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Evaluation of anti-biofouling potential of *Viburnum opulus* extracts

[Evaluación del potencial anti bioincrustante de extractos de *Viburnum opulus*]

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Abstract: In this study the *in vitro* investigation of the inhibitory effect of ethanol extract of *Viburnum opulus* L. bark sample on *Streptococcus mutans* planctonic cells and biofilm has been intended. A Scanning electron microscopy analysis has been performed in order to investigate the inhibitory effect of the extract on *Streptococcus mutans* biofilms. Furthermore, the Exopolysaccharide and dextran production of this bacteria have been identified in the presence of the extract. It has been found out that the bark extract with the concentration of 2,5 mg/mL is able to inhibit more than 50% of the cells in the different times development phases. According to this, the exopolymeric matrix on the biofilm surface disperses and the Exopolysaccharide and dextran production get lowered in the presence of bark extract compared to the control group. It is considered that this extract can be used as an alternative approach for the new chemotherapeutic strategies against tooth decay.

Keywords: *Viburnum opulus*; *Streptococcus mutans*; Antibiofilm; Anti-biofouling; Dental plaque.

Resumen: En este estudio se investigó el efecto inhibitorio *in vitro* del extracto de etanólico de una muestra de corteza de *Viburnum opulus* L. en biopelículas de células planctónicas de Streptococcus mutans. Se realizó un análisis de microscopía electrónica de barrido para investigar el efecto inhibitorio del extracto sobre las biopelículas de *Streptococcus mutans*. Además, se identificó la producción de exopolisacárido y dextrano de esta bacteria en presencia del extracto. Se descubrió que el extracto de corteza con una concentración de 2,5 mg/ml inhibió más del 50% de las células en las diferentes fases de desarrollo. Consecuentemente, la matriz exopolimérica en la superficie de la biopelícula se dispersa y la producción de exopolisacárido y dextrano se reduce en presencia de extracto de corteza en comparación con el grupo de control. Se sugiere que este extracto puede ser usado como un enfoque alternativo para las nuevas estrategias quimioterapéuticas contra la carie dental.

Palabras clave: *Viburnum opulus*; *Streptococcus mutans*; Anti-biofilm; Anti-bioincrustante; Placa dental.

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INTRODUCTION

Dental caries is one of the most common infectious diseases in humans and it is initiated by the formation of dental plaque biofilms. Although different types of bacteria have been found to be associated with pathogenesis of dental caries, the mutans streptococcal group represented by *Streptococcus mutans* is considered to be a major etiologic agent in the pathogenesis of dental caries (Liu *et al.,* 2011). *S. mutans* effectively utilizes dietary sucrose to synthesize large amounts of exopolysaccharides, which play an important role in the accumulation, adhesion, and plaque matrix formation of microorganisms (Goulhen *et al.,* 2003). These bacteria can survive at low pH values that are toxic to many other bacteria species (Bos *et al.,* 1999). The acidogenic and aciduric (associated with acid tolerance) properties of *S. mutans*, with its ability to synthesize extracellular glucans, are the major factors for the development and establishment of cariogenic biofilms (Loesche *et al.,* 1986; Marsh & Bradshaw,,1995). These properties of *S. mutans* could be the primary targets for chemotherapeutic agents to prevent the formation of cariogenic biofilms. The strategies of controlling biofilm aiming at disrupting bacterial virulence offer an attractive and alternative approach to the traditional antimicrobial therapy based on the use of broad spectrum microbiocides (Sagdic *et al.,* 2006).

The genus *Viburnum* L. (Caprifoliaceae) comprises more than 230 species distributed from South America to Southeast Asia and the majority of them are being endemic (Lobstein *et al.,* 1999). The genus is represented by four species in the flora of Turkey; *V. opulus* L., *V. lantana* L., *V. orientale* Pallas, and *V. tinus* L (Davis *et al.,* 1988). In the Central Anatolia, a traditional beverage named gilaburu has been made from *V. opulus* whose fruits have a dark-red color and are edible. *V. opulus* are grown as ornamental plants and the dried fruits are used for complaints of uterine cramps, colicky pains in pelvic organs in many countries (Prajabati *et al.,* 2003).

The species of genus *Viburnum* are known to contain triterpenoids, diterpenoids, sesquiterpenes, iridoids, and polyphenols (Tao *et al.,* 2007). Due to the medicinal properties of this genus, the aim of this study is to investigate the susceptibility of *S. mutans* biofilms to *V. opulus* leaf and bark extracts, with an emphasis on determining the effects of this extract on various biofilm growth phases and architectural organization.

MATERIALS AND METHODS

Bacterial strain

S. mutans ATCC 25175 (Microbiologics KWIK STIK 0266P) was used. The cells containing 25% glycerol were stored frozen at -80ºC. Inocula were prepared from 24 h old cultures in Nutrient Broth (Difco).

Plant extract

V. opulus leaf and bark samples have been collected from Kayseri (Gesi village), Turkey. The plant materials have been dried and powdered. The ethanolic (70%) extracts of each sample have been prepared by stirring on a magnetic stirrer at 37ºC for three days. The extracts have been further dried in a desiccator under vacuum and the residues containing water have been dried by lyophilisation.

Plant extracts susceptibility in S. mutans planktonic cells

Serial two-fold dilutions of plant extracts stock solutions in NB (Nutrient Broth) were prepared in eppendorf tubes over a range of 0.625-10 mg/ml. Inoculum was prepared in NB, and the density was adjusted to 0.5 Mcfarland standards (10^8 CFU ml^{-1}). Then, 100 µl *S. mutans* planktonic cells were mixed with 100 µl extract of various concentrations in a sterile 96-well plates to give a final volume of 200 µl and incubated at 37ºC for 24 h. The data were obtained from three independent experiments. For the positive control, the planktonic cells were incubated in the presence of 100 µl NB without any extract and for the negative control, only 200 µl NB was incubated in wells under the identical conditions. The minimum inhibitory concentrations $(MIC)_{50}$ (minimum inhibitory concentrations) for the planktonic cells were defined as the minimum antibacterial concentration that caused ≥ 50% bacterial inhibition (Pu *et al.,* 2014).

Measurement of biofilm metabolic activity by XTT reduction assay

A semi-quantitative measurement of bacterial biofilm formation was obtained from the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay (Meshulam *et al.,* 1995). NB medium supplemented

with 1% sucrose was used in the study and *S. mutans* biofilm experiments were performed in untreated 96-well plates. All media were autoclaved 15 min at 121ºC. Sugar was seperately autoclaved. The extracted plant was sterilized by the filtration through a 0.45µm membrane filters. The wells of the 96-well plates were incubated for various time periods (8 and 24 h) in 100 µL *S. mutans* suspension adjusted to 0.5 Mcfarland standards at 37ºC. The medium in each well was removed at the indicated time points and the biofilms were washed twice with phosphate-buffered saline (PBS). Following the removal of PBS, 100 µL extract solution of various concentrations (0.3125-5 mg/mL) were added to one well of a 96-well plates and then incubated for additional 48 h at 37ºC. A formazan salt-based XTT reduction assay was performed to assess the metabolic activity. All the tests were performed in duplicate. The positive and negative controls were established as previously designated. Then for the bacterial strains, 100 µL of XTT kit solution (Appli Chem Panreac A80881000) were added to each well. The microtiter plates were incubated at 37ºC for 2 h. The colorimetric change was measured using a microtiter reader (Labsystems Multiskan MS; Labsystems, Finland) at 450 nm.

Culture conditions for exopolysaccharide (EPS) and dextran production

NB medium with 1% (w/v) sucrose was used for the production of EPS and dextran. Moreover, in order to study the influence of the plant extract on EPS and dextran production by strain, *S. mutans* were added to NB medium with 1% (w/v) sucrose medium at the concentration of 5 mg/mL. All media, sugar and plant extract were sterilized as described above.

EPS production of S. mutans

Broth cultures were incubated at 30ºC for 24 h after inoculation. The cultures were boiled at 100ºC for 10 min. After cooling, they were treated with 1.7 µL trichloracetic acid solution (85%) and centrifuged. After the removal of the cells and protein centrifugation, the EPS was precipitated with ethanol (95%). The EPS was recovered by centrifugation at 4ºC at 14.000 g for 20 min. After the centrifugation (12.000 g for 30 min at 4ºC), the pellets were dissolved in the distilled water (Frengova *et al.*, 2000). Total EPS (expressed as mg L^{-1}) was estimated in each sample by phenol sulphuric method (Dubois *et al.*, 1956) using glucose as standard (Torino *et al.*, 2001). All the tests were performed in duplicate.

Dextran production of S. mutans

Isolation of bacterial dextran was done as described by Quader *et al* (Quader *et al.*, 2005). NB medium with 1% (w/v) sucrose (9 mL) was inoculated with 1 mL of inoculum (density was adjusted to 0.5 Mcfarland standards) and incubated at 37ºC for 24h. After the incubation time this culture (10 mL) was transferred to NB medium with 1% (w/v) sucrose (90 ml) and incubated at 37ºC for 24h. pH of this culture medium reduced from 7.5 to 5.5 during the fermentation. The culture medium after 24 h of incubation was precipitated by using chilled ethanol. In the first step, equal amount (100 mL) of ethanol was added, stirred well and centrifuged. The supernatant was decanted. In the second step, chilled ethanol was added with constant stirring and precipitates of dextran appeared. It was allowed to stand for 5-10 minutes and supernatant was again decanted. After standing 10 minutes chilled ethanol was added again and dextran was precipitated. The precipitated dextran was filtered and dried. The yield was calculated on dry weight basis. All the tests were performed in duplicate.

S. mutans biofilm formation with cover slips

Cover slips (0.8 x 0.8 cm) that were used for biofilm growth were soaked into concentrated sulfuric acid overnight. The following day, the concentrated sulfuric acid was washed with flowing water and the clean cover slips were immersed in 95% alcohol overnight. All the cover slips were washed three times with deionized water. For biofilm growth on the treated cover slips, as aforementioned, the cover slips were placed in 24-well plates and immersed in fetal bovine serum (FBS) at 4ºC overnight. Following this pretreatment, the cover slips were washed with PBS (0.01 M) to remove the residual FBS. In order to ensure uniform biofilm formation, the cover slips were immersed in 1 mL standardized cell suspension (0.5 Mc Farland) and incubated at 37ºC for 90 min. The cover slips were lifted carefully using tweezers and gently placed in each well of the 24-well plates containing 1.5 mL fresh NB medium. Samples were incubated at 37ºC for various durations (Pu *et al.*, 2014).

Susceptibility of the biofilms to plant extract

To evaluate the plant extract susceptibility of *S. mutans* cells grown in developing biofilms, the pretreated cover slips were immersed in 1 mL standardized cell suspension (0.5 Mc Farland) and incubated at 37ºC for 90 min. The cover slips were lifted carefully using tweezers and gently placed in each well of the 24-well plates containing 1 mL fresh NB medium. A 5 mg/mL plant extract was added to 24-well plates with cover slips and incubated at 37ºC for an additional 8 and 24 hours (Pu *et al.*, 2014).

Scanning electron microscopy (SEM)

S. mutans biofilms were grown on pretreated cover slips in 24-well plates, as described previously. In the plant extract group, *S. mutans* biofilms were incubated in 5 mg/ml plant extract. For the positive control, *S. mutans* biofilms were incubated in the presence of 200 µL NB without extract for 24 h. The cover slips with biofilms were subsequently washed three times with PBS and transferred to an additional 24-well plate containing 2.5% glutaraldehyde at 4ºC. The samples were prepared using a regular method for an electron microscopy examination (Tsang *et al.*, 2012) and viewed under an S-3000N scanning electron microscope (Hitachi High-Technologies, Tokyo, Japan). Two separate sets of culture were prepared.

Statistical analysis

Statistical analyses were performed using SigmaPlot-SigmaStat (version 12.0; Systat Software, Inc.) Student's *t* test. P values were calculated by the analysis of variance and p values of < 0.05 were considered significant.

RESULTS

MIC values of plant extracts on S. mutans planktonic cells

MIC tests of ethanol extracts of leaf and bark specimens were performed. Figure No. 1 and Figure No. 2 show the results of the experiment assessing the effect of ethanol extracts of leaf and bark specimens at various concentrations on *S. mutans* planktonic cell growth. The leaf extract, at a concentration of > 5mg/mL, was shown to inhibit the activity of *S. mutans* planktonic cells grown for 24 h. In addition, the bark extract, at a concentration of > 2.5 mg/mL, was shown to severely inhibit the activity these planktonic cells. The OD decreased significantly when compared with the positive control ($p < 0.05$). Due to stronger antimicrobial activity of the bark extract on bacteria, the study was continued with only the bark extract.

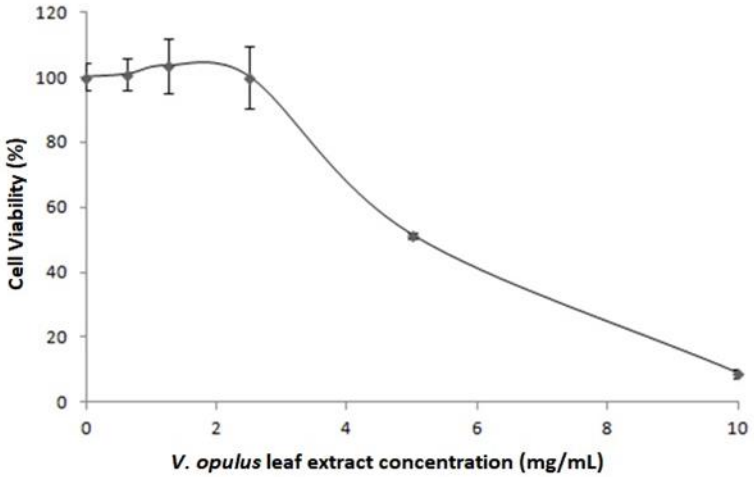


Figure No. 1
Growth curve of *S. mutans* planktonic cells with plant leaf extract. Planktonic cells were co incubated with various concentrations (10, 5, 2.5, 1.25 and 0.625 mg/mL) of plant leaf extract for 24 NB served as a negative control. The line curves show that plant leaf extract (> 5 mg/mL) inhibited the growth of *S. mutans*. Experiments were conducted three times, with similar results each time

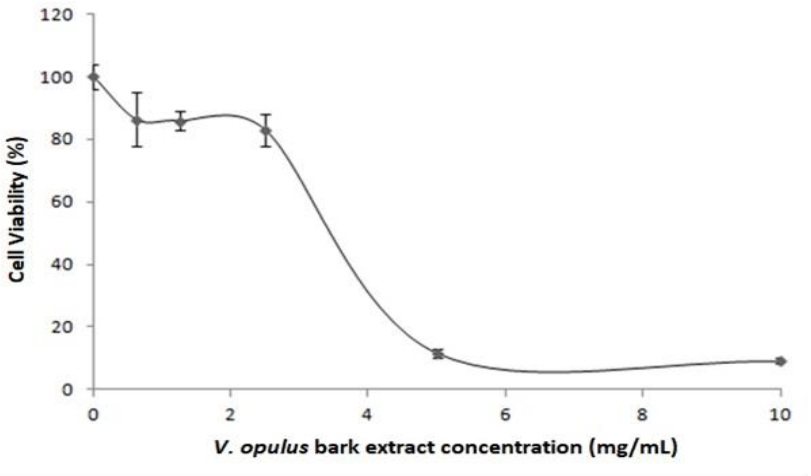


Figure No. 2
Growth curve of S. mutans planktonic cells with plant bark extract. Planktonic cells were co incubated with various concentra¬tions (10, 5, 2.5, 1.25 and 0.625 mg/mL) of plant bark extract for 24 NB served as a negative control. The line curves show that plant bark extract (> 2.5 mg/mL) strongly inhibited the growth of S. mutans. Experiments were conducted three times, with similar results each time

S. mutans biofilm formation

An XTT reduction assay was used to quantify the effects of this plant extract on biofilms produced by *S. mutans*. As shown in Figure No. 3A, Figure No. 3B and Figure No. 4A, Figure No. 4B the different phases (8 and 24 h, respectively) of this bacterial biofilm formation were significantly susceptible to the bark extract. A statistically significant difference was found in the concentration of 1.25-5 mg/L in the biofilm in the 8-hour phase and in all the concentrations studied in the biofilm in the 24-hour phase ($p < 0,05$). In addition, a marked difference was also observed between all the extract groups and the positive control ($p < 0.05$). It has been found that the inhibition is close to each other on the biofilm layer formed as a result of both incubation periods, but in the early phase (8 h), this effect has been found to be stronger at higher concentrations (≥ 2,5 mg/mL) than mature phase (24 h). The results show that biofilms in the mature phase (24 h) have demonstrated less sensitivity to higher concentrations of plant extract compared with those in the early phase (8 h).

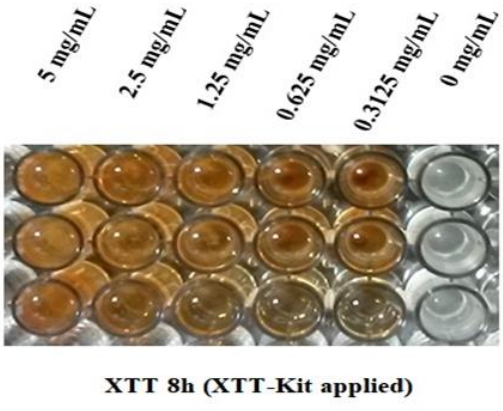
EPS production of S. Mutans

S. mutans was investigated for EPS production at NB medium with 1% (w/v) sucrose (control) and 1% (w/v) sucrose supplemented NB medium containing with 5 mg/mL plant bark extract. It was determined that the EPS production amounts of *S. mutans* were 0.072 and 0.046 mg/mL, respectively. Accordingly, the addition of the extract (5 mg/mL) used to detect the sensitivity of the biofilm indicated a significant reduction in bacterial EPS production (Table No. 1).

Dextran production of S. mutans

The amount of dextran production of *S. mutans* used in the study was determined in a NB medium supplemented with 1% (w/v) sucrose (control) and sucrose (1% (w/v)) supplemented NB medium containing with 5 mg/ml plant bark extract. It was found that the dextran production amounts of *S mutans* were 0.659 and 0.559 mg/mL, respectively. The results showed that the dextran production of the bacteria grown in the plant extract supplemented medium was reduced by about 15% (Table No. 1).

(A)



(B)

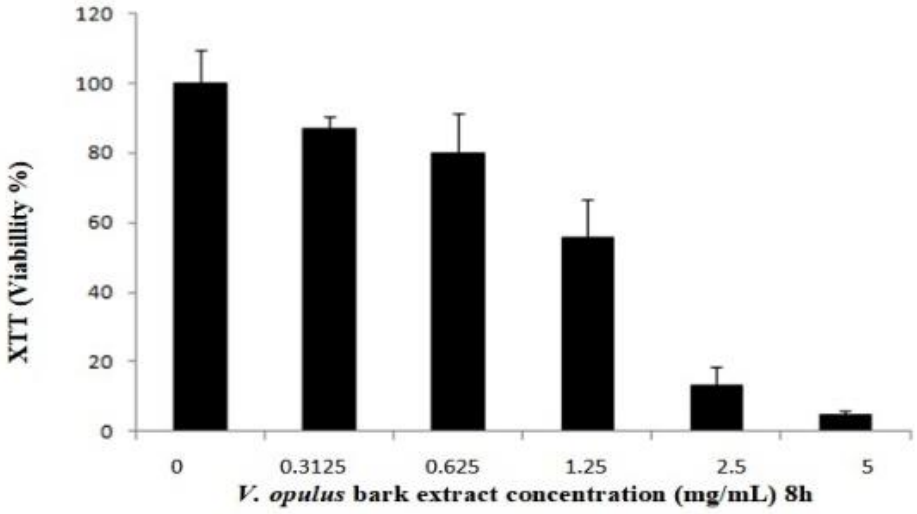
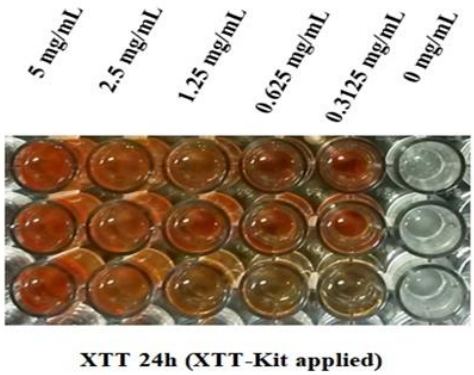


Figure No. 3
***S. mutans* biofilm development with *V. opulus* bark extract resistance; (A) 8 hour XTT study with XTT kit applied, (B) The susceptibility of *S. mutans* biofilms, incubated for 8 h to various concentrations (0.3125, 0.625, 1.25, 2.5 and 5 mg/mL) of plant bark extract are represented as histograms**

(A)



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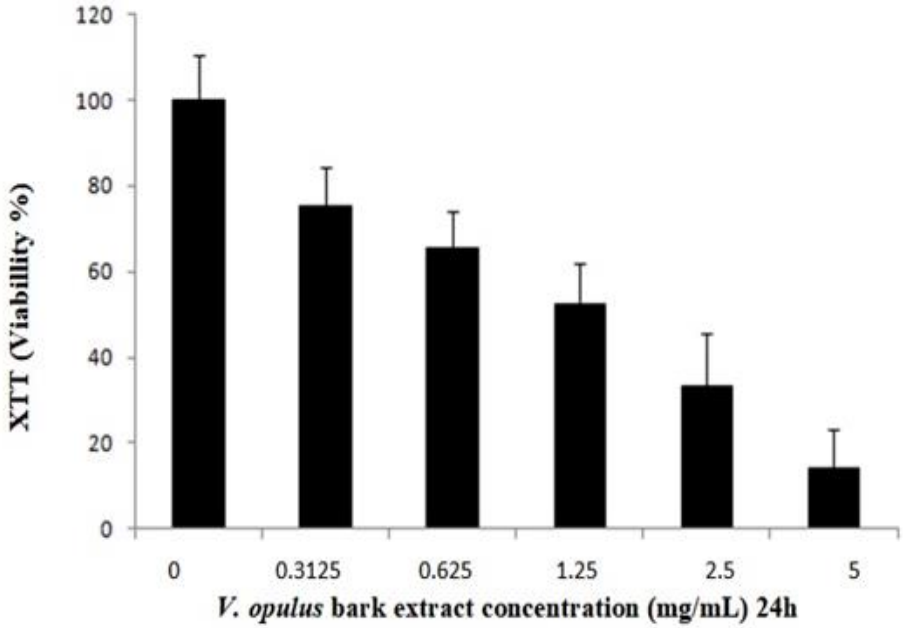


Figure No. 4
***S. mutans* biofilm development with *V. opulus* bark extract resistance; (A) 24 hour XTT study with XTT kit applied, (B) The susceptibility of *S. mutans* biofilms, incubated for 24 h to various concentrations (0.3125, 0.625, 1.25, 2.5 and 5 mg/mL) of plant bark extract are represented as histograms**

Table No. 1
EPS and dextran production after 24 hours incubation of *S. mutans* strain data are mean ± S.D., n = 3

Medium	EPS production (mg/mL)	Dekstran production (mg/mL)
NB medium supplemented with 1% (w/v) sucrose (Control)	0.072±0.008	0.659±0.003
Sucrose 1% (w/v) supplemented NB medium containing with 5 mg/mL plant bark extract	0.046±0.002	0.559±0.005

S. mutans biofilm formation with cover slips and SEM imaging of the susceptibility of the biofilms to plant extract

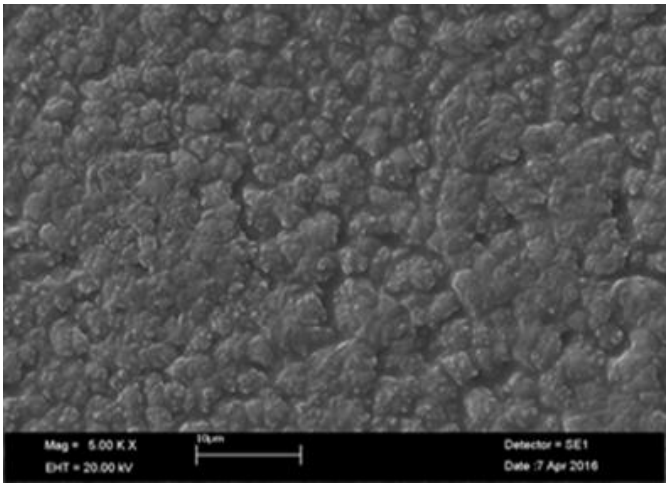
SEM examination was used to visualize the structural differences between control and plant extract-treated (5 mg/mL) *S. mutans* biofilms (8 and 24 h) (Figure No. 5A, Figure No. 5B, Figure No. 6A and Figure No. 6B. Control *S. mutans* biofilms exhibited a dense network layer surrounded by vast amounts of exopolymeric matrix. By comparison, plant extract-treated *S. mutans* expressed a biofilm which lacked a normal network structure and released polysaccharide. It has been noted that control biofilms exhibit a dense network layer and exopolymeric matrix, whereas the network structure and the exopolymeric matrix are visibly dispersed in 8 and 24 hours extract-treated biofilms.

DISCUSSION

A biofilm consists of a gelatinous matrix formed on a solid surface by adhesion of microbes to the surface and subsequent production of extracellular polymers. This process is referred to as biofouling. Dental plaque is an example of a biofilm, and it is considered recalcitrant to antimicrobial agents (Gibbons, 1984). *S. mutans* plays a significant role in dental caries by utilizing sugars and producing large amounts of EPS, which plays an important role in adhesion of bacteria and accumulation of biofilm (Lewis, 2001).

EPS are the key structural and protective matrix components of virulent dental biofilms that act as a supportive framework and barrier to diffusion. *S. mutans* has a number of exoenzymes e.g., glucosyltransferases (GTFs) that make it a essential synthesizer extracellular gummy dextrans (glucans) from sucrose, while it is also both fairly acidogenic and aciduric (Paes *et al.*, 2006; Bowen & Koo, 2011). The persistence of this acidic environment leads to the selection of a highly acid tolerant flora (Marquis *et al.*, 2003; Beighton, 2005), so the low pH environment in the plaque matrix causes demineralization of adjacent enamel and hence initiating the dental caries process. Consequently, EPS and acidification of the biofilm matrix are critical for the formation and establishment of cariogenic dental plaque (Bowen, 2002; Marsh, 2003). *S. mutans* also produces a single fructosyltransferase (FTF), which catalyses the synthesis of fructans from sucrose (Ebisu *et al.*, 1975). Fructans are believed to function primarily as extracellular storage polysaccharides that can be metabolized during periods of nutrient deprivation (Burne *et al.*, 1996). These polysaccharides provide the organisms with a unique microenvironment for their growth, metabolism and survival (Yamashita *et al.*, 1993; Bowden & Hamilton, 1998). Therefore, to prevent the formation and accumulation of cariogenic biofilm assemblies by affecting the synthesis of these polysaccharides is an attractive pathway for preventing both dental caries and plaque formation.

(A)



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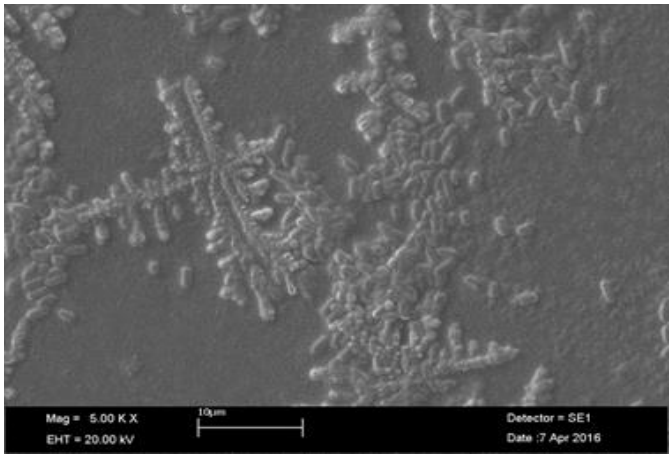
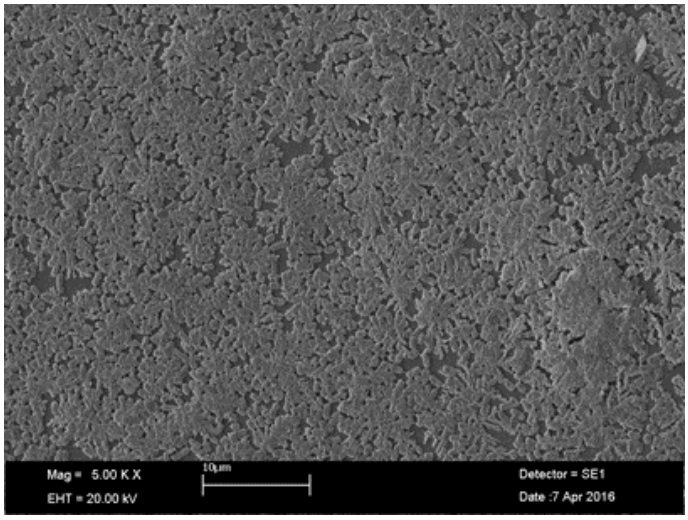


Figure No. 5
(A) *S. mutans* biofilms cultured in NB medium supplemented with 1% (w/v) sucrose (control) for 8 h showed bacterial cells surrounded by large amounts of exopolymeric matrix. (B) By contrast, biofilm co-incubated with sucrose (1% (w/v)) supplemented NB medium containing with 5 mg/mL plant bark extract for 8 h, releasing polysaccharides

(A)



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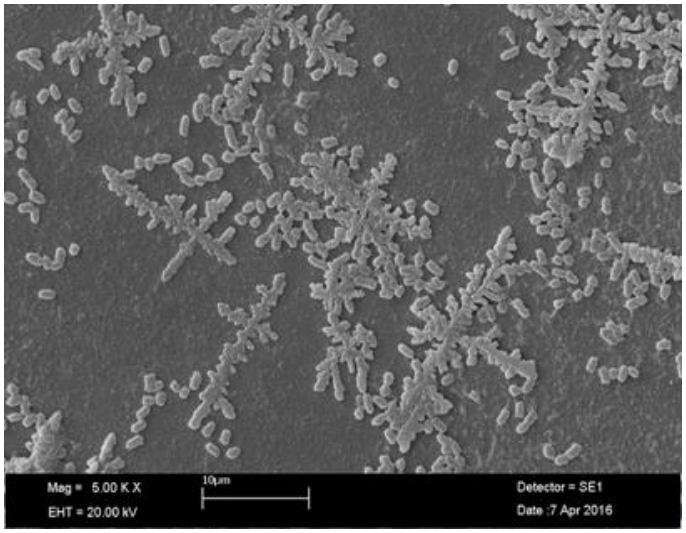

Figure No. 6
(A) *S. mutans* biofilms cultured in NB medium supplemented with 1% (w/v) sucrose (control) for 24 h showed bacterial cells surrounded by large amounts of exopolymeric matrix. (B) By contrast, biofilm co-incubated with sucrose (1% (w/v)) supplemented NB medium containing with 5 mg/mL plant bark extract for 24 h, releasing polysaccharides

Antifouling is the process of removing or preventing the organism accumulation and growth. This process can be used to control biofouling. Many commercially available antiplaque agents have been tested for their ability to interfere with dental biofilm formation or metabolism. However, alternative agents should be sought due to the adverse side effects associated with these agents. Therefore, a proposed approach is to use cheap, effective, stable, new and natural products as anti-biofouling agents (Briand, 2009; Sendamangalama *et al.,* 2011). Plants are valuable sources of new bioactive compounds to combat dental caries, because they produce a wide variety of secondary metabolites, many of which have been found to have biological properties against oral pathogens *in vitro* (Jeon *et al.,* 2011). Several natural products including oolong tea (Nakahara *et al.,* 1993), propolis (Koo *et al.,* 2000; Duarte *et al.,* 2003), green tea (Hattori *et al.,* 1990), cacao extract (Ito *et al.,* 2003), Chinese black tea (Limsong *et al.,* 2004), dark beer (Murata *et al.,* 1995) have been shown to inhibit GTF in vitro.

The genus *Viburnum* is known to contain triterpenoids, diterpenoids, sesquiterpenes, iridoids, and polyphenols (Tao *et al.,* 2007). Due to the medicinal properties of the species of this genus the aim of this study is to identify the effects on *S. mutans* biofilm formation of *V. opulus* bark and leave ethanol extracts. The present study analyzes the role of plant extracts on *S. mutans* using the MIC. The results indicates that the extracts have bacteriostatic activity against these planktonic cells (Figure No. 1 and Figure No. 2). The study was continued with the bark extract only due to its stronger antimicrobial activity on bacteria according to the results obtained in the MIC test. Repeated XTT reduction assay analysis revealed that bark extract at a concentration of 2.5 mg/mL had the potential to kill > 50% of cells in the early (8 h) and mature (24 h) phases of biofilm development. Howewer, this concentration and higher concentration (5 mg/mL) showed better activity in the early phase of biofilm than in the mature phase (Figure No. 3a, Figure No. 3b, Figure No. 4a and Figure No. 4b). The barks of *V. lantanal* have been used medicine for folk as rubefiant and analgesic (Iwai *et al.,* 2004). Khan *et al.* (2012), isolated a phytochemical agent from the leaves of *V. foetens* and demonstrated its potential for antibiotic and anti-adhesion on *S. mutans*. In another study, it was also reported that the crust extract of *V. opulus* strain contains viburnin, tannin, valeric acid esters and flavonoids (Ovodova *et al.,* 2000). Bubulica *et al*., 2012). have suggested that the *V. opulus* extract may be an alternative agent for medical biofilms by studying antibacterial and antibiofilm properties against pathogens of *Staphylococcus aureus* and *Staphylococcus epidermidis* (Bubulica *et al.,* 2012). *Our* study is the first report to show that degenerative effects of the bark extract of *V. opulus* against *S. mutans* biofilms. No paper about this subject has been found in the literature studies so far.

Dextran is an EPS consisting of 1→6 linked α-d-glucopyranose units (Monchois *et al.,* 1999). Dextran could be considered the first remarkable example for a microbial EPS used in pharmaceutical applications (Zorba & Altuğ, 2001). In addition, numerous microorganisms including streptococci synthesize dextran from sucrose (Quader *et al.,* 2005).

S. mutans was determined for EPS and dextran productions at NB medium with 1% (w/v) sucrose (control) and 1% (w/v) sucrose supplemented NB medium containing with 5 mg/mL plant bark extract. It was found that bacterium developing in plant extract supplemented medium showed a decrease in both dextran and EPS productions according to their controls. This result is in agreement with biofilm susceptibility to bark extract. The SEM study revealed the structural differences between *S. mutans* biofilms (8 and 24 h). The results of the present study also revealed that in the overall biofilm formation period, the plant bark extract (5 mg/mL)-treated biofilms exhibited a slower growth compared with those that were untreated (control) (Figure No. 5a, Figure No. 5b, Figure No. 6a and Figure No. 6b).

CONCLUSION

Consequently, the data generally suggest that *V. opulus* ethanol bark extract treatment can provide an option for controlling biofilm development. These data indicate that the *V. opulus* ethanol bark extract is an antimicrobial agent that can be developed on *S. mutans* biofilm diseases, which is one of the most important factors in the formation of dental plaques. However, the plant extract to be a potential antibiofilm agent against *S. mutans* may require further in vivo studies.

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