The Effect of *Pimpinella Anisum* and *Origanum Vulgare* Extracts Against *S. Sanguinis, S. Mutans* and *S. Salivarius*

Fatemeh Lavaee 1; Armin Moqadas 2; Farzan Modarresi 3; Massoumeh Nowrouzi 4;

1 Oral and Dental Disease Research Center, Dept. of Oral and Maxillofacial Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.
2 Undergraduate Students, Student Research Committee, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran.
3 Dept. of Microbiology, School of Medicine, Jahrom University of Medical Sciences, Jahrom Iran.
4 Postgraduate, Dept. of Periodontics, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran.

**KEY WORDS**
Pimpinella anisum; Origanum vulgare; Streptococcus sanguinis; Streptococcus mutans; Streptococcus salivarius;

**Abstract**

**Statement of the Problem:** There are global efforts for introducing new herbal antimicrobial agent with minimal side effects. There are some reports about antimicrobial properties of pimpinella anisum and oregano vulgare.

**Purpose:** In this study the antimicrobial properties of pimpinella anisum and oregano vulgare have been assessed.

**Material and Method:** In this study, the dental plaque samples were collected from children aged 3-5 years old who were referred to a dental office with diagnosis of dental carries. After determination of the bacterial colonies of sanguinis and S.mutans and S.Salivaris, the minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) of ethanolic and methanolic extracts of Pimpinella anisum and Origanum vulgare were measured by macrodilution and microdilution method.

**Results:** The mean MIC and MBC of P.anisum extract and O.vulgare extract and their combination against S.mutans, S.sanguinis and S.salivarius were statistically different ($p<0.001$). The combination of these extracts showed the lowest MIC and MBC.

**Conclusion:** Hydroalcoholic extracts of the P.anisum and O.vulgare were effective antibacterial agent against S.mutans, S.salivarius and S.sanguinis so that the combination of these two extracts showed the highest antibacterial properties on all the bacteria evaluated.

**Corresponding Author:** Modarresi F, Dept. of Microbiology, School of Medicine, Jahrom University of Medical Sciences, Jahrom Iran. Tel: +989173087369 Email: modarresifarzan@gmail.com

**Introduction**

The mouth environment can support the dental plaque formation [1]. PH and temperature, saliva and redox reactions are the main factors related to plaque development [2-4]. Dental plaque is a bacterial biofilm which is formed on different surfaces in the mouth. Dental plaque is a main causative factor for dental carries and periodontal diseases. Biofilm generation first starts with pellicle formation. Loose attachment of some bacteria create micro colonies; ultimately biofilm maturation can cause dental pathologies [5-6]. The development of a biofilm allows aggregation of cell colonies which are increasingly resistant to antibiotics [7-8]. There are many different bacteria responsible for biofilms formation, including gram-positive and gram-negative species [4,8-9]. *Origanum vulgare* is a popular species of *Origanum from* the mint family (Lamiaceae). It is native to western and south-western Eurasia and the Mediterranean region. In Austrian folk medicine, *Oregano* was used as tea or as an ointment for gastrointestinal, respiratory tract, and nervous system diseases [10]. Over 60 different compounds have been extracted. Carvacrol and thymol are dominant compounds (80%) [11]. The antibacterial activity of carvacrol against several bacteria strains such as *Escherichia coli* and *Bacillus cereus has been reported* [12]. The natural biocidal agents such as thymol can decrease the possibility of the bacterial resistance to common antibiotics such as penicillin [13].
Pimpinella anisum (anise), an aromatic plant from Umbelliferae family, has been prescribed as carminative, galactagogue, and disinfectant, in Iranian traditional medicine [14]. The main component of the oil is anethole (80-90%) [15]. Anethole has potent antimicrobial properties, against bacteria, yeast, and fungi [16]. Anise essential oil have antiviral properties, as well as antibacterial, antioxidant and anti carcinogenic properties and antifungal activity [13].

At present, due to the indiscriminate use of antibiotics, different bacteria have developed drug resistance, so new researches are conducted in order to introduce novel antibacterial agents. Herbal agents or their ingredients are widely evaluated [7]. Considering the properties and ingredients of Pimpinella anisum and Origanum, we decided to investigate their antibacterial effects and if possible introduce a new substance with antimicrobial activity with few side effects.

Sarac and Ugur [17] showed that the essential oils of Origanum onites L., Origanum vulgare were effective against some multiple antibiotic-resistant bacteria. Kernanshah et al. [18] showed that P.anisum had growth inhibitory effect against Streptococcus mutans and Lactobacillus rhamnosus. According to this study and other similar researches, and antibacterial effect of these two herbal extracts, we decided to investigate the synergistic antibacterial effect of these extracts against dental plaque bacteria.

Materials and Method
This study is an experimental in vitro study.

Pimpinella Plant extraction
Hydroalcoholic extraction of P.anisum and O.vulgare was obtained by “maceration” method. After preparing air-dried P.anisum, 50gr of its powder was weighted by a digital balance (Sartorius, Germany) and mashed. 1500cc of the solvent (half ethanol and half water) was added to them and shaken (IKA, Germany) for 48h and 90 cycle per minute until the composition reached homogenously. The solution was filtered (Sartorius, Germany) and the solvent was vaporized by using rotary evaporator (KNF, USA). The sterile extract was kept in the refrigerator for microbial evaluations.

Oregano Plants extract
The plant was mixed with the solvent solution (20% methanol: 80% distilled water) by ratio 1:3(1mg powder + 3ml solvent), the mixture was uniformed by electric blender for 30 minutes in room temperature. The solution was filtered for getting transudate solution. It was dried using an incubator (Binder, Germany) at 50°C for 24 hours and the product was kept in a dry place until used. The ethanolic and methanolic extracts of Pimpinella anisum and Origanum vulgare were prepared.

Dental plaque sampling
The samples were collected from 3-5 years old children with dental caries. Dental caries was determined by a cavitation on the tooth surface and evaluation of biting radiographs [20]. The white spot lesions and developmental grooves were excluded. A written consent form was obtained from the parents of participants. The ethics committee of Shiraz University of Medical Sciences has been approved this study (IR.SUMS.REC. 1396.1610); also this evaluation has been conducted according to the Declaration of Helsinki (1975).

The participants should not have any systemic disease, especially one which can affect the rate of dental caries. Samples were taken with a sterile toothpick from dental caries. The toothpick samples were kept into 1.0-mL reduced transport fluid vials for other processes. The plaque samples dilutions were plated onto MM10-sucrose agar [21]. After 3 days of anaerobic incubation (85% N2, 10% CO2, and 5% H2), the colonies were presumed to be S.sanguinis and S.mutans and S. salivarius was selected according to their colony morphology from MM10-sucrose agar [22-23].

Polymerase Chain Reaction (PCR)
The primer pairs of S. mutans and S. sanguinis and S.salivarius were used to detect them by PCR. These primers were 5-GqaGACCACAAACATTGGAAGCTCAGT and 5-GGAATGCGCGCTAAGTCAACA-GGAT for S.mutans that amplified 433bp, GGATAGTGGCTCAGG-GCACACAGTT and GAACAGTTGCTGGACTCTGT-GTC for S.sanguinis and amplicon was 313bp size and MKK-GTGTCACATCCTTACTGCTTCG and MKK-CGGTGATGCTGCTGAAGGGCCACATT for S.salivarius that amplified 544bp. Blast analysis was used for determining the specificity of the sequences of candidate primers in the database (http://www.ncbi.nlm.nih.gov/GenBank). The genomic isolation kit was used for isolating the genomic DNA (Thermo scientific, Lithuania), based on manufacturer instruction. DNA ladder was obtained from Cinnagen Co. (Tehran, Iran). PCR was p-
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Figure 1a: PCR amplification of patient isolated Streptococcus mutans species in this study. The electrophoresis agarose gel was stained with 0.5 µg/ml ethidium bromide and the figure was prepared by UV gel documentation system. Positive control Streptococcus mutans (ATCC 25175) (433bp) is also seen in this figure. b: PCR amplification of patient isolated Streptococcus sanguinis species in this study. Positive control (313bp) Streptococcus sanguinis (ATCC 10556) is also seen in this figure. c: PCR amplification of patient isolated Streptococcus salivarius species in this study. Positive control Streptococcus salivarius (ATCC9759) (544bp) is also seen in this figure.

Performance of PCR amplification

PCR amplification was performed with 1µL DNA template, primer F and R (20µM) 0.7µL, DNA 0.8µL, master mix 8µL, DDW 5.8µL and 3U of LA Taq polymerase. DNA amplification was conducted in temperature gradient thermal cycler (Biometra-T gradient, Germany) (Figure 1).

Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

Microdilution

A culture of bacteria was grown in Brain Heart Infusion (BHI) at 37°C. 100µl of this bacterial culture was placed into the necessary number of 96-well culture plate. Using stock solution of 640µg/ml for both plants’ extract, we prepared a series of 1:2 dilutions of them, 100µl portions was added to each well and incubated overnight at 37°C. Minimum Inhibiting Concentration (MIC) was determined to be the minimum concentration at which no viable cells were observed as evaluated by both microscopic examination and plating on BHI plates. Also minimal bactericidal concentration (MBC) has been assessed.

Macro dilution (Tube dilution)

The MIC, the lowest concentration which causes bacterial growth inhibition, (0.5 McFarland standard in this study) and MBC were determined by macrodilution method. In this study macrodilution method was used to determine MIC and MBC. Serial dilution (dilution by one-half) was used for preparing different concentrations of extracts in BHI broth medium.

In order to obtain bacterial count of 10^6 CFU/mL, the suspension was diluted. 1mL of diluted microbial suspension (1 microbial suspension: 2 culture medium) was added to the tubes containing serially diluted extract. The negative control tube contained culture medium and extract. The positive control tube contained only culture medium and microbial suspension. Also chlorhexidine 0.12% was added in another tube containing microbial suspension as a gold standard of antibacterial activity. After 24 hours of incubation at 37°C, growth and proliferation of microorganisms were evaluated and the MIC value of the evaluated extracts and chlorhexidine for each bacterial strain was determined and repeated in triplicate for each microorganism. MIC and MBC of different concentration of the extracts and chlorhexidine were evaluated. MIC and MBC of each extract of Pimpinella anisum and Oregano were determined alone and in combination with each other against S. sanguinis and S. mutans and S. Salivarius.

Statistical analysis

The data has been assessed in SPSS version 18. The p<0.05 has been considered as significance point. Repeated measurement, ANOVA and sidac post hoc test have been used in this study.

Results

Dental plaque of 60 participants (37 women and 23 men) with mean age of 4.65±1.12 years old, were collected and finally cultured and the intended bacterial species Streptococcus mutans, Streptococcus salivarius and Streptococcus sanguinis were determined as shown in Table 1.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Number of bacteria</th>
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<tbody>
<tr>
<td>S.mutans</td>
<td>15</td>
</tr>
<tr>
<td>S.salivarius</td>
<td>3</td>
</tr>
<tr>
<td>S.sanguinis</td>
<td>2</td>
</tr>
<tr>
<td>All</td>
<td>60</td>
</tr>
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Table 1: The patients’ isolation bacteria distribution
The inhibitory effect of hydroalcoholic extract of *P. anisum* and *O. vulgare* on those extracted bacteria was evaluated by macrodilution and microdilution method and these two methods showed similar results. Based on repeated measure ANOVA and sidac post hoc test, there were significant differences between the MIC and MBC of these two extracts and their combination ($p<0.001$).

The Mean MIC and MBC of *P. anisum*, *O. vulgare* extract, and their combination against *S. mutans* were statistically different ($p<0.001$). The combination of these extracts showed the most potent antibacterial properties (Table 2, Figures 2 and 3). Also, the mean MIC and MBC of *P. anisum*, *O. vulgare* extract and their combination against *S. sanguinis* were significantly different ($p<0.001$). The combination of these extracts showed the most potent antibacterial properties (Table 2, Figures 2 and 3).

In accordance to the antibacterial properties of these extracts against other incubated bacteria, the mean MIC and MBC of *P. anisum* extract, *O. vulgare* extract and their combination against *S. salivarius* were considerably different, too ($p<0.001$). The combination of these extracts showed the most potent antibacterial properties (Table 2, Figures 2 and 3).

According to the findings, the most potent extract was combination of *O. vulgare* and *P. anisum*. Also, *P. anisum* was more potent than *O. vulgare* extract. These extracts showed the best antibacterial effect on *S. mutans*. For better detection of antibacterial effect of the extracts, we decided to evaluate the antibacterial effect of the extracts on standard species bacteria and compare it with chlorhexidine, as a gold standard for antibacterial activity (Table 3 and Figures 4 and 5). According to these findings, a similar trend for antibacterial properties of all evaluated extracts was observed. The most potent extracts were respectively combination

| Table 2: Mean values of MIC and MBC of 3 extract on 3 bacteria in 60 patients |
|-------------------------------|-----------------|-----------------|-----------------|
| Extracts                      | *S. mutans*     | *S. sanguinis*  | *S. salivarius* |
| **MIC (µg/ml)**               | **MBC (µg/ml)**| **MIC (µg/ml)** | **MBC (µg/ml)**|
| *P. anisum*                   | 22.000          | 48.750          | 35.571          |
| (SD = 13.8119)               | (SD = 35.0229)  | (SD = 16.3785)  | (SD = 34.565)   |
| *O. vulgare*                 | 122.000         | 252.00          | 127.14          |
| (SD = 98.793)               | (SD = 197.253)  | (SD = 80.040)   | (SD = 154.618)  |
| Combination                   | 6.4063          | 14.50           | 12.32           |
| (SD = 5.7691)               | (SD = 11.024)   | (SD = 5.69)     | (SD = 18.701)   |

* SD: Standard Deviation

<table>
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<th>Figure 2: Comparison of the MIC of the 3 extracts on 3 bacteria</th>
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<th>Figure 3: Comparison of the MBC of 3 extracts on 3 bacteria</th>
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| Table 3: The MIC and MBC of 3 extracts and chlorhexidine on 3 standard bacteria |
|-------------------------------|-----------------|-----------------|-----------------|
| Extracts                      | *S. mutans*     | *S. sanguinis*  | *S. salivarius* |
| **Bacteria**                  | **MIC (µg/ml)**| **MBC (µg/ml)**| **MIC (µg/ml)** |
| *P. anisum*                   | 10              | 20              | 20              |
| *O. vulgare*                  | 80              | 160             | 160             |
| Combination                   | 2.5             | 10              | 40              |
| Chlorhexidine                 | 50              | 50              | 50              |

* MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration
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Figure 4: Comparison of the MIC of 3 extracts and chlorhexidine on 3 standard bacteria

Figure 5: Comparison of the MBC of 3 extracts and chlorhexidine on 3 standard bacteria

of two extracts, P.anisum and O.vulgare extract. All extracts showed the most antibacterial activity against S.mutans and exhibited lower MIC and MBC antibacterial properties than chlorhexidine except for O.vulgare.

There are some studies on antibacterial properties of P.anisum and O.vulgare against different bacteria. Some studies have reported proper inhibitory effect of P.anisum on different bacteria with different methods [12, 19, 24-27].

Nazia Chaudhry et al. [24] reported antibacterial effect of aqueous extract of aniseed against S.salivarius, S.sanguinis and S.mutans by disc diffusion method. The average diameter of the zone of inhibition of aniseed for S.salivarius was 14mm which was the highest between these three species. In spite of more inhibitory effect of P.anisum extract on S.mutans in the present study, the report of more potent antibacterial effect of this extract against S.salivarius rather than, S.sanguinis and S.mutans is considerable [24].

Kermanshah et al. [25], in a research investigated the cariogenic bacterial inhibitory effect of some native Iranian plant by both broth macrodilution and agar diffusion method. Their results about P.anisum were in the same line with those of this study. Anisum had inhibitory properties against S.mutans in all concentrations of 25, 50, 100, 200, 400 µg/ml. Kermanshah et al. reports of MIC and MBC for anisum against S.mutans were 12.5µg/ml and 200µg/ml. These values are 22µg/ml (MIC) and 48.75µg/ml (MBC) for S.mutans in our study. Different values can be related to diversities of plant extraction concentration, various methodology and difference in cultivated geographic area of plants. Using different solvents exhibit distinct antibacterial potency, while in a study the alcoholic extract exhibited more significant inhibitory properties [27]; another evaluation reported the reverse [26].

Similar to the results of our study and the same as P.anisum, there are some reports on antibacterial properties of O.vulgare [12, 17-19, 28-33].

Nurdan Sarac et al. [17] extracted the essential oils of O.vulgare by hydrodistillation which showed effective antibacterial properties against gram positive and negative bacteria. Inhibition zone of O.vulgare against S.mutans was 19mm which was more than some antibiotics such as penicillin(15mm), ampicillin(12mm) and

these two extracts showed the highest antibacterial properties on all the evaluated bacteria. All extracts showed the most antibacterial activity against S.mutans and exhibited lower MIC and MBC antibacterial properties than chlorhexidine except for O.vulgare.

Discussion

Hydroalcholic extract of the P.anisum and O.vulgare was an effective antibacterial agent against S.mutans, S.salivarius and S.sanguinis so that the combination of all the 3 species bacteria isolated from 60 patients were compared with standard bacteria. In all comparisons, the clinically isolated bacteria were more resistant to the extracts (p< 0.05) except for standard S.sanguinis which was more resistant than patients’ isolated species against O.vulgare extract (p Value for MIC<0.05).

The MIC and MBC value of O.vulgare extract on S.salivarius (p= 0.418, p= 0.136) and the MBC values of the combinational extract on S.sanguinis (p=0.321) were not significantly different from the standard bacteria species.

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cefoprazone (14 mm). Some studies listed the biochemical composition of *O. vulgare* and determined carvacrol and thymol as the most effective ingredients of this plant against bacteria [12, 18, 28-31, 33]. Also, Shams Tabrez Khan et al. [28] regarded *O. vulgare* as a green alternative to control dental caries. They showed a significant decrease in viability, metabolic activity and biofilm formation of *S. mutans* by thymol and carvacrol. Even a study introduced these two extracts as a more potent antimicrobial agent than beneficial probiotic bacteria lactobacillus [12].

A research exhibited this potency in comparison to traditionally used essential oil in the dentistry clove oil [31]. The present evaluation revealed proper antibacterial (bacteriostatic/bactericidal) effects for *O. vulgare* though its MIC and MBC like other studies were relatively high and more than *P. anisum* values.

Magi et al. [25] reported a range of MIC between 256-512 μg/ml for essential oil thyme of Origano and 64-250 μg/ml against Group A Streptococci for carvacrol. Although these values were high for MIC, they can be indicative of antibacterial effects in accordance to the finding of this study (MIC for *S. mutans, S. sanguinis* and *S. salivarius* was 122, 127, 145 μg/ml).

In this regard, Birol Ozkalp Zuloet et al. [32] argued about the strong antibacterial activity of *O. vulgare* oil and its higher activity against gram positive bacteria.

The findings of our study have confirmed the general aspects of previous studies. Although there were few studies in which dental caries responsible bacteria had been evaluated, similar trend of the results was considerable.

In addition to antibacterial activity of *P. anisum* and *O. vulgare*, the noticable synergistic activity of these two extracts in combination to each other is very practical and usable.

Different plants with distinct composition show various properties with diverse degree of antibacterial activities. Diversities of geographical areas of plants and their climate play a very important role in determining the dominant composition of plants. For example, Saudi Origanum oil is carvacrol dominant, but Jordanian Origanum is thymol dominant [25].

In the literature, antibacterial activity of different *O. vulgare* extracts has been assumed to be related to essential oils, flavonoids and triterpenoids. Essential oils contain a high percentage of phenolic ingredients including carvacrol, eugenol, E2-methoxy-4-(2-propenyl) phenol and thymol which are the most potent antibacterial agents [34-35].

In addition to some other properties of thymol and carvacrol, such as immunity enhancement against virus and tumor and anticancer activities, some mechanisms for their antibacterial activity have been proposed. These essential oils permiabilize and depolarize the cytoplasmic membrane which causes a decrease in pH and consequently disturbs the proton motive force, reduces the intracellular ATP level, and finally causes cell death [28, 30].

Carvacrol and thymol mediated cell lysis can also be related to over-expression of autolysin gene which can affect the cell wall [31]. In addition, down-regulation of glycosyl transformase B gene mediated by thymol and carvacrol justified their antibiofilm properties [36].

Method selection for evaluating the antibacterial properties is important. Confounding factors which can affect disk or agar diffusion method, are more prominent in broth dilution method. Chemical composition of essential oils, their agar diffusion rate and chemical volatility can affect the size of inhibition zone. Different solvents with various polarities can extract diverse compositions. Compatibility of the solvent’s polarity and plant composition in order to extract all ingredients as much as possible should be considered. Since thymol and carvacrol are not water soluble [37], hydroalcholic solvent was selected for the present study.

Except thymol and carvacrol, other ingredients of *O. vulgare*, such as α-pinen, terpinene, β-pinen, can destroy the cytoplasmic membrane structure and blocking electron transport. Other compositions like limolool, terpinen-4-ol and terpineol denature proteins and solve or dehydrate the cells [18].

Some important compositions of aniseeds essential oil are trans-anethole (84-94%), estragol, γ-hymachalen, p-anisaldehyde and methyl chavicol [34], Trans-anethole, methyl chavicol, limolool, anisaldehyde, limomene, α-pinene, methyl eugenol, and bomeol [19].

According to what LaGow and ESCOP (European Scientific Cooperative on Phytotherapy) said, anethol, β-caryophyllene and flavonoids are effective compositions against dental caries responsible bacteria, especially *S. mutans*. Kermanshah indicated that the hy-
hydrophylic agents of P. anisum are responsible for antibacterial effect [39].

Selecting proper solvent, evaluating the synergistic activity of using two effective extracts and using macrodilution and microdilution methods for evaluation to use patient isolated bacteria instead of standard species are the strong points of our research. Studying the patient -isolated bacteria can emerge more practical horizons for introducing antimicrobial agents for resistant bacteria.

On the other hand, introducing a new combinational powerful green and natural antibacterial agent for decreasing dental caries bacteria can be considered for commercial usage, bypassing antibiotic resistance and some side effects of public mouthwashes. These extracts or their effective essential oils can be used in mouthwashes and toothpastes in order to control the bacterial growth and biofilm formation.

Selecting more accurate methods for evaluating antibacterial properties, studying other solvents, performing GC-MS analysis on Iranian P. anisum and O. vulgare, and determining the most effective ingredients of these plants can be considered in future studies.

Conclusion
The hydroalcoholic extract of P. anisum and O. vulgare on patient isolated dental caries bacteria S. mutans, S. sanguinis, and S. salivarius showed proper antimicrobial properties. The combination of these extracts exhibited very significant potency in antibacterial activity.

Conflict of Interest
The authors declare that they have no conflict of interest.

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