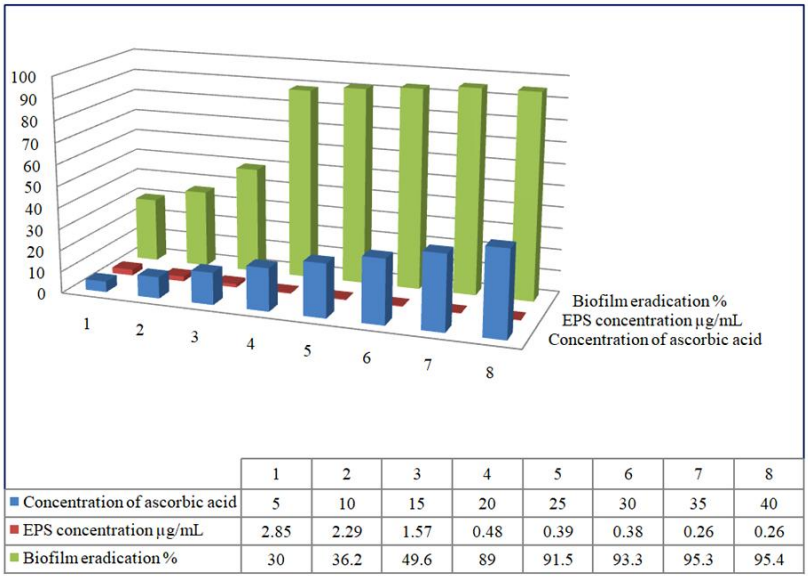


Table 3 shows the results of exopolysaccharide extraction and estimation (EPS analysis). EPS concentration in µg/mL and biofilm eradication percentage are provided in the table corresponding to different concentrations of ascorbic acid ranging from 5-40 mg/ml. The EPS concentration was 0.48 µg/mL and biofilm eradication percentage was 89% at 20 mg/ml of ascorbic acid. At 40 mg/ml, the EPS concentration was 0.26 µg/mL with biofilm eradication percentage as high as 95.4%.

Table 3: Exopolysaccharide concentration and biofilm eradication percentage at different concentrations of ascorbic acid ranging from 15-40 mg/ml through EPS extraction and estimation

Concentration (mg/ml)	OD at 490 nm	EPS concentration µg/mL	Biofilm eradication %
-Ve control (Only medium)	0.020	0.098	-
+Ve control (Chlorhexidine)	0.787	3.86	0
5	0.581	2.85	30.0
10	0.467	2.29	36.2
15	0.321	1.57	49.6
20	0.098	0.48	89.0
25	0.081	0.39	91.5
30	0.078	0.38	93.3
35	0.054	0.26	95.3
40	0.054	0.26	95.4

Graph 2: Bar chart depicting the EPS concentration and biofilm eradication percentage at different concentrations of ascorbic acid ranging from 5-40 mg/ml



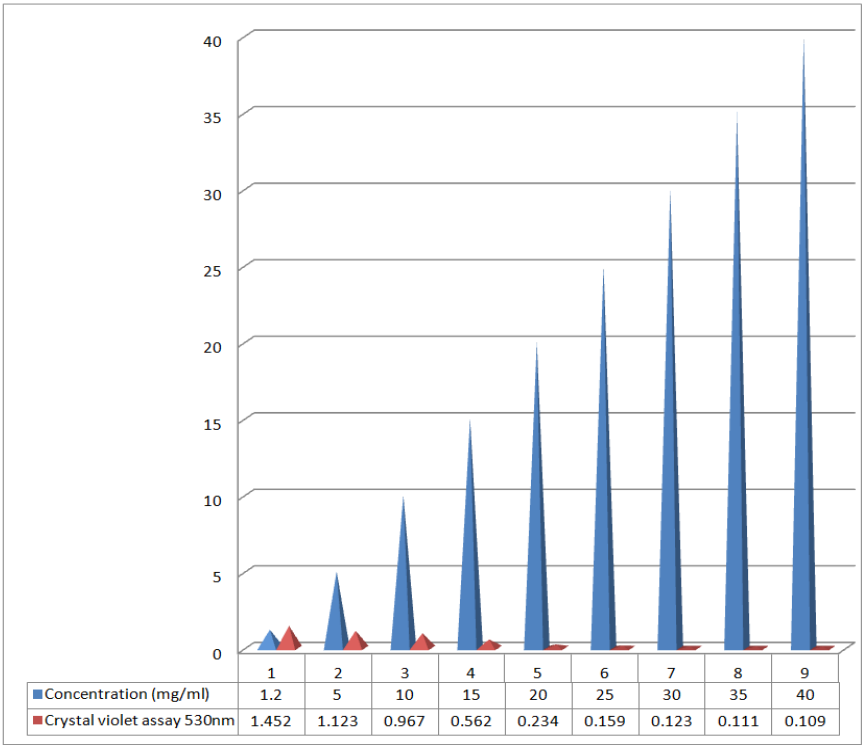
	1	2	3	4	5	6	7	8
Concentration of ascorbic acid	5	10	15	20	25	30	35	40
EPS concentration µg/mL	2.85	2.29	1.57	0.48	0.39	0.38	0.26	0.26
Biofilm eradication %	30	36.2	49.6	89	91.5	93.3	95.3	95.4

Table 4 show the results of crystal violet biofilm assay. Biofilm formation was quantified spectrophotometrically at 530 nm. At 20 mg/ml, the crystal violet assay exhibited a value of 0.234. The EPS analysis and crystal violet biofilm assay showed correlating results.

Table 4: Results of crystal violet biofilm assay at different concentrations of ascorbic acid ranging from 15-40 mg/ml at an optical density of 530 nm

Concentration (mg/ml)	**Crystal violet assay 530nm**
-Ve control(Only medium)	0.043
+Ve control(Chlorhexidine)	1.452
5	1.123
10	0.967
15	0.562
20	0.234
25	0.159
30	0.123
35	0.111
40	0.109

Graph 3: Chart depicting the Crystal violet biofilm assay reading at an optical density of 530 nm at different concentrations of ascorbic acid ranging from 5-40 mg/ml



IV. Discussion

The importance of Vitamin C, also known as ascorbic acid, was first realized more than 250 years ago, when a shortage of it was discovered to be the cause of scurvy. Ascorbic acid is vital in the formation of collagen and also works as a potent antioxidant. Since primates and humans lack the required enzyme to produce vitamin C, it has to be obtained in adequate amounts from external sources.

Though the anti oxidant properties of ascorbic acid are well known, the antimicrobial and anti biofilm activities have largely remained underexplored. This study attempts to offer invitro experimental evidence that ascorbic acid has associated anti cariogenic potential.

From the results obtained, the average diameter of zone of inhibition was 14.5 mm, 16 mm, 19 mm and 21.5 mm at concentrations of 15, 20, 25 and 30 mg/ml respectively. As the concentrations of ascorbic acid increased, there was a proportional increase in the zone of inhibition diameter against Streptococcus mutans.

Serial dilutions of ascorbic acid from 5-30 mg/ml were then employed for determination of MIC, MBC, biofilm prevention concentration and biofilm eradication concentration. MIC and MBC were analyzed using MTT dye and subsequent colonial formation on TSA agar. MIC was observed spectrophotometrically at optical density of 660nm and MBC was observed as Colony forming units per ml.

The MIC value of ascorbic acid was quite significant at 19 mg/ml (0.020). At 25 mg/ml the value was observed at 0.012 and at 30 mg/ml it was observed at 0.003. However, the observed value is higher when compared to the study done by Isela S R et al(2013)[6], where ascorbic acid was shown to inhibit microbial growth at 10 mg/ml.

The biofilm prevention percentage was as high as 97.9% at 19 mg/ml which elevated to 98.7% at 25 mg/ml and 99.6% at 30 mg/ml. The MBC was observed at 4 CFU/ml at 19 mg/ml of ascorbic acid. It was revealed to be 2 CFU/ml at 25 mg/ml of ascorbic acid and at increasing concentrations the colony forming units almost zeroed out. The results suggest that ascorbic acid could be utilized in low but effective concentrations to prevent caries formation in the oral cavity. In the study done by Eydou Z et al(2020)[11], the biofilm prevention concentration was observed at 5.61 mg/ml which was much lower than the findings of the current study.

It is well documented that dental plaque is significantly associated with the initiation of caries development. Dental plaque is considered to be an oral biofilm with Streptococcus mutans being the key causative microorganism of biofilm formation. Hence the anti biofilm properties of ascorbic acid were also assessed with the help of exopolysaccharide (EPS) analysis and Crystal violet assay.

Microbes produce a biofilm matrix consisting of proteins, extracellular DNA, and polysaccharides that is integral in the formation of bacterial communities. The polysaccharide component of the matrix can provide many diverse benefits to the cells in the biofilm, including adhesion, protection, and structure. Aggregative

polysaccharides act as molecular glue, allowing the bacterial cells to adhere to each other as well as surfaces[15]. Hence quantification of exopolysaccharides would provide a direct measure of biofilm formed and the adhesion potential of microorganisms.

In the EPS analysis, at 20 mg/ml of ascorbic acid, EPS concentration was observed to be 0.48 µg/mL with a biofilm eradication of 89%. At 40 mg/ml, EPS concentration was 0.26 with biofilm eradication of 95.4%.

In the crystal violet biofilm assay, biofilm formation was quantified using spectrophotometric method at 530 nm. At 20 mg/ml, the value stood at 0.234 while at 40 mg/ml the biofilm formation exhibited a value of 0.109.

As compared to the positive control, which was chlorhexidine(1.2 mg/ml) in this case, the results obtained in association with ascorbic acid were significantly higher as compared to the standard concentration of chlorhexidine currently used to prevent microbial proliferation in the oral cavity.

The study clearly indicates that ascorbic acid is associated with effective caries prevention potential. The social acceptability, cost effectiveness and non toxic nature of ascorbic acid makes it a better alternative to the current anti caries agents.

The limitations of the study must be taken into consideration. The study is primarily invitro in design and thus requires further invivo studies to support or refute its findings. Moreover, oral biofilm is a complex structure involving a community of microorganisms. This study has focused only on one species involved in biofilm formation, Streptococcus mutans. Further studies are needed to assess the efficacy and efficiency of ascorbic acid against other microorganisms forming as essential part of the dental plaque.

V. Conclusion

19 mg/ml of Ascorbic acid shows MIC and reduction in biofilm. EPS and crystal violet assay also shows correlating results. The study thus concludes that ascorbic acid could be considered as an effective anti microbial and anti biofilm agent. Further studies are needed to establish the role of ascorbic acid as an effective anti cariogenic agent. If proven cost effective, ascorbic acid could turn out to be a very important alternative to conventional preventive strategies.

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