

Original Article

Inhibitory effects of ethanolic, methanolic, and hydroalcoholic extracts of olive (*Olea europaea*) leaf on growth, acid production, and adhesion of *Streptococcus mutans*

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ABSTRACT

Background: Usage of chemical mouthwashes for controlling dental caries can end to some side effects such as oral biological imbalance. Recently, using natural derivatives such as herbs, presented to overcome such adverse effects. Due to antibacterial property of olive leaf extracts (*Olea europaea*), this study conducted in order to evaluate bacteriocidal, anti-acid production, and anti-adhesion effects of olive leaf ethanolic, methanolic, and hydroalcoholic extracts on *Streptococcus mutans*.

Materials and Methods: In this *in vitro* study, nine strains of *S. mutans* (PTCC1683) were used. Maceration methods were done in order to provide the olive leaf ethanolic, methanolic, and hydroalcoholic extracts. The antibacterial activities evaluated by macrodilution and disc diffusion method in different concentrations (3.25%–100%). Acid production and adhesion of bacterial strains also were evaluated. The obtained data were analyzed by analysis of variance method using SPSS software. $P < 0.05$ was considered as statistically significant.

Results: The minimum inhibitory concentration and minimum bactericidal concentration for ethanolic, methanolic, and hydroalcoholic olive leaf extracts on *S. mutans* are 12%–25%, 50%–75%, and 12%–25%, respectively. In addition, inhibition zone of *S. mutans* significantly increased in higher concentration (ethanolic and methanolic extracts: $P = 0.004$; hydroalcoholic extract: $P = 0.003$). The acid production and adhesion significantly decreased by increase in the concentration ($P < 0.001$).

Conclusion: In general, olive leaf ethanolic, methanolic, and hydroalcoholic extracts induce growth inhibition, acid production, and adhesion of *S. mutans*. Consequently, it can be used as a natural preservative in the food industry, as well as in the production of commercial products such as chewing gum, chocolate, and toothpaste to prevent dental caries.

Key Words: Bacterial adhesion, microbial sensitivity tests, *Olea*, *Streptococcus mutans*

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INTRODUCTION

Dental caries is the most prevalent chronic bacterial disease observed in people worldwide. It forms through a complex interaction over time between acid-producing bacteria and fermentable carbohydrate,

and the host factors including saliva and teeth.^[1,2] Dental caries is caused by the bacterial activity that can efficiently create an acidic environment to remove

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the dental minerals. This gelatinous mass of bacteria that binds to the surface of the tooth is called dental plaque.^[3] Several (above 330) bacterial species may colonize the buccal cavity of the adults; however, only a small group of bacteria can produce acid and cause caries. The main strains involved in this process are Streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) and *Lactobacillus*.^[4,5] *S. mutans* is known to be the main etiologic factor of decay^[6] and is naturally present in the mouth as a small group of oral microbial complexes.^[7]

The routine methods of decay prevention include mechanical methods (toothbrush–dental floss) and the use of chemical agents with antimicrobial features, such as mouthwashes. The use of chemical agents may lead to microbial resistance and can also cause certain complications by disturbing the biological equilibrium of the mouth.^[8]

In recent years, due to the increasing resistance of pathogenic microorganisms to the chemical antimicrobials and the side effects of these compounds, researches on medicinal plants have been considered in order to discover new antibacterial sources.^[9] Medicinal plants are a rich source of active biological compounds with advanced antibacterial properties.^[10] The most known cause of antimicrobial activity in medicinal plants is the presence of phytoalexins compounds, which are small antibiotics with <500 molecular weight, and are divided to several groups including polyphenols, flavonoids, terpenoids, and glycosteroids.^[11] Recently, efforts have been increased to identify new therapeutic strategies against oral microbes, using plant compounds. Regarding the presence of secondary metabolites, plants have significant pharmacological effects toward different microorganisms and their synergistic patterns.^[10]

Olive (*Olea europaea*) leaf have been widely used in folk medicine over centuries.^[12] Olive leaf extract contains a noticeable amount of polyphenols, which is responsible for its various medicinal properties.^[13] Several studies have reported the antimicrobial activity of different medicinal plants and its biological compounds.^[14-17] However, very limited studies have been done on antimicrobial effects of olive leaf on bacteria in oral flora.^[10]

Consequently, the aim of this study was to investigate the inhibitory effects of ethanolic, methanolic, and hydroalcoholic extracts of

O. europaea leaf on growth, acid production, and adhesion of *S. mutans*.

MATERIALS AND METHODS

Preparation of extract

This study was approved by the research and ethics committee of Isfahan university of medical sciences No: 295045. In this *in vitro* study, the fresh olive leaves were collected from olive gardens in Shahin Shahr, Isfahan province, Iran, in 2015. The obtained olive leaf dried in 3 days at room temperature and then powdered. The preparation of plant extracts was performed by maceration method.^[18] About 500 ml of solvent was added to 100 g of olive leaf powder and stirred for 5 days at room temperature. The obtained solutions were filtered and the remaining plant was pressed by press instrument. To prevent chemical changes of extracts, due to exposure to sunlight, preparation of extracts was carried out in a dark place. The obtained extracts were kept at sterilized vials at 4°C.

Preparation of bacterial strains

In this study, nine strains of *S. mutans* PTCC1683 (Persian Type Culture Collection, Tehran) were purchased from microbial collection of Iranian Research Organization for Science and Technology, and bacterial suspension was prepared according to the 0.5 McFarland standard (1.5×10^8 CFU/ml).

Evaluation of antimicrobial activity by macrodilution method

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination, was performed according to the Clinical and Laboratory Standards Institute (CLSI) guide.^[19,20] The Brain Heart Infusion broth (BHI) (Merck, Darmstadt, Germany) was added (1 ml) to each tube. Then, 100% concentration of leaf extracts was added to the first well and other dilutions (10 concentrations) were prepared by serial macrodilution method. Following this, 1 ml bacterial suspension equivalent (1.5×10^8 CFU/ml) was added to each tubes. Furthermore, two tubes were considered as positive (medium with bacterial suspension) and negative (medium with extracts) controls, respectively. The tubes were incubated for 18–24 h at 37°C and then its opacity was evaluated.

The first tube without opacity was considered as MIC and was cultured on a BHI broth and incubated for 18–24 h at 37°C. The lowest concentration at

which an antimicrobial agent will kill a particular microorganism was considered MBC. This experiment was repeated three times for each ethanolic, methanolic, and hydroalcoholic extracts.

Evaluation of antimicrobial activity by disc diffusion

According to the CLSI guidelines,^[21] using a sterile inoculating loop, four or five isolated colonies were cultured uniformly on Mueller–Hinton agar. The blank discs containing prepared concentrations of extracts are placed on inoculated Mueller–Hinton agar and incubated at 37°C during the night. The diameter of inhibition zone was measured and result read from the Kirby–Bauer chart as sensitive, intermediate, or resistant.^[22]

Evaluation of acid production

The method described by Matsumoto *et al.*^[23] was used to investigate the effect of ethanolic, methanolic, and hydroalcoholic extracts of olive leaf on acid production by *S. mutans*. The sterile extracts of olive leaf (0.1–0.5 mg/ml) and bacterial suspension (0.05 ml) were added to 0.95 ml phenol red solution containing 1% glucose. After 24 h incubation at 37°C, the pH was measured. The solution of sodium fluoride 1% was used as a positive control. This experiment was repeated three times for each ethanolic, methanolic, and hydroalcoholic extracts.

Evaluation of adhesion

The method described by Köhler *et al.*^[24] was used to investigate the effect of ethanolic, methanolic, and hydroalcoholic extracts of olive leaf on adhesion by *S. mutans* to the glass. The suspension of *S. mutans* was cultured in test tubes containing ethanolic, methanolic, and hydroalcoholic extracts of olive leaf (3.125%–100%) for 18 h at 37°C. Then, the contents of the tubes were discarded and the sticks to bacteria to the tube were suspended in distilled water by a shaker. The bacterial colonies were measured by WPA Biowave II spectrophotometer (Biochrom UK) at 550 nm. This experiment was repeated three times for each ethanolic, methanolic, and hydroalcoholic extracts.

Statistic analysis

Statistical analysis of obtained data was performed using the Statistical Package for the Social Sciences software version 22.0 (Chicago, IL, USA). The experiments were repeated three times and obtained data were analyzed by analysis of variance (ANOVA) method and Bonferroni test. $P < 0.05$ was considered as statistically significant.

RESULTS

In this study, the antibacterial effects of ethanolic, methanolic, and hydroalcoholic extracts of olive leaf were determined using MIC, MBC, and inhibition zone diameter. The MIC and MBC values of the extracts are shown in Table 1.

Analysis of various concentrations effects of ethanolic, methanolic, and hydroalcoholic extracts of olive leaf on bacterial growth (inhibition zone) of *S. mutans* was performed using one-way ANOVA. The results showed [Table 2] that the inhibition zone had a significant correlation with concentration in all three extracts at higher than 6.25% concentrations (ethanolic and methanolic extracts: $P = 0.004$; hydroalcoholic extract: $P = 0.003$).

Effects of extract type on diameter of inhibition zone were analyzed using the Bonferroni test. The results showed that inhibition zone of ethanolic and methanol extracts was equal ($P > 0.05$) and higher than hydroalcoholic extract ($P < 0.05$), at 100% concentration. Furthermore, diameter of inhibition zone of methanolic and hydroalcoholic extracts was equal ($P > 0.05$) and less than ethanolic

Table 1: The minimum inhibitory concentration and minimum bactericidal concentration of olive leaf ethanolic, methanolic, and hydroalcoholic extracts on *Streptococcus mutans* (%)

Extract type	MIC (%)	MBC (%)
Ethanolic extracts	12	25
Methanolic extracts	50	75
Hydroalcoholic extracts	12	25

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration

Table 2: The mean and standard deviation of *Streptococcus mutans* inhibition zone in the effects of different concentrations of ethanolic, methanolic, and hydroalcoholic extracts of olive leaf (mm)

Concentrations (%)	Ethanolic extracts		Methanolic extracts		Hydroalcoholic extracts	
	Mean	SD	Mean	SD	Mean	SD
100	21.93	2.12	20.50	1.55	14.63	1.24
50	14.97	4.06	9.73	0.46	8.39	0.80
25	9.07	1.63	6.97	0.34	6.76	0.43
12.5	6.77	0.26	6.45	0.03	6.45	0.57
6.25	6.41	0.17	6.40	0.01	6.40	0.00
3.125	6.40	0.00	6.40	0.00	6.40	0.00
Negative control	6.41	0.12	6.40	0.00	6.40	0.00
Statistically significant	<0.004		<0.004		<0.003	

SD: Standard deviation

extract ($P < 0.05$), at 25% and 50% concentrations. There was no significant difference in inhibition zone of ethanolic, methanolic, and hydroalcoholic extracts, at 1.625%, 3.125%, 6.25%, and 12.5% concentrations ($P > 0.05$), respectively [Figure 1].

Analysis of various concentrations effects of ethanolic, methanolic, and hydroalcoholic extracts of olive leaf on adhesion of *S. mutans* was performed using one-way ANOVA. The results showed that increase of concentration of ethanolic, methanolic, and hydroalcoholic extracts decreases adhesion of *S. mutans*, significantly ($P < 0.001$) [Table 3].

Effects of extract type on adhesion were analyzed using one-way ANOVA. The results showed that there was a significant difference in inhibition of bacterial adhesion by ethanolic, methanolic, and hydroalcoholic extracts ($P = 0.003$). The highest and the lowest inhibition of bacterial adhesion are related to ethanolic and methanolic extracts, respectively [Table 3].

Analysis of simultaneous effect of the concentration and type of extract on adhesion of *S. mutans* was performed using two-way ANOVA. The results showed that the difference of inhibition of bacterial adhesion mean by different concentrations of ethanolic, methanolic, and hydroalcoholic extracts was statistically significant ($P < 0.001$).

Effects of extract type on adhesion of *S. mutans* were analyzed using Bonferroni test. The results showed that inhibition of bacterial adhesion by methanolic extracts was significantly more than hydroalcoholic and ethanolic extracts ($P < 0.05$), at 50%, 25%, 12.5%, and 6.25% concentrations. Furthermore,

inhibition of bacterial adhesion by methanolic and hydroalcoholic extract was equal ($P = 0.053$) and less than ethanolic extract ($P < 0.05$), at 3.125% concentration [Figure 2].

Effects of different concentrations of ethanolic, methanolic, and hydroalcoholic extracts of olive leaf on acid production by *S. mutans* were analyzed using one-way ANOVA. The results showed that increase of concentration of ethanolic, methanolic, and hydroalcoholic extracts decreases acid production by *S. mutans*, significantly ($P < 0.001$) [Table 4].

Effects of extract type on inhibition of acid production were analyzed using one-way ANOVA. The results showed that there was a significant difference in inhibition of acid production by ethanolic, methanolic, and hydroalcoholic extracts ($P = 0.003$). The highest and the lowest inhibition of acid production are related to ethanolic and methanolic extracts, respectively [Table 3].

Analysis of the simultaneous effect of the concentration and type of extract on an acid production was performed using two-way ANOVA. The results

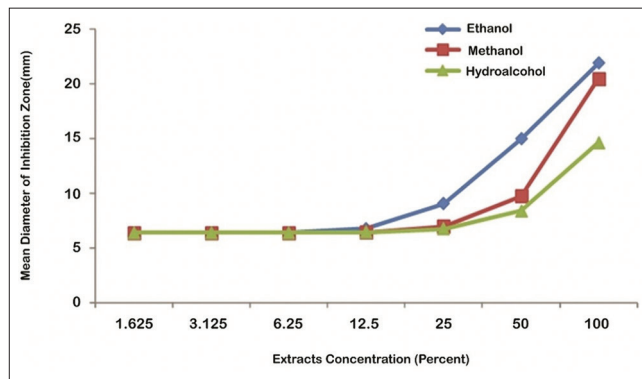


Figure 1: The mean of *Streptococcus mutans* inhibition zone in the effects of different concentrations of ethanolic, methanolic, and hydroalcoholic extracts of olive leaf.

Table 3: The mean and standard deviation of *Streptococcus mutans* adhesion in the effects of different concentrations of ethanolic, methanolic, and hydroalcoholic extracts of olive leaf (colony number)

Concentrations (%)	Ethanolic extracts		Methanolic extracts		Hydroalcoholic extracts	
	Mean	SD	Mean	SD	Mean	SD
Negative control	1.73	0.10	1.73	0.10	1.73	0.10
3.125	1.17	0.06	1.51	0.12	1.35	0.06
6.25	0.79	0.07	1.44	0.16	1.10	0.06
12.5	0.75	0.01	1.36	0.01	0.91	0.03
25	0.61	0.07	1.14	0.10	0.87	0.04
50	0.52	0.04	0.94	0.10	0.79	0.02
Adhesion mean	0.93	0.43	1.36	0.29	1.12	0.34
Statistically significant	<0.001		<0.001		<0.001	

SD: Standard deviation

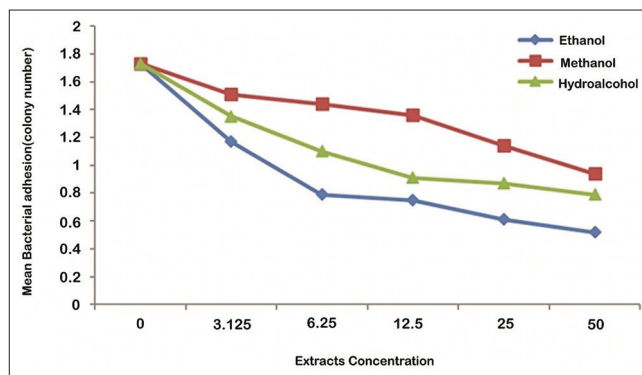


Figure 2: The mean of *Streptococcus mutans* adhesion in the effects of different concentrations of ethanolic, methanolic, and hydroalcoholic extracts of olive leaf.

showed that the pH mean of medium was statistically significant at different concentrations ($P < 0.001$). The results showed that the difference of pH mean in different concentrations of ethanolic, methanolic, and hydroalcoholic extracts was statistically significant ($P < 0.001$).

Effects of extract type on inhibition of acid production were analyzed using the Bonferroni test. The results showed that inhibition of acid production by ethanolic and hydroalcoholic extracts was equal ($P > 0.05$) and more than methanolic extract ($P < 0.05$), at 50%, 25%, 12.5%, and 3.125% concentration. Furthermore, inhibition of acid production by ethanolic extracts was significantly more than hydroalcoholic and methanolic extracts ($P < 0.05$), at 6.25% concentrations [Figure 3].

DISCUSSION

The results of the present study showed that ethanolic,

Table 4: The mean and standard deviation of *Streptococcus mutans* acid production in the effects of different concentrations of ethanolic, , and hydroalcoholic extracts of olive leaf (pH)

Concentrations (%)	Ethanolic extracts		Methanolic extracts		Hydroalcoholic extracts	
	Mean	SD	Mean	SD	Mean	SD
Negative control	5.43	0.03	5.43	0.03	5.54	0.03
3.125	5.81	0.01	5.38	0.23	5.77	0.06
6.25	6.23	0.05	5.40	0.01	5.93	0.14
12.5	6.66	0.23	5.87	0.12	6.35	0.21
25	7.08	0.03	6.27	0.25	7.00	0.00
50	7.17	0.03	6.90	0.17	7.07	0.06
pH mean	6.40	0.66	5.87	0.58	6.25	0.63
Statistically significant	<0.001		<0.001		<0.001	

SD: Standard deviation

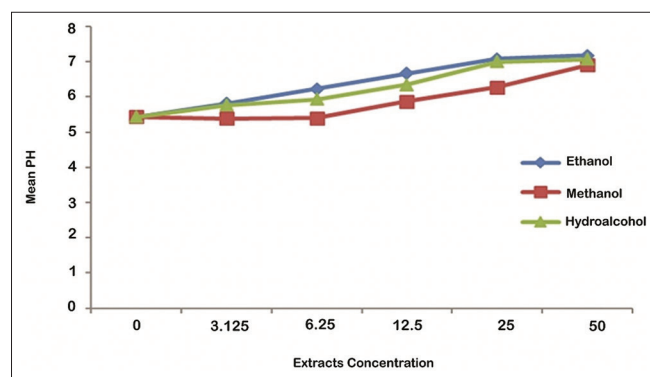


Figure 3: The mean of *Streptococcus mutans* acid production in the effects of different concentrations of ethanolic, methanolic, and hydroalcoholic extracts of olive leaf.

methanolic, and hydroalcoholic extracts of olive leaf inhibit growth, adhesion, and acid production by *S. mutans*. So far, several studies have investigated the antimicrobial effect of olive fruit on various bacterial strains, such as *S. mutans*;^[10,25,26] however, very limited studies have investigated the antibacterial activity of olive leaf on *S. mutans*.^[10] Olive leaf extract was effective against Gram-negative pathogens such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum*. Sudjana *et al.* reported antibacterial action of olive leaf against *Campylobacter jejuni*, *Helicobacter pylori*, and *Staphylococcus aureus*.^[13]

Olive fruit and leaf have high antibacterial activity due to the presence of phenolic compounds in its extracts, including oleuropein and hydroxytyrosol.^[27,28] In the study of Karygianni *et al.*, oleuropein content of olive leaf and fruit extracts was reported 60% and 15%, respectively.^[10] Regarding different levels of phenolic compounds in olive fruit and leaf, their antibacterial properties are also different, probably.

Pereira *et al.* reported an appropriate antimicrobial effects for olive leaf on Gram-positive bacteria, including *Bacillus cereus*.^[29]

The cause of cell wall destruction of Gram-positive bacteria by olive extracts is probably due to the presence of antiquorum sensing (QS) compounds.^[30] Other plants compounds with antibacterial effect against *S. mutans* are *Thymus deanesis*, *Thymus vulgaris*, thymol, and carvacrol.^[31]

Contrary to the present study, the results of Karygianni *et al.* showed that the olive leaf extract does not have a significant bactericidal effect on *S. mutans* and only inhibits its growth.^[10] This variation may be due to differences amount of phenolic compounds in the studied olive leaf. Studies have shown that climate, weather conditions, and harvest time of a plant could effect on type and amount of extracts compounds.^[32]

The results of this study showed that an increase in antibacterial properties of the studied extracts was associated by an increase in its concentration. However, this association was observed at higher than 6.25% concentrations. This is probably due to an increase in the amount of the extract compounds by increasing its concentration, which leads to a stronger antibacterial effect. Korukluoglu *et al.* showed that type of used solvent could effect on the amount of phenolic compounds and also antibacterial properties of extracts.^[32] In the present study, it was observed

that the type of used solvent (ethanol, methanol, and hydroalcohol) effects on inhibition of growth, acid production, and adhesion of *S. mutans*, so that similar concentrations of different extracts show a different effects on growth, acid production, and adhesion of *S. mutans*. *S. mutans* gradually cause damage to tooth enamel and dental caries, because of adhesion to dental surfaces and the production of acidic lactic after metabolizing foodstuffs.^[33-35]

Casagrande *et al.* reported that the solvent type could effect on antimicrobial properties of plants extract, and ethanol is the best solvent to extract phenolic compounds.^[36] The present study also showed that the ethanolic extract causes the most inhibition of adhesion and acid production of *S. mutans*. This is probably due to the difference in the amount of polyphenols extraction by various solvents.

Šikić Pogačar *et al.* showed that the extract of olive leaf (with 3.125–200 µg/mL-1 concentration) decreased 10% to 23% of *C. jejuni* adhesion.^[37] The polyphenolic compounds can interact with proteins, enzymes, and membranes of microbes, and thus changes in cell permeability, and loss of protons, ions and macromolecules, which can lead to inhibition of bacterial adhesion.^[38]

Contrary to the present study, Rafiei *et al.* showed that the methanolic extracts of olive leaf contain the highest amount of phenolic compounds and also has the highest bactericidal effects on *S. aureus* and *Escherichia coli* strains.^[39]

The limitations of the present study are failure to determine of minimum bactericidal time, as well as efficacy of olive leaf extracts in inhibition of bacterial adhesion to the tooth surface (hydroxyapatite), denture prosthesis, and oral applicators. Furthermore, the present study performed *in vitro* and cannot show accurate inhibition of acid production by olive leaf extracts in a saliva-containing medium. Therefore, it is suggested that the bacterial growth curve based on time, as well as inhibition of adhesion to mentioned dental structures and inhibition of acid production in the presence of saliva, will be investigated by other studies.

CONCLUSION

In general, it can be concluded that methanolic, ethanolic, and hydroalcoholic extracts of olive leaf extracts have appropriate antibacterial activities due to their high phenolic content and can also reduce

bacterial adhesion in dental plaque formation, as well as increases pH of the oral environment. Therefore, olive leaf extracts can be used as a natural preservative in the food industry, as well as in the production of commercial products such as chewing gum, chocolate, and toothpaste to prevent dental caries.

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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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