ORIGINAL ARTICLE

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 12.5 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 substantivity 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. 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*.

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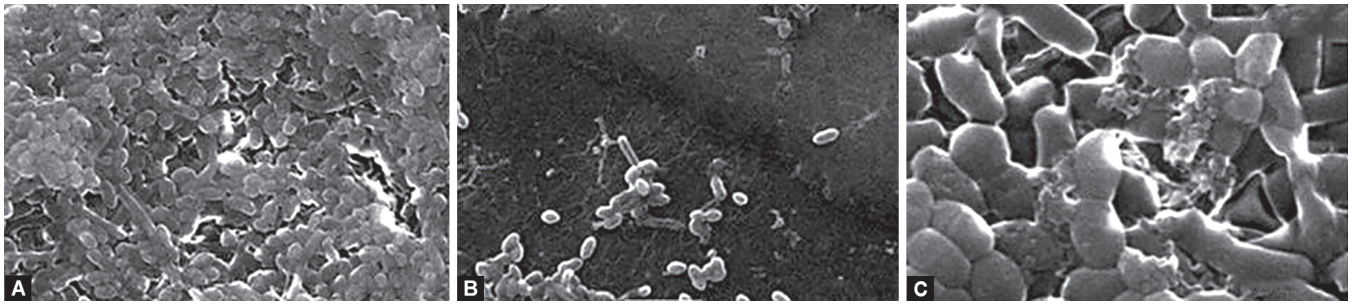
Materials and Methods

Preparation of Extract

Fresh cranberry (*Vaccinium macrocarpon*) was collected from a local market of Mumbai in July–August 2016. The fruits were stored



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

at −80°C until used. The extract was prepared as prescribed by Mbata et al., substituting methanol with a hydroalcoholic solvent as mentioned below.[10]

The frozen fresh fruits were rinsed under running tap water and thawed to remove the water and transversely sectioned into two halves. The water content was removed by blotting with a tissue paper. The fresh fruits were dried under shade till constant weight. 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 was added to all the 10 tubes. Microcentrifuge tubes were incubated in an incubator aerobically for 24 hours.[11]

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 in an incubator aerobically for 24 hours. The MBC values were determined as the lowest concentrations at which no colony formation occurred.[11]

Time Kill Assay

To measure the substantivity of the cranberry extract, a starting inoculum was prepared by inoculating a tube of sterile water with a 24-hour growth of the microorganism and adjusting it to the turbidity of a 0.5 McFarland standard. This inoculum was added to the cranberry extract at MIC and MBC to give an inoculum of 10^5 colony-forming unit (CFU)/mL. The BHI agar was streaked with the above aliquot at predetermined time intervals of 0, 3, 6, 12, and 24 hours. 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 $\geq 3\log_{10}$ decrease in CFU/mL was reached compared with the initial inocula. Colony counts were performed in triplicates, and means were taken.[12]

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 BHI and the sucrose (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.[13,14]

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 100 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 BHI and sucrose broths 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 serious of ethanol (30%, 50%, 70%, 80%, 85%, 90%, 95%, and 100%), and coated with gold. The samples were then examined by a SEM.[15,16]

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 $3\log_{10}$ 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, 1× MIC and 1× 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 1× MIC reduced the number of CFUs by approximately 50% and 1× 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

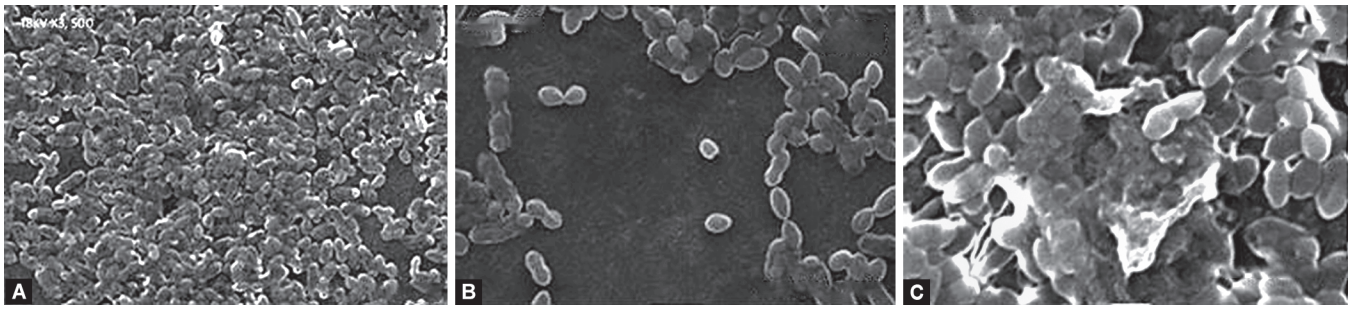
	0 hour mean (±SD)	*3 hours* mean (±SD)*	*6 hours** mean (±SD)*	*12 hours*** mean (±SD)*	*24 hours**** mean (±SD)*	*p value#*
Lactobacillus acidophilus						
MIC	5.35 (±0.01)	4.59 (±0.064)	3.23 (±0.019)	1.19 (±0.01)	0.23 (±005)	0.016
MBC	5.30 (±0.077)	4.42 (±0.006)	2.91 (±0.039)	0.6027 (±0.006)	0.46 (±0.05)	0.02
Control	5.18 (±0.007)	5.41 (±0.006)	5.62 (±0.003)	5.73 (±0.041)	5.5 0.04)	0.018
p^{α} value	0.061	0.026*	0.027**	0.025***	0.024****	
Streptococcus mutans						
MIC	4.98 (±0.006)	2.33 (±0.023)	1.89 (±0.059)	1.44 (±0.01)	0.115 (±0.005)	0.05
MBC	4.97 (±0.006)	2.06 (±0.008)	1.55 (±0.023)	0.99 (±0.011)	0.12 (±0.005)	0.02
Control	4.99 (±0.002)	4.98 (±0.006)	5.59 (±0.085)	5.78 (±0.006)	0.067 (±0.00)	0.017
$p^{@}$	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^{α} values at different time intervals 3, 6, 12, 24 hours

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 ($A_{600\ nm}$)

% inhibition of bacteria	*Control (%)*	*MIC* ($MBIC_{50}^{\dagger}$) (%)*	*MBC* ($MBIC_{70}^{\dagger}$) (%)*
Streptococcus mutans	0	58	70
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



Figs 2A to C: Effect of the cranberry extract on biofilm morphology of *L. acidophilus* incubated for 24 hours in the BHIS broth at control (without cranberry), MIC, and MBC as observed under scanning electron microscopy. Magnification was 3,500×, 7,000×, and 11,000×, respectively, for each concentration

Flavonols also have a potent effect on gram-positive bacteria.[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 *S. mutans* and *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.[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.[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 *S. mutans* cells. Furthermore, biofilm development and acidogenicity were significantly affected by topical applications of PAC and FLAV.[23]

There was almost 50% and 70% reduction in the biofilm of *S. mutans* and *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 *S. mutans* to adhere to the hydroxyapatite decreased when exposed to the cranberry juice and the biofilm was reduced to 80–95%.[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.[25] The present study revealed that the number of viable CFU/mL of *S. mutans* and *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 MBC, *S. mutans* and *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 *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.[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 *S. mutans* and *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

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.

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