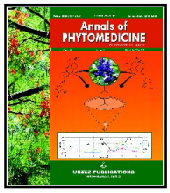
Annals of Phytomedicine 8(2): 99-104, 2019

DOI: http://dx.doi.org/10.21276/ap.2019.8.2.11

Annals of Phytomedicine: An International Journal
http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885



Original article

Anticariogenic effect of *Vaccinium macrocarpon* Aiton against causative organisms of dental caries

S. Sumathi◆, K. Suganya, A. Vishali and P.R. Padma
Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore-641043, Tamil Nadu, India

Received October 1, 2019: Revised November 21, 2019: Accepted November 25, 2019: Published online December 30, 2019

Abstract

Oral diseases continue to be a major health problem world wide. It can be treated with antibiotics which lead to side effects so an alternatives use of natural herbal extracts. The aim of the study is to evaluate the antibacterial and antibiofilm activity of *Vaccinium macrocarpon* Aiton fruit extract. Then, we attempted to formulate the juice in the form of a polymer gel to aid topical application. *V. macrocarpon* (Cranberry) components possess potential anti-caries agents and, hence they inhibit acid formation, attachment and biofilm formation by microorganisms. *V. macrocarpon* exhibited bacteriocidal effect against oral microbes. Cranberry extract acted as a potent antibiofilm properties and prevents biofilm formation. The methanolic extract of cranberry was found to becytotoxic against the causative microorganisms. The time kill assay also confirmed the bacteriostatic action of the extract. The cranberry extract was formulated into a gel for applying to treat dental caries.

Key words: *Vaccinium macrocarpon* Aiton, oral cavity, microbes, biofilm, natural herbal extracts

1. Introduction

Dental caries, normally referred to as cavities, may be a common dental problem in the world. Cavity may be a chronic dental illness that damages the hard tissue of teeth and happens due to building up of plaque on teeth surfaces formed by acid-producing microorganism. Microorganism interact with carbohydrates which will be fermented after long duration, forming acids thereby lowering pH below essential and leading to demineralization of arduous tissue of teeth (Aneja *et al*., 2015).

The main investigation and progress of dental caries involves acidogenic and aciduric activity of gram-positive bacteria and gram-negative bacteria, such as *Streptococcus, Lactobacillus, Staphylococcus, Pseudomonas, Klebsiella* and *Actinomycetes* colonizing the supragingival biofilm which impede with usual nutrition intake, verbal communication, self-worth and daily habitual behavior. The experience of pain, problem with eating, chewing, smiling and communication due to missing, discolored or spoiled teeth have a foremost impact on people's everyday life (Kidd and Fejerskov, 2016).

Organisms synthesize exopolysaccharides and it forms a biofilm which will inhibit the growth of the organism in the culture, and it protects us from the unfavourable environmental conditions (Basting *et al*. 2016). The biofilms are attached to the substratum and they are embedded using an extracellular polymeric matrix. Due to the growth of the bacteria, the biofilm attaches to the surface and it is hydrated (Aneja *et al*., 2015). Oral biofilm for instance is one of the most prevalent sources of chronic human infectious disease widely spread over all age groups. Although, various methods are established and are presently in use to manage oral biofilms, the pursuit for natural and effective antibiofilm agents still continues (Slobodnikova *et al*. 2016).

Phytochemical studies have shown that *Vaccinium macrocarpon* (Family: Ericaceae) has active components include flavanols, anthocyanins, proanthocyanidins are potential anti-caries agents (Sanoner *et al*. 2019). Phytochemicals found in cranberries (flavanols and benzoic and cinnamic acid derivatives) have a great deal of attention mainly because of their antioxidant, antibacterial, anti-inflammatory and antimutagen properties. Cranberries can play a role in preventing certain infectious diseases, such as urinary tract disorders, dental decay, as well as stomach ulcers and cancers (Kim *et al.* 2016).

The present study was designed to analyse the anticariogenic property of *V. macrocarpon* extracts against different bacterial strains.

2. Materials and Methods

2.1 Extraction of cranberry (Organic extraction)

Fresh cranberries were purchased from a local country drug store at Coimbatore in the month of January. Methanol extraction of the

Author for correspondence: Dr. S Sumathi
Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore-641043, Tamil Nadu
E-mail: sumii.venkat@gmail.com; sumathi_venkat@yahoo.co.in
Tel.: +91-9843021733

cranberry was carried out by using methanol according to method described by Ulrey *et al*. (2014). 10 g of dried fruit was placed in 100 ml of methanol in a conical flask, and then kept on a rotary shaker at 190-220 rpm for 24 h. After 24 h, the extract was filtered and centrifuged at 4500 rpm for 15 min. The supernatant was collected and the solvent was evaporated by using rotary evaporator (40°C) to get rid of methanol and then stored at 4°C in airtight bottles.

2.2 Microorganism

Bacterial strains of four different species (*Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeuroginosa, Klebsiella pneumoniae*) which are known for enhancing activity in caries formation were selected. All tested strains were obtained from the microbiological laboratories and were sub-cultured on Mueller-Hinton Agar slants and were maintained at 4°C.

2.3 Antibacterial test

2.3.1 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was the lowest concentration of the juice that prevents visible growth of bacteria. The antibacterial assay was performed using the broth micro dilution method (Mutmainnah *et al*., 2019). A two fold serial dilution of the juice was prepared in Mueller-Hinton Broth (MHB). For every experiment, a negative control (distilled water, medium, and inoculums) was included. The negative control consists of broth and bacterial cell suspension without the agent, and the blank control contained only the medium. 100 l of Mueller-Hinton broth plus different concentrations of cranberry extracts were prepared and transferred to each microplate well to obtain dilutions of the active extract, ranging from 1.0 to 25 mg/ml. Then, 10 l of a fresh culture of *Streptococcus mutans* (4.3×10^6 cfu/ml), *Staphylococcus aureus* (3.8×10^6 cfu/ml), *Pseudomonas aeuroginosa* (3.5×10^6 cfu/ml), *Klebsiella pneumoniae* (2.0×10^6 cfu/ml) was added. Microplates were incubated at 37°C for 24 h. The MIC end-point was defined as the lowest concentration of the test agent that completely inhibited growth or produced at least 90 % reduction of absorbance in comparison with the negative control. All experiments were performed in triplicate and the average values were reported as MIC. Microbial growth in each well was determined by observing and comparing the well with the negative control. The absence of microbial growth was interpreted as an antibacterial activity.

2.3.2 Time kill assay

Bactericidal activity of cranberry extract was examined using a time kill assay. It is the most appropriate method for determining the bactericidal effect. The time kill test reveals a time dependent or a concentration dependent antimicrobial effect (Kidd and Fejerskov, 2016). Growing cultures of each representative strain (*Streptococcus mutans, Klebsiella pneumoniae, Pseudomonas aeuroginosa, Staphylococcus aureus*) were added to appropriate medium and exposed to 1× 2× and 4× the MIC of cranberry extracts. Samples were taken for colony counts at 0th, 30th min and 2, 4, 6, 8, 10, 12 and 24 h. The viable counts were determined after appropriate incubation and each experiment was performed in triplicates. Ciprofloxacin (0.1%) and extract free medium were used as the positive and negative controls, respectively.

2.4 Antibiofilm activity

The effect of extract on biofilm formation of each representative strain, (*Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeuroginosa, Klebsiella pneumoniae*) was examined using the modified microdilution method (Sanchez *et al*., 2016). Overnight culture of *Streptococcus mutans* (6.5×10^6 cfu/ml), *Staphylococcus aureus* (4.8×10^6 cfu/ml), *Pseudomonas aeuroginosa* (4.2×10^6 cfu/ml), *Klebsiella pneumoniae* (2.2×10^6 cfu/ml) in trypticase soy broth was diluted in the ratio 1:100 in respective fresh medium and allowed to grow for another hour. 100 µl of the diluted strains were added to 96 well titre plate and the different concentrations of *V. macrocarpon* extracts were added and incubated at 37ºC for overnight. After the incubation, the medium was removed and 100 µl of crystal violet solution was added and incubated at room temperature for 30 min. The dye was removed after staining and the wells were washed thoroughly with distilled water and finally incubated with 95% ethanol for 15 min and read in spectrophotometer at 595 nm. Inhibition mediated reduction of biofilm formation was calculated by the following formula

$$\% \text{ of inhibition} = \frac{\text{OD of control} - \text{OD oftreatment} \times 100}{\text{OD of control}}$$

2.5 MTT assay

MTT assay is the most sensitive method for measuring cytotoxicity (Subaramenium *et al*., 2015). The cytotoxicity of the cranberry was assessed using microorganisms (*Streptococcus mutans, Staphylococcus aureus, Pseudomonas aeuroginosa, Klebsiella pneumoniae*). Overnight culture of each bacterial strainwere prepared in nutrient broth and 100 µl of the overnight culture were added to 96 well titre plate and the different concentrations (12.5, 25, 50 mg/ml) of *V. macrocarpon* extracts were added. The cells were incubated for one hour. And added 50 µl of MTT to 100 µl of treated cells of strain and incubated at 37ºC for 3 h. Then 200 µl of acid-propanol was added to it after incubation and left overnight in dark room. The cell viability was noted at 650 nm in a microtitre readerby fixing the control group as 100 % viability and the percentage of viable cells were calculated relative to other treatment groups.

$$\% \text{ of cell viability} = \frac{\text{OD of control} - \text{OD Oftreatment} \times 100}{\text{OD of control}}$$

2.6 Formulation of cranberry extract into gel

The evaluation of antimicrobial potency was studied prior to gel formulation to compare the changes in activity after incorporation in polymer gel (Vinita *et al*., 2013). The topical formulations were developed using different concentrations of polymers. Carbopol 934 gels were prepared by soaking carbopol in water and by neutralizing with triethanolamine to pH 6.4. Weighed amount of methyl and propyl paraben were added to water prior to the addition of carbopol 934. In another beaker, the required quantity of propylene glycol was taken in another test tube to which accurately measured amount of cranberry fruit extract corresponding to its MIC was incorporated and finally this mixture was added to the beaker, containing carbopol with stirring. Sweetening agent aspartame was also added to the polymer dispersion and stirred continuously till it forms a homogeneous product. The volume was

made up with distilled water and stirring was done vigorously. The single formulated gels were then subjected to evaluation tests in order to select the best formulation. The composition of gel formulation is shown in Table 1.

Table 1: Composition of gel formulation

Ingredients	Quantity
Vaccinium macrocarpon extract	100 µl
Carbapol 934	0.3 g
Propylene glycol	15 ml
Glycerin	5 ml
Methyl paraben	0.18 g
Propyl paraben	0.02 g
Aspartame	0.4 g
Distilled water	Required quantity

3. Results

3.1 Determination of minimum inhibitory concentration (MIC)

The antimicrobial activity of *V. macrocarpon* was investigated against the dental caries causing microbes, namely; *S. mutans, S. aureus, P. aeruginosa* and *K. pneumoniae.* Table 2 lists the results obtained for the assays against causative agents of dental caries. *V. macrocarpon* afforded MIC value of 60 mg/ml against *S. mutans*, which characterized a bactericidal effect. *V. macrocarpon* also exhibited bacteriocidal effect against *S. aureus* and *P. aeruginosa* with MIC value of 40 mg/ml, respectively. For *K. pneumoniae*, MIC value was 20 mg/ml, respectively.

Table 2: Determination of minimum inhibitory concentration using *V. macrocarpon* extract against dental caries causing microorganisms

Test organisms	MIC (mg/ml of *Vaccinium macrocarpon*)
Streptococcus mutans	60 ± 3
Staphylococcus aureus	40 ± 1.8
Pseudomonas aeruginosa	40 ± 2
Klebsiella pneumonia	20 ± 1.5

3.2 Antibiofilm assay

To investigate whether *V. macrocarpon* extract reduced biofilm formation, antibiofilm assay was done. *V. macrocarpon* was found to exhibit antibiofilm effect against *S. mutans, S. aureus, P. aeruginosa, K. pneumoniae.* The results depicted in the Figure 1, showed that *V. macrocarpon* exhibited antibiofilm activity, by inhibiting the growth of microorganisms. Higher concentration of the extract 50 mg/ml showed maximum percentage of inhibition for all the four bacterial cultures, *S. mutans, S. aureus, P. aeruginosa, K. pneumoniae* at 95.2%, 83.07%, 97.3%, 84.35% after 24 h. It confirmed that the phytochemicals of *V. macrocarpon* caused bacterial cell death and cell damage of dental caries causing microbes by binding to bacterial cell membrane. Thus, *V. macrocarpon* acted as a potential antimicrobial agent.

3.3 Assessment of cytotoxicity of *V. macrocarpon*

For evaluating the intracellular activity of the cell and for determining the cell viability the MTT assay is carried out. The results of MTT cell viability assay was depicted in Figure 2 clearly showed that the methanolic extract of *V. macrocarpon* at a concentration of 20 mg/ml shows less cytotoxic effect. But, as the concentration increased, the cytotoxicity of the methanolic extract of cranberry also increased. When the concentration is 50 mg/ml the percentage of the bacterial cell death of the microbes *S. mutans, S. aureus, P. aeruginosa, K. pneumoniae* observed was 76%, 70%, 80%, 64%. The percentage of dead cells increased at the 60 mg/ml and still the percentage can reach upto 100 % by increasing the concentration of the methanolic extract of *V. macrocarpon.*

3.4 Time kill assay

In the present study, the time kill test was carried out to evaluate the ability of methanolic extract of *V. macrocarpon* to reduce the microbial population ability and to assess its efficiency against selected dental bacterial strains. Time kill curves were constructed for all the four pathogens, namely; *S. mutans, S. aureus, P. aeuroginosa, K. pneumoniae* was studied. The results showed the methanolic extract of cranberry was more effective in reducing the microbial count and the reduction was in a concentration and time dependent manner. A graph was plotted with incubation time against log cfu/ml which is shown in Figure 3.

3.5 Formulation of *V. macrocarpon* into gel

The methanolic extract of the *V. macrocarpon* exhibited effective antibacterial activity. The oral gel formulation of *V. macrocarpon* extract was developed with carbapol 934. The formulated gel was pinkish white in colour and the homogeneity of the gel was good. The pH of the gel was within 6-7, the normal pH range of the buccal cavity which substantiates that the prepared gels will be irritation free. The gel formation is shown in Figure 4.

4. Discussion

Aneja *et al.* (2015) examined the acetone, methanol and ethanol extracts of *Piper cubeba* against both two species, namely; *S. aureus* and the caries causing *S. mutans* with an MIC of 50 mg/ml. Antibacterial agent kuwanon G isolated from root bark of *Moras alba* has showed action against *S. mutans* at an MIC of 8.0 g/ml. The bactericidal test showed that kuwanon G completely inactivated *S. mutans* at the concentration 20 g/ml in 1 min was reported by Park *et al.* (2003). These findings were similar to our results.

Liu *et al.* (2017) evaluated the effect of *Bergenia crassifolia* leave extracts on biofilms. Compared with control groups, the percentages of remaining bacteria in the biofilms were initially reduced in proportion to the concentration of extracts and then entered a stable phase at a higher level. Teanpaisan *et al.* (2014) reported the higher concentrations of *Artocarpus lakoocha* extract are required to significantly inhibit existing biofilm cells. These results are in line with our findings observed in our study. Inorder to be used in formulations, the extract should not be cytotoxic. So, cytotoxicity was tested in *V. macrocarpon*.

Veiga Junior *et al.* (2017) assessed CRO cytotoxicity in mouse peritoneal macrophage cultures by the MTT assay. Oleoresin concentrations did not alter the viability of the macrophages. Kreling *et al.* (2016) examined the percentage metabolism of HaCat and OBA-9 cells. After exposure for 24 h, D6-17 did not affect the cell

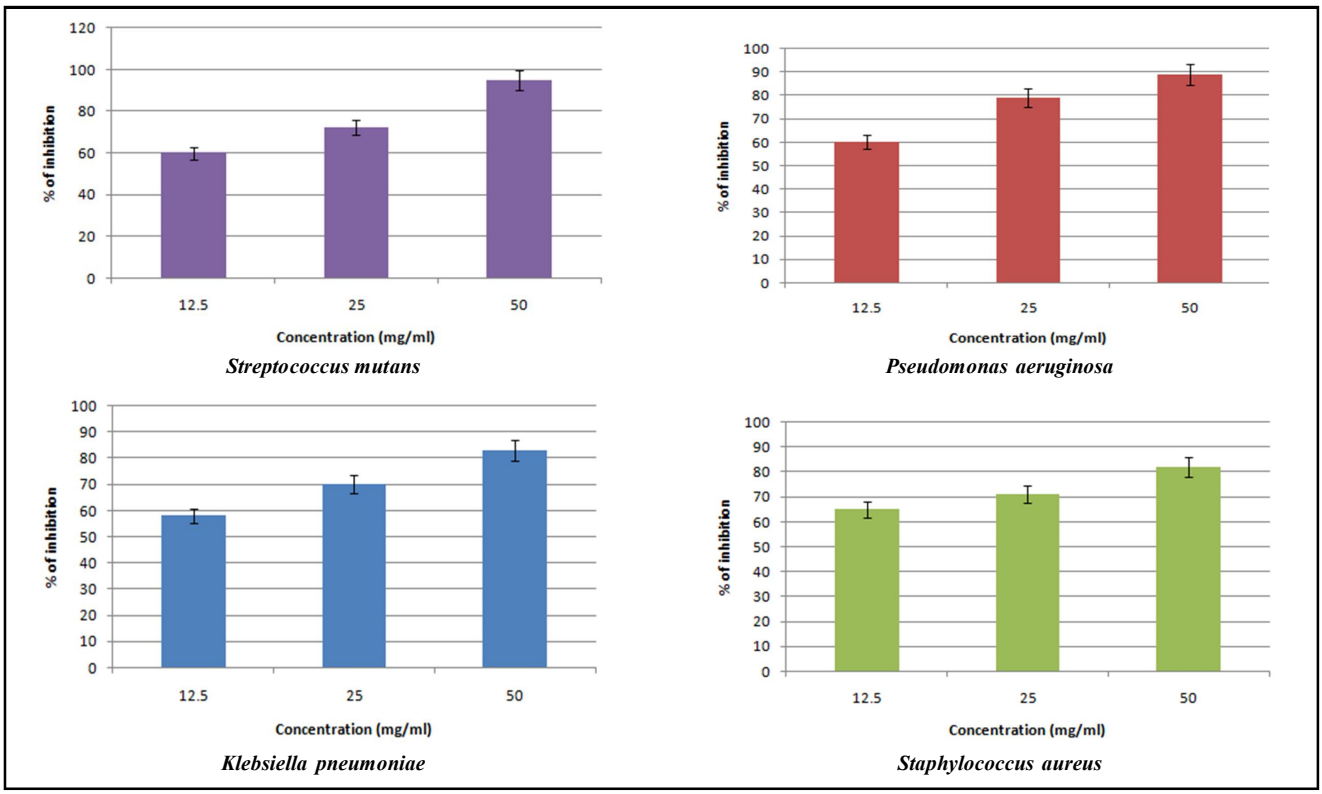


Figure 1: Antibiofilm activity of *V. macrocarpon*.

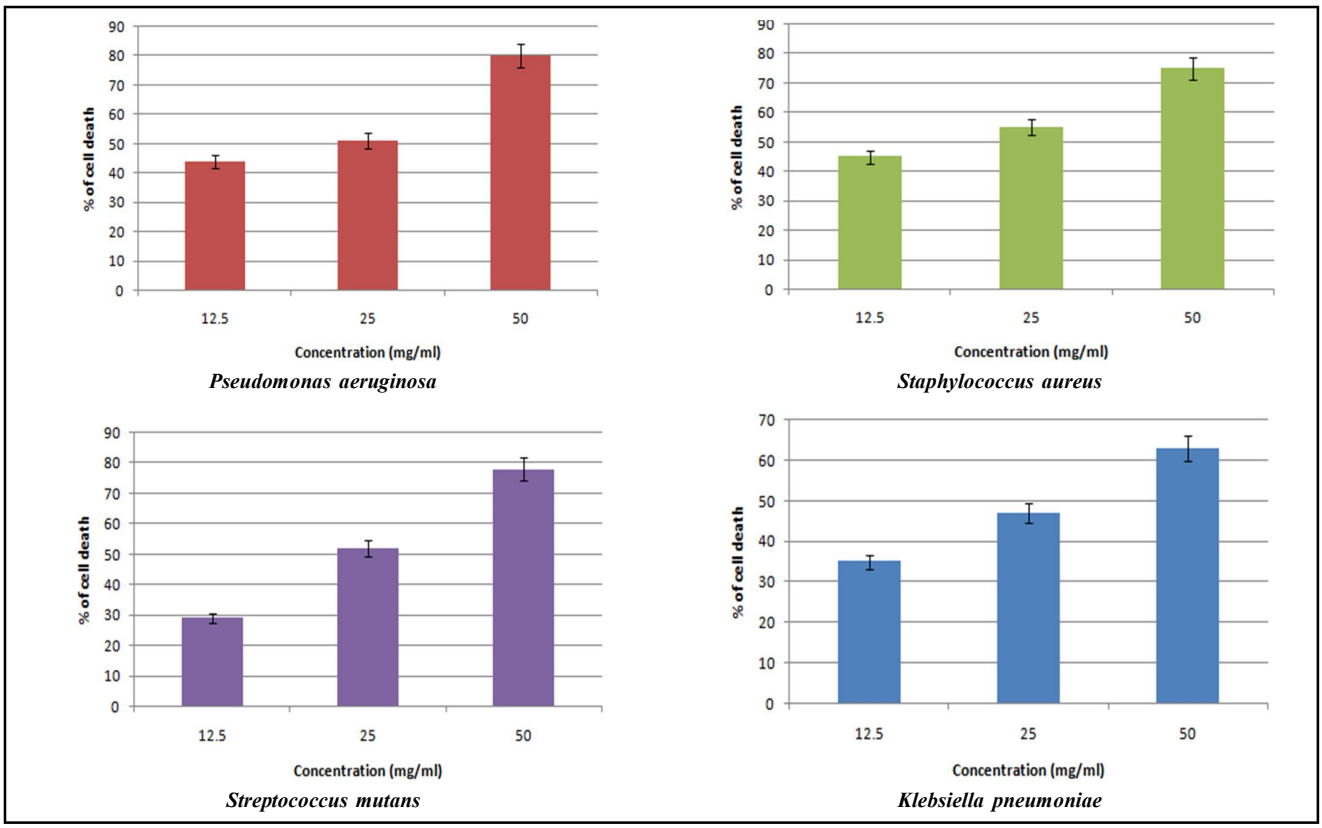


Figure 2: Antimicrobial activity of *V. macrocarpon* by MTT cytotoxicity assay.

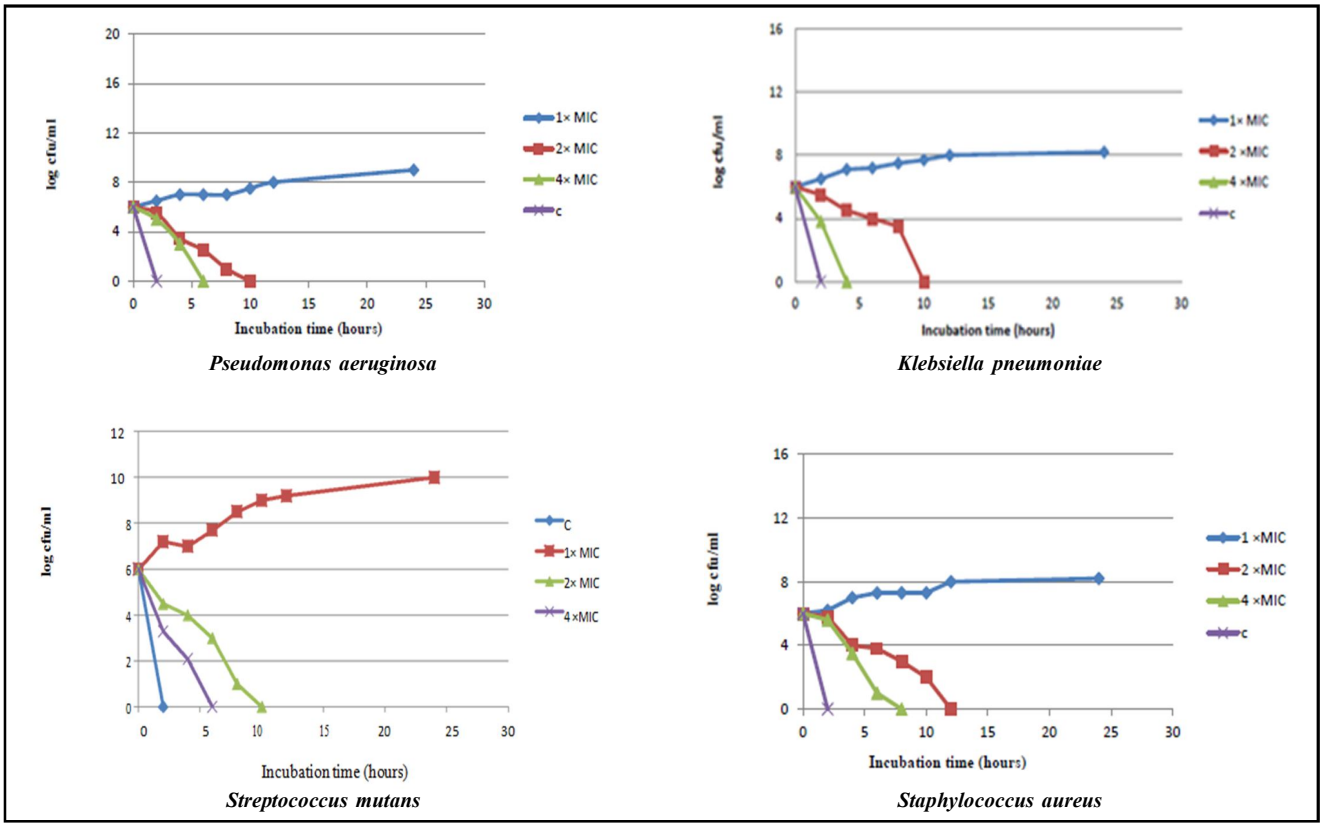


Figure 3: Time kill kinetics of *V. macrocarpon*.

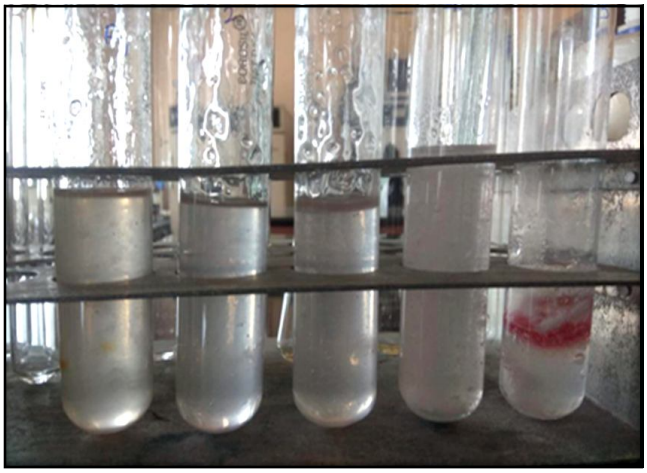


Figure 4: Formulated *V. macrocarpon* gel with different concentrations of carbapol.

metabolism of either epithelial line at the concentrations tested. D1-23 showed toxicity at concentrations >0.2 mM for both epithelial cells. LL-37 and CHX were the most cytotoxic peptides, demonstrating toxicity at concentrations >0.02 mM for both cells. *V. macrocarpon* extract was toxic to the tested microorganism.

Taweechaisupapong *et al.* (2018) reported the results of time-kill assay showed that 1% lemongrass oil killed all 105 CFU/ml of *C. albicans* ATCC 10231 within 1 min. Diarra *et al.* (2013) reported the combination of the cranberry fraction FC111 at its MIC (1 mg/ml) and 1/8×MIC of amoxicillin induced a strong bactericidal effect (>3 Log10 CFU/ml) against a bovine mastitis strain *S. aureus*. In our present study too, *V. macrocarpon* extract was found to inhibit the causative organisms.

Khushali *et al.* (2018) reported the formulation of papain and clove oil based chemo-mechanical caries removing gel. Thombre *et al.* (2018) reported the formulation of guava leaves extract into gel responsible for mouth ulcer and formulations that canbe used to treat mouth ulcer infection. The gel prepared using *V. macrocarpon* extract was found to be good in appearance and stability. The antimicrobial activity of the gel needs to be assessed, and the pH of the gel formulation was compatible with normal pH range.

The present study reported the antibiofilm efficacy of *V. macrocarpon* extracts against *S. mutans, S. aureus, P. aeruginosa, K. pneumoniae*. The results of cytotoxicity assay also revealed that the formulation is to be effective againstthe organisms tested. The time kill assay also confirmed the bacteriostatic action of the extract.

5. Conclusion

V. macrocarpon extracts have been attracting ever-growing attention bydental researchers. *V. macrocarpon* components are potential anti-caries agents since they inhibit acid production, attachment,

and biofilm formation by *S. mutans*. Glucan-binding proteins, extracellular enzymes, carbohydrate production, andbacterial hydrophobicity, are all affected by cranberry components. The above-listed effects suggest that *V. macrocarpon* components, especially those with high molecular weight, could serve as bioactive molecules for the prevention and treatment of oral diseases.

Acknowledgements

Authors are thankful to Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, Tamil Nadu, India for providing infrastructure facilities for completion of this study.

Conflict of interest

The authors declare that there are no conflicts of interest in the course of conducting the research. All the authors had final decision regarding the manuscript and decision to submit the findings for publication.

References

Aneja, K.R.; Joshi, R.; Sharma, C. and Aneja, A. (2015). Antimicrobial efficacy of fruit extracts of two Piper species against selected bacterial and oral fungal pathogens. Braz. J. Oral. Sci., **9**:421-426.

Basting, R. T.; Gonçalves, F. R.; França, F. M. G.; do Amaral, F. L. B. and Florio, F. M. (2016). Antimicrobial potential of papain chemomechanical agent on *Streptococcus mutans* and L*actobacillus casei* followed by the use of self-etching adhesive systems. J. Clin. Pediatr. Dent., **40**:62-68.

Diarra, M.S.; Block,G; Rempel, H.; Oomah, B. D.; Harrison, J.; McCallum, J.; Boulanger, S.; Brouillette, E.; Gattuso, M. and Malouin, F. (2013). *In vitro* and *in vivo* antibacterial activities of cranberry press cake extracts alone or in combination with β-lactams against Staphylococcus aureus. BMC. Complement. Altern. Med., **13**:90.

Khushali, C.; Rupali, K. and Satish, S. (2018). Formulation optimization and performance evaluation of papain and clove oil based chemo-mechanical Caries Removing Gel. J. Young. Pharmacists., **10**:24.

Kidd, E.A. and Fejerskov, O. (2016). Essentials of dental caries. Oxford University Press.

Kim, J. and Cvitkovitch, D.G. (2016). Compositions and methods for reducing caries-causing oral bacteria. U.S. Patent Application. **14**/668,142.

Kreling, P. F.; Aida, K. L.; Massunari, L.; Caiaffa, K. S.; Percinoto, C.; Bedran, T. B. L. and Duque, C. (2016). Cytotoxicity and the effect of cationic peptide fragments against cariogenic bacteria under planktonic and biofilm conditions. Biofouling., **32**:995-1006.

Liu, Y.; Xu, Y.; Song, Q.; Wang, F.; Sun, L.; Liu, L.; Yang, X.; Yi, J.; Bao, Y.; Ma, H. and Huang, H. (2017). Anti-biofilm activities from *Bergenia crassifolia* leaves against *Streptococcus mutans*. Front. Microl., **8**:1738.

Mutmainnah, B. and Baktir, A. (2019). Antimicrobial activity of ethanol extract of *Abrus precatorius* L. roots against planktonic cells and biofilm of urine and blood methicillin sensitive *Staphylococcus aureus* (MSSA) Isolate. In IOP Conference series: Earth and Environmental Science. IOP Publishing., **217**:012027.

Park, K. M.; You, J. S.; Lee, H. Y.; Baek, N. I. and Hwang, J. K. (2003). Kuwanon G: An antibacterial agent from the root bark of *Morus alba* against oral pathogens. J. Ethnopharmacol., **84**:181-185.

Sanchez, E.; Rivas Morales, C.; Castillo, S.; Leos-Rivas, C.; García-Becerra, L. and Ortiz Martínez, D. M. (2016). Antibacterial and antibiofilm activity of methanolic plant extracts against nosocomial microorganisms. Evid. Based Complement. Alternat. Med., **17**:233-243.

Sanoner, P.; Bochard, V.; Charissou, L.; Lastique, B.; Jacob, M and Thomas, P. (2019). Cranberry extract useful in the treatment and prevention of urinary Infections. U.S. Patent Application. **16**:144-246.

Slobodnikova, L.; Fialova, S.; Rendekova, K.; Kovac, J. and Mucaji, P. (2016). Antibiofilm activity of plant polyphenols. Molecules, **21**:1717.

Taweechaisupapong, S.; Boonmars, T.; Satthanakul, P.; Chatchanayuenyong, V.; Nitinon, S.; Soodtoetong, U. and Khunkitti, W. (2018). Effect of lemongrass gel against *Candida albicans* in rat model of oral candidias.

Teanpaisan, R.; Ruangkiatkul, P.; Thammasitboon, K.; Puripattanavong, J. and Faroongsarng, D. (2013). Effectiveness of *Artocarpus lakoocha* extract, poloxamer 407, on *Enterococcus faecalis in vitro*. J. Investig. Clin. Dent., **4**:219-224.

Thombre, K. P.; Sharma, D. and Lanjewar, A. M. (2018). Formulation and evaluation pharmaceutical aqueous gel of powdered *Cordia dichotoma* leaves with guava leaves. Am. J. Pharmatech. Res., **8**:268-277.

Ulrey, R. K.; Barksdale, S. M.; Zhou, W. and Van Hoek, M. L. (2014). Cranberry proanthocyanidins have anti-biofilm properties against *Pseudomonas aeruginosa*. BMC. Complement. Altern. Med., **14**:499.

Veiga, Jr V.F.; Rosas E.C.; Carvalho, M.V.; Henriquesb, M.G and Pinto, A.C. (2017). Chemical composition and anti-inflammatory activity of copaiba oils from *Copaifera cearensis* Huber ex Ducke, *Copaifera reticulata* Ducke and *Copaifera multijuga* Hayne: A comparative study. J. Ethnopharmacol., **112**:248e254.

Vinita, P. A.; Trupti, B.B.; Mitesh, T. R.; Nitin, M.D. and Khandelwal, K.R. (2013). Formulation and evaluation of dental gel containing essential oil of coriander against oral pathogens. Int. Res. J. Pharm., **4**:48-55.

Citation: S. Sumathi, K. Suganya, A. Vishali and P.R. Padma (2019). Anticariogenic effect of *Vaccinium macrocarpon* Aiton against causative organisms of dental caries. Ann. Phytomed., 8(2):99-104.