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An in Vitro Study on the Antibacterial Effect of *Ferula Assa-Foetida L.* and *Quercus Infectoria Olivier* Extracts on *Streptococcus Mutans* and *Streptococcus Sanguis*

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Background: From the ancient times, medicinal herbs have been regarded as efficient resources for the treatment of diseases. Among the diseases that can be treated by medicinal herbs, infectious diseases like oral ones are of notable importance.
Objectives: The aim of this study was to evaluate the antibacterial effects of *Ferula assa-foetida L (F. assa-foetida L.)* and *Quercus infectoria Olivier (Q. infectoria Olivier)* aqueous and ethanolic extracts on *Streptococcus mutans (S. mutans)* and *Streptococcus sanguis (S. sanguis)*.
Materials and Methods: The studied plants were *F. assa-foetida L.* and *Q infectoria Olivier*. Their extracts with different concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 mg/mL were prepared in culture medium, with well diffusion method, and, for control, their antibacterial effects were compared with chlorhexidine. For each extract, the antibacterial ability was determined based on the created inhibition zone diameter in the microbial culture medium.
Results: The aqueous and ethanolic extract of *F. assa-foetida L.* lacked the inhibitory effect against the growth of *S. mutans* and *S. sanguis* bacteria. There was a significant difference among the inhibitory zones created by dissimilar concentrations of *Q. infectoria* ($P = 0.025$). Also, the aqueous and ethanolic extracts' minimum inhibitory concentration (MIC) for *S. mutans* bacterium was calculated to be 12.5 mg/mL, while its value for *S. sanguis* bacterium was 6.25 mg/mL.
Conclusions: The results of our study, regarding the observed effects based on differences in concentrations, suggest that further and more comprehensive studies should be undertaken to determine the appropriate concentration for obtaining the effect of the extract of *F. assa-foetida L.* and *Q infectoria Olivier* on *S. mutans* and *S. sanguis*.

Keywords: Herbals; Ferula; *Streptococcus mutans*; *Streptococcus sanguis*

1. Background

Dental caries, or tooth decay, represents one of the most prevalent chronic human diseases worldwide. It is a multifactorial disease that starts with the bacterial change inside the dental plaque (1). Dental caries are affected by consumption of sugar, salivary flow, contact with fluoride, and preventive behaviors (1, 2). In its preliminary stage, this disease is created by the decays of the dental surface that is caused by the acids that, as a result of carbohydrate metabolism, are produced by bacteria which cause dental cavity. The aggregation of bacterial plaque and normal oral bacterial flora on the dental surfaces are the primary causes of dental diseases and cavity. Reproduction of effectual bacteria on dental caries is one of the most dangerous factors in the spread of dental diseases, and among those bacteria *Streptococcus mutans (S. mutans)* is important in dental decay (3, 4). Moreover, other types of Streptococcus also can bring about decay, even in the absence of *S. mutans* (5). Such a bacterial agent promoting dental decay is *Streptococcus sanguis* (*S. sanguis*) (6, 7). This bacterium is among the dominant types in human dental plaque and is among the first microorganisms that colonize on the plaque of the newly erupted teeth. Even in the absence of carbohydrate fermentation, this bacterium is able to survive and remain active through the hydrolysis of arginine (3, 7). Epidemiological studies showed that in Western Europe, the rate of tooth decay has decreased during recent decades (8). However, reportedly in many developed countries, dental caries prevalence is increasing among young children (9). Moreover, in many developing countries, dental decay prevalence has been increasing (10). Therefore, dental caries are still common among children and adults (10, 11) and they are reported to afflict 46% of 4-year-old

children and 80% of 15-year-old ones. Additionally, dental decay is a common health problem, because it has a widespread situation, which imposes high treatment expenses and it also affects the quality of life in older ages (11). *Ferula assa-foetida (F. assa-foetida)* belongs to the family of *Apiaceae* and it reaches heights of up to 2 meters (12). The *F. assa-foetida* is a herbaceous plant and has a strong, thick and fibrous stem, The part of the plant that is used is a resin that exudates from it. Disulfide, along with three and tetrasulfide, have been isolated from the resin of *F. assa-foetida* (13). This plant is being used as antispasmodic, aromatic, carminative, digestive, expectorant, laxative, sedative, nervine, analgesic, anthemintic, aphrodisiac and antiseptic in Iranian folk medicine (13, 14). *Quercus infectoria Olivier* (*Q. infectoria Olivier*) belongs to the *Quercus* genus, *Fagaceae* family and *Fagales* phylum (1). The *Q. infectoria* (*Fagaceae*) is a small tree or a shrub, mainly present in Greece, Asia Minor, Syria and Iran (15). The galls of *Q. infectoria* are the result of a non-sexual activity of the Andricus Sternlicht insect from the *Cynipidae* family. Due to high tannin content in the galls of *Q. infectoria*, it has many applications in folk medicine. The constituents of galls comprise gallic acid, syringic acid, ellagic acid, β-sitosterol, amentoflavone, hexamethyl ether, isocryptomerin, methyl betulate, methyl oleanate and hexagalloyl glucose (15). It is used as astringent in anti-diarrhea preparations and ulcerative colitis, and its dry extract is used as analgesic, hyperglycemic and has sedative hypnotic efficacy (16).

The use of herbs as drugs for the treatment of several diseases has been prevalent. However, by progress in sciences and technologies, researchers have paid attention to their chemical ingredients for pharmaceutical manufacturing. Nevertheless, due to the side effects of the mentioned drugs and their inefficiencies, scientists have returned to herbal ingredients in drug manufacturing (17). Antibiotic resistance still remains a dilemma. Segregation of microbial types, which are less resistant to antibiotic, and also prevention of resistant strains to antibacterial treatments have been intensified all over the world, This fact shows that there is a need for novel principles in the treatment of bacterial infections (18). Due to the inappropriate use of antibiotics in treatment of bacterial infections, most bacterial species have developed increasing resistance to different antibiotics (19). Therefore, the use of medicinal herbs, due to fewer complications and therapeutic effects has become more important (18, 20). Because oral diseases have become prevalent and it is necessary to prevent them, there has been a continuous effort to produce new antibacterial agents, which are natural and safe (21, 22). Multiple studies have been conducted on the biological activities of plants and their natural derivatives (23-25).

2. Objectives

The purpose of this study is to investigate the antibacterial effects of *F. assa-foetida L.* and *Q. infectoria* extracts on *S. mutans* and *S. sanguis*, and also to compare the results with previous reports on other bacterial species (26-30).

3. Materials and Methods

This study was conducted experimentally in the Department of Bacteriology and Virology, Shiraz University of Medical Sciences, Shiraz, Iran.

3.1. Plant Species

The *F. assa-foetida L.* and *Q. infectoria* were purchased from grocery and characterized by the Department of Pharmacognosy, Shiraz School of Pharmacy, Shiraz, Iran.

3.2. Bacterial Strains

To accomplish the objectives of this study, we used the standard strains of *S. mutans* bacterium (PTCC: 1683) and *S. sanguis* (PTCC: 1449), obtained from the Iranian Research Organization for Science and Technology, Tehran, Iran.

3.3. Soxhlet Extraction Method

The plant materials were powdered separately and extracts were prepared through the Soxhlet extraction method. To produce aqueous and ethanolic extracts, distilled water and ethanol 96% were used as solvents, respectively. The amount of 200 gr of each of *F. assa-foetida* gum resin and *Q. infectoria* fruits were dipped in the solvents and left overnight at room temperature. Soxhlet extraction was carried out for 4 hours for each plant sample, and the extracts were filtered and the solvent was removed on a rotary evaporator under reduced pressure, to afford a gummy residue, and afterwards the resulting product was placed in a vacuum desiccator to remove traces of solvent and moisture. The resulted extracts were stored at -20°C prior to analysis.

3.4. Preparation of Diluted Extracts

To prepare the solution of plant extracts, one gram of each extract was added to 10 cc of relevant solvent [water for aqueous extract and dimethyl sulfoxide (DMSO) for ethanolic extract], using a 0.22 micron syringe filter.

3.5. Antibacterial Assays

3.5.1. Well Diffusion Method

In order to test antimicrobial susceptibility with the well diffusion method, bacterial strains were cultivated in blood agar environment for 24 hours. Then, a suspension in brain heart infusion broth (BHI) environment was prepared. The turbidity of the suspension was adjusted to 0.5 McFarland standards. In this situation, the number of bacteria was around 1.5×10^8 cfu/mL. The bacterial strands resulting from the suspension were cultured in Mueller–Hinton-Agar, using the spreading method. In

every 25 mm distance in these bacterial environments, wells were dag with a diameter of 6 mm and height of 5 mm and then 150 μL of extracts were injected in concentrations of 100, 50, 25, 12.5, 6.26, 3.125 mg/mL into the wells. For control, in the center of the plate, a well was dug and 150 μL of 0.2% chlorhexidine added. In the next step, the plates were stored at 37˚C for 24 hours and incubated under microaerophil (CO_2 5%). The inhibition zone diameters created by different concentrations of extracts and also chlorhexidine were measured. In this way, the bacterial sensitivity or resistance against different extract dilutions were tested. All experiments were performed in triplicate.

3.5.2. Broth Dilution Method

In order to perform antibacterial tests with the broth dilution method, we prepared a suspension in BHI environment with the turbidity adjusted to 0.5 McFarland standards. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) levels were measured, using the microdilution method, and we added 100 μL of BHI to each well of culture plate of 96 cells. Then, 100 μL of the extract was added to the first well and the dilutions in each well reached half of the previous well by passaging, and different dilutions were prepared from the extracts. After that, five of the bacterial suspensions equal to 0.5 McFarland were added. In this test, the BHI environment was considered to be a negative control and the BHI with bacterial suspension was considered as a positive suspension. After mixing with the shaker (20 seconds with 300 cfm), the samples were left in an incubator for 18-24 hours at 37˚C and then their optical densities (OD) were measured in 450 nm wavelength using the VMax Kinetic ELISA Microplate Reader (Molecular Devices,California, USA). In case of no turbidity, the MIC was determined. Then, the samples without turbidity were passed on Mueller–Hinton-Agar culture environment and the MBC was determined. Each experiment was repeated three times.

3.6. Statistical Analysis

As data distribution was not normal, we used non-parametric tests like the Mann-Whitney and Kruskal-Wallis. Data analysis was performed using the SPSS v.17 software (SPSS Inc., Chicago, IL, USA). The significance level was considered for a $P < 0.05$.

4. Results

The aqueous and ethanolic extracts of *Q. infectoria* had inhibitory effects on *S. mutans* and *S. sanguis,* whereas *F. assa-foetida* extracts did not have such effects. The Mann-Whitney test did not show any significant difference from the inhibitory zone prospect for the two bacterial groups ($P = 0.939$).

Statistical analysis using Kruskal-Wallis showed that there were significant differences among diameters of inhibitory zones for different dilutions of *Q. infectoria* ($P = 0.025$), whereas the Mann-Whitney did not show any significant difference between the growth inhibitory zone emanating from aqueous and ethanolic extracts of *Q. infectoria* ($P = 0.472$) (Table 1).

According to the results of the microdilution method, the MIC of aqueous and ethanolic extracts of *Q. infectoria* were measured to be 12.5 mg/mL for *S. mutans* and 6.25 mg/mL for *S. sanguis* (Table 2).

Table 1. Average Growth Inhibitory Zone (mm) Concentrations (mg/mL) for Different Extracts [a]

Bacteria	Extract Concentration	Average Growth Inhibitory Zone (mm)			
		Aqueous *Q. infectoria*	**Ethanolic** *Q .infectoria*	**Aqueous** *F. assa-foetida*	**Ethanolic** *F. assa-foetida*
S. mutans					
	100	11.33	12.66	0	0
	50	9.0	10.0	0	0
	25	7.33	8.0	0	0
	12.5	0	2.66	0	0
	6.25	0	0	0	0
	3.125	0	0	0	0
	100	11.66	14.33	0	0
S.sanguis					
	50	9.0	10.33	0	0
	25	7.66	8.66	0	0
	12.5	0	5.0	0	0
	6.25	0	0	0	0
	3.125	0	0	0	0

[a] The diameter of growth inhibitory zone for chlorhexidine 0.2% is 15 mm.

Table 2. Average Minimum Inhibitory Concentration and Minimum Bacterial Concentration (mg/mL) Determined for Various Extracts Against the Bacterial Strains [a]

Bacteria	Group	*Q. infectoria* Aqueous Extract	*Q. infectoria* Ethanolic Extract	*F. assa-foetida* L. Aqueous Extract	*Assa-foetida* L. Ethanolic Extract
S. mutans					
	MIC	12.5	12.5	0	0
	MBC	25	12.5	0	0
S.sanguis					
	MIC	6.25	6.25	0	0
	MBC	12.5	12.5	0	0

[a] Abbreviations: MBC, Minimum Bacterial Concentration; MIC, Minimum Inhibitory Concentration.

5. Discussion

Due to the geographical and climate variation, there is a rich and varied source of herbal species in Iran. Several of these species have medicinal effects, especially antibacterial effects. In this study, two types of these herbs indigenous in Iran, *F. assa-foetida L.* and *Q. infectoria*, were examined for the above-mentioned effects. Herbs are considered as an important alternative treatment. In many cases, they are cheaper and have less side effects, as compared to synthetic drugs (31). Multiple of these herbs grow in desert areas of Iran (32). Araghizadeh et al. showed that all clinical isolates of *S. mutans* (100%) were sensitive to green tea extract, at concentrations of 6.25, 12.5, 25, and 50 mg/mL, producing inhibition zones ranging from 10 to 38 mm in diameter. The MIC of green tea extract for *S. mutans* was found to be 3.28 ± 0.7 mg/mL (33). Also, in another study, Fani et al. have reported that all of the *S. mutans* strains were sensitive to 62.5 µg/mL concentration of *Myrtus communis* oil. The MIC of *Myrtus Communis Oil* (MCO) for *S. mutans* was 31.25 ± 0 (34).

The extract of *F. assa-foetida L.* has many beneficial effects, including tranquilizing, carminative, gastrointestinal, antispasmodic, laxative, pain killing, diuretic, and disinfecting activities (35). Besides these pharmacological properties, *Q. infectoria* affects *S. mutans*, as declared in other reports (25). In this study, statistical tests of the effects of the extracts of *F. assa-foetida L.* and *Q. infectoria* on the growth of *S. mutans* and *S. sanguis* did not show any significant difference. However, Haghighati et al. compared the in vitro antibacterial effects of 10 herbal extracts with chlorhexidine on three harmful microorganisms and showed that Q. infectoria has antifungal effects (26). Moreover, Lens et al. also reported that *Q. infectoria* affects *S. mutans* (36). Furthermore, Kavoosi et al., in their study on the antioxidant and antibacterial effects of *F. assa-foetida L.*, found that this herb has inhibitory effects on gram positive bacteria (29). Regarding solvents used for extraction, ethanol was more efficient, as it helps to extract more active ingredients, while the butanolic extract showed no significant results in this study.

In the present study, determination of zone diameter based on concentrations of 100, 50, 25, 12.5, 6.25, 3.125 mg/mL indicated a significant difference ($P < 0.05$). In their study on the effects of *F. assa-foetida L.* oil on positive and negative gram bacteria, Siddiqui et al. showed that the antibacterial effects of *F. assa-foetida L.* against positive gram bacteria differ, based on the concentration, so that the effects of 50 µg of this herb's oil on gram negative bacteria showed no significant difference from the standard antibiotics, whereas when the concentration was doubled, the effects of *F. assa-foetida L.* were better than for standard antibiotics (30), which is consistent with the findings of Haghighati et al. (26).

Finally, in order to establish the effects of these two herbs on *S. mutans* and *S. sanguis*, based on concentration difference, it is suggested that more comprehensive studies are required to determine the active concentrations of each extract. Moreover, extensive collaboration between research centers will facilitate this process.

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