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Original Article

Evaluation of biofilm removal activity of *Quercus infectoria* galls against *Streptococcus mutans*

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ABSTRACT

Background: Dental caries is one of the most prevalent infectious diseases affecting humans of all ages. *Streptococcus mutans* has an important role in the development of dental caries by acid production. The purpose of this study was to evaluate the antibacterial and biofilm disinfective effects of the oak tree *Quercus infectoria* galls against *S. mutans*.

Materials and Methods: The bacterial strain used in this study was *S. mutans* (ATCC: 35668). Two kinds of galls, Mazouj and Ghalghaf were examined. Galls were extracted by methanol, ethanol and acetone by Soxhlet apparatus, separately. Extracts were dissolved in sterile distilled water to a final concentration of 10.00, 5.00, 2.50, 1.25, 0.63, 0.31, and 0.16 mg/ml. Microdilution determined antibacterial activities. The biofilm removal activities of the extracts were examined using crystal violet-stained microtiter plate method. One-way ANOVA was used to compare biofilm formation in the presence or absence of the extracts.

Results: The methanolic, ethanolic, and acetonic extracts of *Q. infectoria* galls showed the strong inhibitory effects on *S. mutans* ($P < 0.05$). The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values for the Mazouj and Ghalghaf gall extracts against *S. mutans* were identical. The MIC values ranged from 160 μg/ml to 320 μg/ml, whereas the MBC values ranged from 320 μg/ml to 640 μg/ml. All extracts of *Q. infectoria* galls significantly ($P < 0.05$) reduced biofilm biomass of *S. mutans* at the concentrations higher than 9.8 μg/ml.

Conclusion: Three different extracts of *Q. infectoria* galls were similar in their antibacterial activity against *S. mutans*. These extracts had the highest biofilm removal activities at 312.5 μg/ml concentration. The galls of *Q. infectoria* are potentially good sources of antibacterial and biofilm disinfection agent.

Key Words: Biofilm removal activity, dental plaque, oak gall, plant extracts, *Streptococcus mutans*

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INTRODUCTION

Despite many efforts toward its control and prevention, dental caries is still one of the most prevalent infectious diseases affecting humans. Dental plaque as a biofilm, structured microbial communities attached to a surface, is one of the significant medical concerns. *Streptococcus mutans* has been implicated as a primary etiological agent of dental caries worldwide. *S. mutans* is a Gram-positive coccus found in the mouth normally. *S. mutans* can make a polysaccharide capsule and is

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involved in biofilm formation. The bacterium ferments simple carbohydrates in food and releases organic acids, mainly lactic acid. These organic acids demineralize the teeth and cause the development of dental caries.[1-3]

The key to prevention and treatment of oral infectious diseases is the effective control of these cariogenic bacteria. However, the elimination of bacteria is difficult because oral microorganisms form plaque that shelters pathogens and enhances the resistance to antimicrobial agents. The Mechanical removal of dental biofilms is the preferred method for the prevention of caries and periodontal diseases.[4] Dental biofilms cannot be eliminated thoroughly ; therefore, the aim of the antimicrobial agents is to control rather than eliminating dental plaque. Antibiotics and antiseptics are used for the prevention and treatment of oral infections, but a major problem has been the emergence of resistant bacteria.[5-7]

The *Quercus infectoria* (*Fagaceae*), the oak tree is widely distributed throughout the Western provinces of Iran and is an important source of wood and fibers. *Q. infectoria* Olivier is a small tree native of Greece, Asia, and Iran. The galls arise on branches of this tree as a result of an attack by the gall-wasp.[8,9] The galls can be seen as abnormal growth caused by an increase in the number (hyperplasia) or size (hypertrophy) of plant cells formed as a response to the insect's stimulus caused by egg-laying, larvae, or nymph feeding. Two kinds of galls are locally known as Mazouj and Ghalghaf in Iran and have been shown to have many medicinal properties such as astringent, antibacterial, antifungal, antiviral, antidiabetic, local anesthetic, larvicidal, and anti-inflammatory activities.[10,11] The Mazouj and Ghalghaf gall types are caused by two different gall-wasp species, that is, *Cynips tinctoria* and *Andricus quercustozae,* respectively.

Plants synthesize secondary metabolites that have potential activity against bacteria. Previous research has shown that *Q. infectoria* gall extracts can inhibit oral pathogen.[9,12] So far no studies have determined the anti-biofilm and biofilm removal activities of *Q. infectoria* gall extracts against oral bacteria. The present study was to evaluate the biofilm removal activity of acetone, ethanol, and methanol extracts of *Q. infectoria* gall against *S. mutans*.

MATERIALS AND METHODS

Preparation of plant materials

Galls of *Q. infectoria* were collected from the oak trees of Lorestan in 2012 fall. Galls (Mazouj and Ghalghaf) were identified by the Herbarium of Research Institute of Agriculture Jihad of Lorestan, Iran.

All galls were washed with distilled water, cut into small pieces, and dried at room temperature for 2 weeks. Then galls were powdered in an electric grinder aseptically. For preparation of ethanolic, methanolic and acetonic extracts of *Q. infectoria* galls, the dried powdered of galls were extracted in a Soxhlet apparatus. The ground gall (approximately 50 g) was weighed in a flask, followed by adding 100 ml of solvent (ethanol, methanol or acetone) and storing for 5 h. All the extracts were sterilized by passing through a 0.45 µm membrane filter. These extracts were vacuum dried using rotary evaporator. The extracts were stored at −20°C and freshly dissolved in 10% dimethyl sulfoxide (DMSO, Merck, Germany) before using.[13]

Bacterial strains

The *S. mutans* (ATCC: 35668) strain was obtained from the Iranian Research Organization for Science and Technology, Tehran, Iran. *S. mutans* was revived by streaking onto Brain Heart Infusion (BHI) agar (BHI, Merck, Germany) supplemented with blood and incubated at 37°C for 24 h. The fresh inoculums of *S. mutans* were standardized by adjusting the optical density (OD) of the bacterial suspension to a turbidity corresponding to spectrophotometric absorbance OD = 0.08-0.1 at 630 nm. This bacterial suspension was equal to 1.5×10^8 bacterial cells per ml (cfu/ml).

Determination of minimum inhibitory concentration and minimal bactericidal concentration values

The minimum inhibitory concentration (MIC) of the extracts for *S. Mutans* was determined using the two fold serial microdilution method as recommended by the National Committee for the Clinical Laboratory Standard.[14] Serial dilutions of the extracts were done by dissolving powder of each extract in Muller Hinton broth medium to a final concentration ranging from 10.00 mg/ml to 0.16 mg/ml. The test wells of a 96-well microtiter plate were filled with 100 µl of bacterial suspensions (about 1.5×10^8 cfu/ml) and 100 µl of different concentrations of gall extract. The bacterial suspension was used as positive control and extracts in broth were used as negative control [Figure 1]. The absorbance of each well was determined using an automatic ELISA tray reader (Stat Fax-2100, Awareness Technology, USA) adjusted at 630 nm.

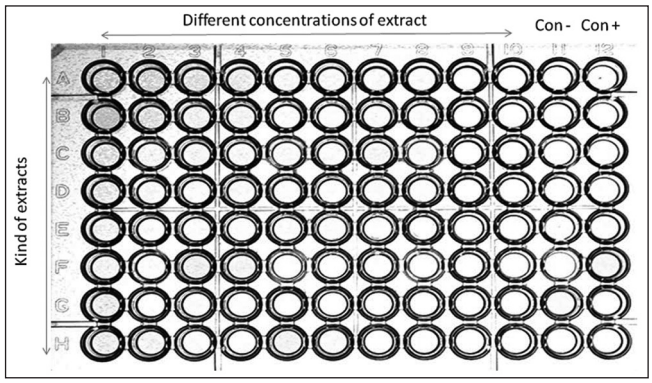

Figure 1: Schematic picture of the microtiter plate.

The plate was incubated at 37°C for 24 h, agitated, and the absorbance was read again in the reader at the same wavelength. These absorbance values were subtracted from those obtained before incubation. The MIC values were taken as the lowest concentration of the extracts in the test tubes that showed no turbidity after 24 h of incubation at 37°C.

To determine the minimal bactericidal concentration (MBC), 10 µl of each well was transferred to Mueller Hinton agar (Merck, Germany) plates and incubated at 37°C for 24 h. The MBC was considered as the lowest concentration of the extracts associated with no visible growth of bacteria on the agar plates. All samples were tested in triplicate.[9]

Biofilm removal assay

Screening of the extracts for the prevention of the attachment and inhibition of further growth and development of a preformed biofilm was followed. The assay used in this study to assess the biomass production of the biofilm was the crystal violet (CV)-stained microtiter plate method. To avoid the biofilm disinfection in response to antimicrobial activities, sub-MIC concentrations of the extracts was tested in the biofilm assay.[15,16] *S. mutans* was grown in BHI broth containing 1% glucose, for 24 h at 37°C and then diluted to obtain a suspension, the OD of which at 630 nm was about 0.08-0.1. The diluted suspension was added into the wells (250 µl/well) of a sterile 96-well polystyrene microtiter plate and incubated for 24 h at 37°C. Then 100 µl of each well was removed from and 100 µl of BHI broth containing 1% glucose added to wells. The microtiter plate incubated for 72 h at 37°C. After 72 h, the wells were washed 3 times with 250 µl of sterile phosphate-buffered saline and air-dried at 37°C. Each of the wells was filled with 100 µl of BHI broth, except the positive control wells. Final concentrations ranging from 4.9 µg/ml to 312.5 µg/ml of *Q. infectora* gall extracts was added to each as well. The plates were incubated for 1 h at 37°C. Following this incubation period, the medium was removed, and the plates were air-dried. The wells with adhered biofilms were fixed with 250 µl of 96% ethanol per well and after 15 min, the plates were made empty and left to dry. Each well was then stained for 5 min with 200 µl of 2% CV (CV Gram-stain, Merck, Germany). The stain was rinsed off by placing the plates under running tap water. CV stain is suitable for determining the amount of biofilm.[17] After drying the stained plates, the biofilms were visible as purple rings formed on the sides of each well. The quantitative analysis of biofilm production was performed by adding 200 µl of 33% (v/v) glacial acetic acid (Merck, Germany) per well. Then OD of the stain was measured at 495nm by an ELISA tray reader (Stat Fax-2100, Awareness Technology, USA).[15,17]

$$\text{Reduction percent (RP)} = \text{RP} = \frac{(C-B)-(T-B)}{C-B}\times 100.$$

C = Mean of OD of positive control wells.

B = Mean of OD of negative control wells.

T = Mean of test wells.

Statistical analysis

One-way ANOVA was used to compare biofilm removal in the presence or absence of the extracts. All the assays were repeated 3 times. Data were expressed as a mean ± standard deviation and $P \leq 0.05$ was considered to be statistically significant.

RESULTS

The broth microdilution method was used to determine MIC and MBC of different concentration extracts of *Q. infectoria* galls. The MIC and MBC values of the ethanol, methanol, and acetone extracts of galls of *Q. infectoria* against *S. mutans* are shown in Table 1. There is no difference between the MICs and MBCs of Mazouj and Ghalghaf galls. MIC value of acetone extracts was obtained 160 µg/ml, whereas MIC value of ethanol and methanol extracts were 320 µg/ml same. As we expect, MBC values are greater than related MIC.

Biofilm disinfection activity of different concentration extracts of *Q. infectoria* galls was determined by CV staining method. RP of biofilms biomass in the presence of extracts are presented in Figures 2 and 3.

The intensity of the biofilm biomass production was significantly reduced in the presence of all the extracts ($P > 0.05$). According to the diagram, the positive correlation between the biofilm reduction and increased concentration of all gall extracts was seen. Maximum and minimum biofilm reductions of Mazouj gall were 84.9% (ethanol extract) and 36.1% (acetone extract), respectively. Acetone and ethanol extracts of Ghalghaf gall resulted in 100% reduction of the biofilm biomass. Minimum biofilm biomass reductions of Ghalghaf gall was 26.3%. Results analysis revealed a significant difference between biofilm biomass reductions and increased the concentration of all gall extracts ($P > 0.05$).

DISCUSSION

Streptococcus mutans in dental plaque plays an important role in tooth decay. Inhibition of *S. mutans* adherence to tooth surfaces is important for the prevention of plaque formation.[18,19] Our study showed that the extracts from the galls inhibited the *S. mutans to grow*very well.

The MBC values for all gall extracts of *Q. infectoria* were higher than the MIC values of the same extracts against *S. mutans*. The MIC and MBC values for the Mazouj and Ghalghaf gall extracts against *S. mutans* were identical. The MIC and MBC values of ethanolic and methanolic extracts were 2 times greater than that of acetonic extract for *S. mutans* [Table 1]. Basri reported that the MIC ranges for methanolic and acetonic extracts of *Q. infectora* galls were the same, between 0.16 and 0.63 mg/ml.[9] This suggests that the bioactive compound in the extracts of the galls of *Q. infectoria* has the same bactericidal and bacteriostatic activity against *S. mutans* as reported previously.[9] It is well known that tannin is a phenolic compound that is soluble in water, alcohol, and acetone and precipitates with protein.[20] The similarity of the antimicrobial activity of the ethanolic, methanolic, and acetonic extracts suggests that these extracts may have high total tannin content. The antimicrobial activity seemed to depend on the contents of tannin in the plant extracts. High content of tannin in the galls of *Q. infectoria* is responsible for the antibacterial activity.[21-23]

In the present study, we tested the disinfective effect of different extracts of two kinds of *Q. infectoria* galls on *S. mutans* biofilm removal of. At the MIC value, *Q. infectoria* extract significantly reduced the biofilm biomass formation of *S. mutans*. At sub-MIC level, while the bacterial growth was not inhibited, the biofilm-forming ability of the organisms was significantly reduced. Anti-biofilm activity of *Q. infectoria* Olivier against methicillin-resistant *Staphylococcus aureus* was established by Chusri *et al*. their results indicated that *Q. infectoria* ethanolic extract affected the Staphylococcal biofilm formation at 0.50 mg/ml (lower concentration of MIC).[24] Mansouri showed methanolic extracts of

Table 1: MICs and MBCs of *Quercus infectoria* gall extract against *Streptococcus mutans* (µg/ml)

Galls extracts	Mazouj		Ghalghaf	
	MIC	MBC	MIC	MBC
Acetone	160	320	160	320
Ethanol	320	640	320	640
Methanol	320	640	320	640

MICs: Minimum inhibitory concentrations; MBCs: Minimal bactericidal concentrations

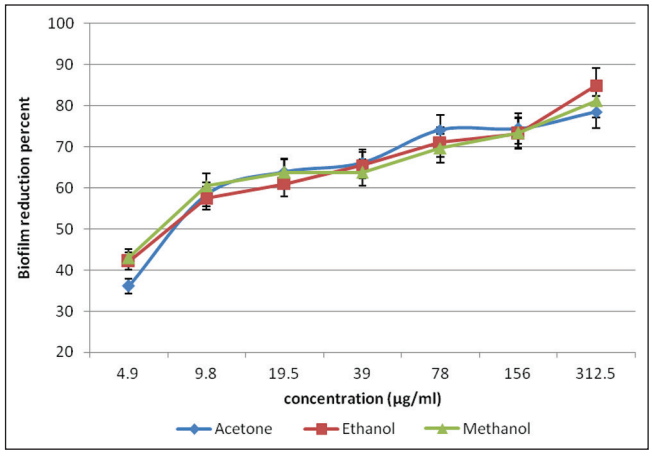

Figure 2: Biofilm reduction percent by *Streptococcus mutans* in the presence of Mazouj gall extracts.

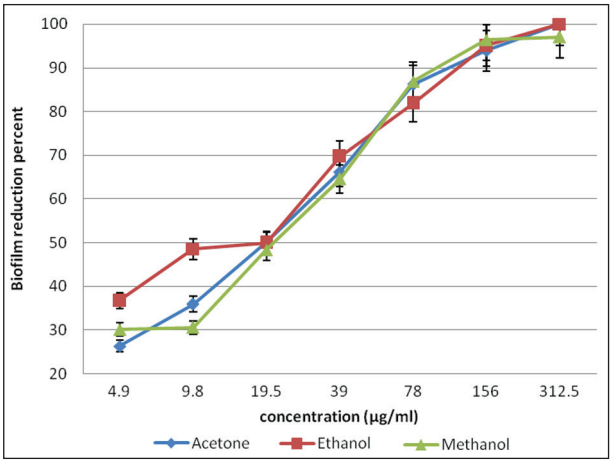

Figure 3: Biofilm reduction percent by *Streptococcus mutans* in the presence of Ghalghaf gall extracts.

Q. infectoria inhibited growth and biofilm formation of *Pseudomonas aeruginosa* at 500 μg/ml.[25]

Biofilm biomass formation of *S. mutans* in the presence of different concentrations of the extracts was significantly reduced. Reduction biomass percentage value ranges from 30.6% to 87.0%. Maximum inhibition reached to 312.5 μg/ml of methanol extract of Ghalghaf gall and minimum reduction showed 9.8 μg/ml of the same extract. Reduction biomass percent showed a dose dependent manner as well.

CONCLUSION

The Mazouj and Ghalghaf extracts of *Q. infectoria* are high potent as antibacterial and biofilm removal agents. These findings highlighted that the bioactive materials of *Q. infectoria* galls may be useful for the prevention and treatment of dental caries. Further work to understand the relation between antibacterial and biofilm biomass formation is needed for the development of new means to fight the infections caused by *S. mutans* in future. Also, further studies should be conducted to determine these extracts have no effect on oral microflora or human tissues.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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