Antimicrobial and Antibiofilm Effect of Cranberry Extract on Streptococcus mutans and Lactobacillus acidophilus: An In Vitro Study

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Abstract

Background: Nature has been a source of medicinal treatments since millennia and plant-based systems continue to play an essential role.

Aim: To study the antimicrobial and antibiofilm effect of cranberry on Streptococcus mutans and Lactobacillus acidophilus.

Materials and methods: The ethanolic extract of cranberry was tested against standard MTCC strains of S. mutans (MTCC 25175) and L. acidophilus (MTCC 8129) for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The time kill assay was performed to check the time-dependent bactericidal effect of the cranberry extract on microorganisms. Percentage of cell adhesion and biofilm inhibition of the dental microorganism at various doses of cranberry extract was measured by a spectrophotometer and biofilm morphology characteristics were observed under scanning electron microscopy. All the tests were carried out in triplicates. Data were computed in the SPSS software and mean/SD was determined. The results are presented in a descriptive manner; Kruskal–Wallis analysis of variance (ANOVA) and the Friedman's test were applied for comparative evaluation of the groups. p value <0.05 was considered statistically significant.

Results: The results showed that MICs of cranberry extract against S. mutans and L. acidophilus are 12.5 mg/dL and 6.125 mg/dL, respectively, and MBCs are 25 mg/dL and 125 mg/dL, respectively. A significant decrease in the biofilm formation and cell adhesion of microorganisms at MIC (50%) and MBC (70%) was observed as compared to control as observed under a spectrophotometer and a scanning electron microscope.

Conclusion: This study has identified bactericidal, bacteriostatic, and antibiofilm effects of cranberry extract against S. mutans and L. acidophilus in a time-dependent and dose-dependent manner.

Keywords: Cranberry, Minimum bactericidal concentration, Minimum inhibitory concentration.

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Introduction

The oral cavity is an open growth system with an uninterrupted introduction and removal of microbes and their nutrients. The dental biofilm, found on hard surfaces in the oral cavity, harbors cariogenic bacteria, cell-free enzymes, polysaccharides, and host constituents.1

Biofilms have been implicated in the etiopathogenesis of dental caries. The pioneer microorganisms implicated in the initiation and progression of dental caries are Streptococcus mutans and Lactobacilli acidophilus, respectively. Furthermore, with time biofilms have the potential to calcify into dental calculus, which is difficult to remove and requires professional help.2

There are various chemicomechanical agents used for biofilm or plaque removal. Mechanical agents such as toothbrush and other interdental aids have numerous drawbacks such as varying individual manual dexterity, force, lack of motivation, and inability to reach proximal surfaces.3

The most effective chemical plaque-control agent is chlorhexidine that is considered as the gold standard. Its effectiveness can be attributed to its bactericidal and bacteriostatic properties and its substantive properties within the oral cavity; despite all this, chlorhexidine has a lot of side effects like extrinsic tooth staining, loss of taste, discoloration, parotid swelling, and microbial resistance, which make its long-term use unfavorable.4–7

So the search is on for natural antimicrobial agents that have less side effects and are equally effective. Deep red and luscious-tasting cranberry is a native, North American fruit. It is widespread throughout the cool temperate northern hemisphere, including northern Europe, northern Asia, and northern North America.

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Preparation of Extract

Cranberry is used as fresh fruit, juice, sauce, and also as a medicinal agent to prevent diabetes, stomach pain, diarrhea, atherosclerosis, cholesterol, etc. Cranberry juice is most recommended against UTI agents and is safe to use.8

It contains polyphenols, vitamins, proteins, flavonoids, and other rare phytochemicals that contribute to its antimicrobial, anti-inflammatory, and antitumor activities.9

This study aims to explore its benefits against dental microorganisms and their biofilm in vitro.

Materials and Methods

Preparation of Extract

Fresh cranberry (Vaccinium macrocarpon) was collected from a local market of Mumbai in July–August 2016. The fruits were stored
Antimicrobial and Antibiofilm Effect of Cranberry Extract on *Streptococcus mutans* and *Lactobacillus acidophilus* under sterile conditions (Fig. 1).

The residue left was semisolid, reddish-pink in color, which was to be further evaporated and to obtain a semisolid consistency. Whatman filter paper no. 40. The filtrate was evaporated under a vacuum rota-evaporator. This filtrate was kept on the water bath to be further evaporated and to obtain a semisolid consistency. The residue left was semisolid, reddish-pink in color, which was soluble in aqueous solution. It was stored at 4°C in a vacutainer.

Around 20 g of the dried fruit was macerated with 200 mL of a hydroalcoholic solvent with a ratio of ethanol (70%):water (30%) in a conical flask. The extract was kept on a rotary shaker at 190–220 rpm for 48 hours and the macerated liquid was filtered through Whatman filter paper no. 40. The filtrate was evaporated under a vacuum rota-evaporator. This filtrate was kept on the water bath to be further evaporated and to obtain a semisolid consistency. The residue left was semisolid, reddish-pink in color, which was soluble in aqueous solution. It was stored at 4°C in a vacutainer under sterile conditions (Fig. 1).

**Antibacterial Test**

**Minimum Inhibitory Concentration**

Around 200 μL of the plain brain heart infusion (BHI) broth was added from 2nd to 10th tube by using a micropipette. It was followed by 200 μL of respective cranberry extract that was added to first and second tubes. The broth and cranberry extract solution were thoroughly mixed using a micropipette. Further 200 μL of diluted cranberry extract was pipetted and added to the third tube. Like this, serial dilution started from the second tube and was continued till the ninth tube. Finally, 20 μL of 0.5 McFarland standard inoculums of respective microorganisms were added to all the 10 tubes. Microcentrifuge tubes were incubated in an incubator aerobically for 24 hours.

**Minimum Bactericidal Concentration**

After the incubation periods, the lowest concentrations of the extract that did not produce any bacterial growth on the solid medium were regarded as MBC values for this extract. Plates were incubated at 35°C for 24–48 hours and then scanned and counted. Colonies were counted as CFU/mL and converted to log_{10} values over a period of time and compared with the control. Bacterial carryover was minimized by dilution. Extracts were considered bactericidal when a ≥3 log_{10} decrease in CFU/mL was reached compared with the initial inocula. Colony counts were performed in triplicates, and means were taken.

**Effect of Cranberry Extract on Biofilm Formation**

The effect of the cranberry extract on *S. mutans* and *L. acidophilus* biofilm formation was measured by the method modified from that of Li et al. Overnight-grown *S. mutans* and *L. acidophilus* were diluted in the BHI broth to obtain an optical density (OD) of 0.2 (about 10 CFU/mL). The wells of a sterile 24-well tissue culture plate, each contained 10 μL of such cell suspension and 190 μL of BHIS broth with different cranberry concentrations at MIC and MBC. Each concentration contained three parallel samples, and the BHIS broth without the cranberry extract was used as a control. After 24 hours, the supernatant from each well was aspirated, and the biofilm in each well was mixed with methanol for 15 minutes, stained with crystal violet (0.5%) for 30 minutes, and washed three times with distilled deionized water to remove the unbound crystal violet. After that, 200 μL of 100% ethanol was added to each well to dissolve the crystal violet on the biofilm. The dead bacterial cells were washed away and the live bacterial cell took up the crystal violet stain that absorbed the light. The plate was rocked at room temperature for 20 minutes, and the absorbance was read at 600 nm by a spectrophotometer.

**Effect of Cranberry Extract on Biofilm Morphology**

The structures of *S. mutans* and *L. acidophilus* biofilms formed in the presence of the cranberry extract were observed by a scanning electron microscope (SEM) (Bitoun et al. and Jongsma et al.). Around 10 μL of overnight-grown *S. mutans* suspension diluted in fresh BHI at an initial OD of 0.2 and 1900 μL of the BHIS broth with different concentrations of the cranberry extract at MIC and MBC were added to the wells of a 24-well tissue culture plate. Each concentration contained three parallel samples, and BHIS and sucrose broth without the cranberry extract were used as a control. Glass coverslips (5 mm in diameter) were prepared in each well. After incubation for 24 hours, the biofilm-coated glass coverslips were immersed in 2.5% glutaraldehyde at 4°C overnight, washed three times with distilled deionized water, dehydrated using ascending graded series of ethanol (30%, 50%, 70%, 80%, 85%, 90%, 95%, and 100%), and coated with gold. The samples were then examined by a SEM.

Figs 1A to C: (A to C) Effect of the cranberry extract on biofilm morphology of *S. mutans* incubated for 24 hours in the BHIS broth at control (without cranberry), MIC, and MBC as observed under scanning electron microscopy. Magnification was 5,500×, 4,300×, and 20,000×, respectively, for each concentration.
RESULTS

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The results of the present study show that the ethanolic extract of cranberry has MICs of 12.5 mg/dL and 6.125 mg/dL and MBCs of 25 mg/dL and 12.5 mg/dL against S. mutans and L. acidophilus, respectively (Table 1).

Time Kill Assay

The number of viable CFU/mL of S. mutans and L. acidophilus showed greater than 3log_{10} drop in viability over the period of 24 hours compared to the control at both MIC and MBC of the cranberry extract (Table 2). The killing activity depended on time and concentrations of the cranberry extract. The control group showed progressive increase in CFU/mL, whereby, 1x MIC and 1x MBC reduce the number of CFUs by approximately 50% after 10 hours of incubation. Although, complete sterility was not achieved, reduction in the bacterial count was statistically significant at both the concentrations compared to the control.

Biofilm Inhibition

When bacteria were allowed to attach and form biofilms for 24 hours before treatment, exposure to the cranberry extract for an additional 24 hours resulted in a 50% (p < 0.05) reduction of preformed biofilm (compared to untreated control), whereby 1x MIC reduced the number of CFUs by approximately 50% and 1x MBC led to reduction of biofilm to 70% after 24 hours of incubation. Control cell suspensions without the cranberry extract showed no drop in viability over the same period. The concentrations of the cranberry extract required to inhibit >50% biofilm formation (MBIC50) of S. mutans and L. bacillus were 16.67 (±7.21) and 8.33 (±3.60) mg/dL, respectively, and for >70% inhibition of biofilm growth (MBIC70) the concentrations were 20.83 (±7.21) and 10.416 (±3.60) mg/dL, respectively (Table 3).

Scanning Electron Microscope

In addition, under scanning electron microscopy, it was observed that a biofilm was relatively thick and homogeneous without the cranberry extract as compared with the samples treated with cranberry at different concentrations (Figs 1A and 2A). As the concentration of the extract increased, the biofilm integrity and structure was gradually disrupted. Moreover, this disruption to biofilm integrity was also dosage dependent. At MIC, S. mutans and L. bacillus biofilms (Figs 1B and C and 2B and C) became very sparse and could not cover the surface of the slips, and at MBC the bacteria cluster became much smaller showing clumping and cell degradation compared with the control group.

DISCUSSION

With the emergence of antimicrobial resistance to the currently available drugs, there has been a rise in the demand of new natural and safe antimicrobial agents for the control of the plaque/biofilm and its harmful implications like caries and periodontal diseases in the oral cavity.\(^{17}\) Disruption or removal of biofilm becomes a decisive component in the overall outcome of any good antiplaque agent and prognosis of the dental diseases.\(^{18}\)

Cranberries offer a rich source of plant-based polyphenols particularly proanthocyanidins and flavonols. Polyphenols contribute to the various properties like bitterness, astringency, color, flavor, odor, and oxidative stability. Meta-analyses strongly suggested that long-term consumption of diets rich in plant polyphenols offered protection against many diseases like cancers, cardiovascular diseases, diabetes, osteoporosis, and neurodegenerative diseases.\(^{19}\)

| Table 1: The MIC and MBC of the cranberry extract against MTCC of S. mutans and L. acidophilus |
|-----------------|-----------------|-----------------|
| Microorganisms  | MIC (mg/dL) mean (±SD) | MBC (mg/dL) mean (±SD) |
|-----------------|-----------------|-----------------|
| 1 Streptococcus mutans (MTCC-25175) | 16.67 (±7.21) | 20.83 (±7.21) |
| 2 Lactobacillus acidophilus (MTCC-10307) | 8.33 (±3.60) | 10.416 (±3.60) |

MIC, minimum inhibitory concentration
MBC, minimum bactericidal concentration
MTCC, microbial type culture collection

| Table 2: Comparative evaluation of effectiveness of the cranberry extract using TKA at MIC, MBC, and control at 0, 3, 6, 12, and 24 hours |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Lactobacillus acidophilus | 0 hour mean (±SD) | 3 hours* mean (±SD) | 6 hours** mean (±SD) | 12 hours*** mean (±SD) | 24 hours**** mean (±SD) |
| MIC | 5.35 (±0.01) | 4.59 (±0.064) | 3.23 (±0.019) | 1.19 (±0.01) | 0.23 (±0.005) |
| MBC | 5.30 (±0.077) | 4.42 (±0.006) | 2.91 (±0.039) | 0.6027 (±0.006) | 0.46 (±0.05) |
| Control | 5.18 (±0.007) | 5.41 (±0.006) | 5.62 (±0.003) | 5.73 (±0.041) | 5.5 (0.04) |
| p* value | 0.061 | 0.026* | 0.027** | 0.025*** | 0.024**** |
| Streptococcus mutans | 4.98 (±0.006) | 2.33 (±0.023) | 1.89 (±0.059) | 1.44 (±0.01) | 0.115 (±0.005) |
| MIC | 4.97 (±0.006) | 2.06 (±0.008) | 1.55 (±0.023) | 0.99 (±0.011) | 0.12 (±0.005) |
| MBC | 4.99 (±0.002) | 4.98 (±0.006) | 5.59 (±0.085) | 5.78 (±0.006) | 0.067 (±0.00) |
| p* value | 0.071 | 0.025* | 0.027** | 0.02*** | 0.02 |

p*, Kruskal Wallis ANOVA; p*, Friedman’s test; *p <0.05), statistically significant
MIC, minimum inhibitory concentration
MBC, minimum bactericidal concentration
MTCC, microbial type culture collection
* **, **** indicate p* values at different time intervals 3, 6, 12, 24 hours
Flavonols also have a potent effect on gram-positive bacteria.\textsuperscript{20} Moreover, considering that the microbial resistance has become an increasing global problem, there is a compulsory need to find out new potent antimicrobial agents as accessories to antibiotic therapy.

The results of the present study show that ethanolic extract of cranberry has MICs of 12.5 mg/dL and 6.125 mg/dL and MBCs of 25 mg/dL and 12.5 mg/dL against \textit{S. mutans} and \textit{L. acidophilus}, respectively.

This effect can be attributed to the fact that 65% polyphenols found in the nondialyzable material (NDM) component of the cranberry extract have shown antimicrobial properties. The high-molecular-weight NDM fraction is shown to reverse the coaggregation of the majority of bacterial pair. The NDM component of cranberry seems to affect the phosphorylation and expression of various intracellular proteins that are implicated in MMP production, which is responsible for biofilm formation and thus can be exploited as antibiofilm agents.\textsuperscript{21}

A study done by Neto et al. revealed upon analysis that NDM and subsequent subfractions revealed the presence of A-type proanthocyanidin (PAC) oligomers.\textsuperscript{22} Proanthocyanidin and flavonoid (FLAV) components of cranberry, alone or in combination, inhibit the surface-adsorbed glucosyl transferases and F-ATPases activities, and the acid production by \textit{S. mutans} cells. Furthermore, biofilm development and acidogenicity were significantly affected by topical applications of PAC and FLAV.\textsuperscript{23}

There was almost 50% and 70% reduction in the biofilm of \textit{S. mutans} and \textit{L. acidophilus} formation at both MIC and MBC. These result are similar to the study done by Yamanka et al. who found that the ability of \textit{S. mutans} to adhere to the hydroxyapatite decreased when exposed to the cranberry juice and the biofilm was reduced to 80–95%.\textsuperscript{24}

The persistence of action, or substantivity, of antimicrobial agents in the mouth appears to be a major variable influencing plaque inhibition. Such substantivity can be assessed by measuring the duration and magnitude of suppression of salivary bacterial numbers produced by antimicrobial agents.\textsuperscript{25} The present study revealed that the number of viable CFU/mL of \textit{S. mutans} and \textit{L. acidophilus} decreased over a period of 24 hours as compared to the control at both MIC and MBC of the cranberry extract. But complete sterility could not be achieved.

These results were further supported by SEM images showing progressive thinning and disruption of the biofilm at both MIC and MBC of the cranberry extract as compared to the control, which showed dense and thick growth of microorganisms indicating the dose-dependent response of biofilms. At MIC, \textit{S. mutans} and \textit{L. acidophilus} biofilm became very sparse, and the bacteria cluster became much smaller compared with the control group.

This study opens new vistas for research of \textit{L. acidophilus} biofilm inhibition due to scarcity of study and literature. Loesche et al. in 1984 discovered the colonization of lactobacilli bacteria on occlusal fissures. However, sustained colonization of lactobacilli in the oral cavity seems to be possible only in the presence of caries.\textsuperscript{1} Hence, cranberry might act as not only an adjunct to the preventive therapy but also as a curative agent by aiding in the conventional treatment protocol by being incorporated in dental varnishes, sealants, and restorative materials.

So, further research is indicated for the effective extraction process and its incorporation into the currently available oral hygiene products such as toothpaste, mouthwash, chewing gums, etc., for an effective drug delivery system of the cranberry components, making its use easy and beneficial among all age groups.

**Conclusion**

The results of the present study indicate that there is sufficient evidence to prove that cranberry can act as not just an antimicrobial agent but also as an antibiofilm agent in vitro against \textit{S. mutans} and \textit{L. acidophilus}.

**Clinical Significance**

Due to continued exploitation and increased rise in microbial resistance to currently available chemical formulations, a new therapeutic agent like cranberry can be a boon in preventing certain

### Table 3: Percentage inhibition of microbial cell adhesion and reduction in biofilm formation at various concentrations of the cranberry extract as observed under a spectrophotometer ($A_{600\text{ nm}}$)

| % inhibition of bacteria | Control (%) | MIC* (MBIC$_{50}$) (%) | MBC* (MBIC$_{70}$) (%) |
|--------------------------|-------------|-------------------------|-------------------------|
| \textit{Streptococcus mutans} | 0           | 58                      | 70                      |
| \textit{Lactobacillus acidophilus} | 0           | 50                      | 70                      |

*MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration

†MBIC$_{50,70}$, concentration of the cranberry extract for 50% and 70% biofilm inhibition
oral diseases with its antimicrobial and antibiofilm properties as a part of mouthwashes, chewing gums, lozenges, toothpaste, etc. Due to the sweet and tangy flavor of the cranberry, its use can be popularized among pediatric patients.

REFERENCES

1. Chandki R, Banthia P, Banthia R. Biofilms: a microbial home. J Indian Soc Periodontol 2011;15(2):111. DOI: 10.4103/0972-124X.84377.

2. Lang NP, Mombelli A, Attström R. Oral Biofilms and Calculus. In: Lindhe J, Lang NP, Karring T, ed. Clinical Periodontology and Implant Dentistry, 5th ed., Oxford: Blackwell-Munksgaard; 2008. pp. 183–267.

3. Axellson P, Albandar JM, Rams TE. Prevention and control of periodontal diseases in developing and industrialized nations. Periodontol 2000 2002;29:235–246. DOI: 10.1034/j.1600-0757.2002.290112.x.

4. Gunsolley JC. A meta-analysis of six-month studies of antiplaque and antigingivitis agents. J Am Dent Assoc 2006;137(12):1649–1657. DOI: 10.14219/jada.archive.2006.0110.

5. Moshrefi A. Chlorhexidine. J West Soc Periodontol Periodontal Abstr 2002;50(Suppl):5–9.

6. Santos A. Evidence based control of plaque and gingivitis. J Clin Periodontol 2003;30(Suppl 5):13–16. DOI: 10.1034/j.1600-051X.30.s5.5.x.

7. Pizzo G, Cara ML, Licata ME, et al. The effects of an essential oil and an amine fluoride/stannous fluoride mouthrinse on supragingival plaque regrowth. J Periodontol 2008;79(7):1177–1183. DOI: 10.1902/jop.2008.070583.

8. Kaviya Srinidhi A. Cranberry and its antibacterial activity - A review. J Pharm Sci Res 6(1 2014; 41–44.

9. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12(4):564–582. DOI: 10.1128/CMR.12.4.564.

10. Mbata TI, Debiao LU, Saïka A. Antibacterial activity of the crude extract of Chinese green tea (Camellia sinensis) on Listeria monocytogenes. Afr J Biotechnol 2008;7(10):1571–1573.

11. NCCLS. National Committee for Clinical Laboratory Standards. Approved Standard M7-AS: Methods for Dilution Antimicrobial Susceptibility Test for Bacteria that Grow Aerobically, 5th ed., 2000. pp. 4–9.

12. Cruishank R, Duguid JP, Marmion BP, et al. Medical microbiology. In: The Practice of Medical Microbiology, 12th ed., London, UK: Churchill Livingstone; 1975.

13. Li M-Y, Huang R-J, Zhou X-D, et al. Role of sortase in Streptococcus mutans under the effect of nicotine. Int J Oral Sci 2013;5(Suppl 4): 206–211. DOI: 10.1038/jios.2013.86.

14. Assaf D, Steinberg D, Shemesh M. Lactose triggers biofilm formation by Streptococcus mutans. Int Dairy J 2015;42:51–57. DOI: 10.1016/j.idairyj.2014.10.008.

15. Bitoun JP, Liao S, Xie GG, et al. Deficiency of BrpB causes major defects in cell division, stress responses and biofilm formation by Streptococcus mutans. Microbiology 2014;160(Suppl 1):67–78. DOI: 10.1099/mic.0.072884-0.

16. Jongsmra MA, van der Mei HC, Atema-Smit J, et al. In vivo biofilm formation on stainless steel bonded retainers during different oral health-care regimens. Int J Oral Sci 2015;7(Suppl 1):42–48. DOI: 10.1038/jios.2014.69.

17. Roberts AP, Mullany P. Oral biofilms: a reservoir of transferable, bacterial, antimicrobial resistance. Expert Rev Anti Infect Ther 2010;8(12):1441–1450. DOI: 10.1586/eri.10.106.

18. Teitelbaum AP, Czulinski GD. Control of Dental Biofilm and Oral Health Maintenance in Patients with Down Syndrome.

19. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev 2009;2(5):270–278. DOI: 10.4161/oxim.2.5.9498.

20. Daglia M. Polyphenols as antimicrobial agents. Curr Opin Biotechnol 2012;23(2):174–181. DOI: 10.1016/j.copbio.2011.08.007.

21. Sethi R, Govila V. Inhibitory effect of cranberry juice on the colonization of Streptococci species: An in vitro study. J Indian Soc Periodontol 2011;15(1):46. DOI: 10.4103/0972-124X.82271.

22. Neto CC, Penn dorf KA, Feldman M, et al. Characterization of non-dialyzable constituents from cranberry juice that inhibit adhesion, co-aggregation and biofilm formation by oral bacteria. Food Funct 2017;8(5):1955–1965. DOI: 10.1039/C7FO00109F.

23. Duarte S, Gre-goire S, Singh AP, et al. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of Streptococcus mutans biofilms. FEMS Microbiol Lett 2006;257(1):50–56. DOI: 10.1111/j.1574-6968.2006.00147.x.

24. Yamanaka A, Kimizuka R, Kato T, et al. Inhibitory effects of cranberry juice on attachment of oral streptococci and biofilm formation. Oral Microbiol Immunol 2004;19(3):150–154. DOI: 10.1111/j.0902-0055.2004.00130.x.

25. Elworthy A, Greenman J, Doherty FM, et al. The substantivity of a number of oral hygiene products determined by the duration of effects on salivary bacteria. J Periodontol 1996;67(6):572–576. DOI: 10.1902/jop.1996.67.6.572.