The Effect of Hydro Alcoholic Extract of Seven Plants on Cariogenic Bacteria - An in Vitro Evaluation

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Abstract

Aim: To compare the antibacterial effect of hydro alcoholic extract of Salvia officinalis, Pimpinella anisum, Satureja hortensis, Rhus coriaria, Carum copticum, Mentha longifolia, Achillea millefolium against Streptococcus mutans, Lactobacillus rhamnosus and Actinomyces viscosus through two in vitro methods.

Methods: In this experimental study, hydro-alcoholic extracts have been prepared from the shoot of Salvia officinalis, Satureja hortensis, Mentha longifolia and Achillea millefolium, the seed of Pimpinella anisum and Carum copticum and the fruit of Rhus coriaria with maceration method. Their antibacterial activity against Streptococcus mutans, Lactobacillus rhamnosus and Actinomyces viscosus have been evaluated with broth macrodilution and agar diffusion methods.

Results: In Broth macrodilution method MIC (Minimum Inhibitory Concentration) of Pimpinella anisum, Salvia officinalis, Mentha longifolia, Achillea millefolium, Satureja hortensis, Carum coticum and Rhus coriaria for Streptococcus mutans were respectively 12.5, 6.25, 12.5, 50, 12.5, 12.5 and 12.5 µg/ml. For Lactobacillus rhamnosus MIC of 12.5, 1.56, 3.12, 12.5, 6.25, 6.25 and 6.25 µg/ml except for Actinomyces viscosus. The lowest MIC of 12.5, 100, 50, 100, 25 and 25 µg/ml was observed for the three bacteria in the broth macrodilution method. In Agar diffusion method Pimpinella anisum, Salvia officinalis and Rhus coriaria against Streptococcus mutans, Pimpinella anisum, Carum coticum and Rhus coriaria against Lactobacillus rhamnosus and Mentha longifolia, Rhus coriaria and Carum coticum against Actinomyces viscosus had antibacterial effects.

Conclusion: All seven extracts had growth inhibitory effects on all three bacteria. Salvia officinalis had the greatest inhibitory effect on growth of all three bacteria. All of the extracts except Carum coticum had bactericidal effect in the range of concentration. By agar diffusion method Rhus coriaria had antibacterial effect against all three cariogenic bacteria.

Key words: Dental caries, Antibacterial agents, Cariogenic bacteria

Introduction

Dental caries is a multi-factorial disease with a microbial nature. Although dental caries is probably one of the most common chronic diseases in the world [1], there has never been any preventive programs to eradicate this disease like programs to fight against polio and small pox. The destructive effects of this disease are the loss of teeth, pain and esthetic defects. Using antibiotics and steroids against cariogenic bacteria to treat or prevent dental caries may change the oxidation reduction potential of saliva, weaken the activity of lysozyme, facilitate the allergic reactions and reduce the body resistance to pathogenic factors [2]. On the other hand, attraction to traditional medicine and herbal drugs in different fields of medicine has progressed because plants have been used as drugs since a few centuries ago [3]. The use of traditional medicine is one way to make new drugs. Nowadays there are 119 drugs with plant origin that have been brought out of only 90 species from 250000 known species [4]. But it is almost impossible, very expensive and time consuming to search all effective ingredients of all plants against every disease [4]. Therefore one of the best and the most acceptable strategies in the world to discover, use and research about drug plants is to rely on local (native) knowledge.

Taking care of oral and dental health was important in Iran's traditional medicine and there was a chapter about oral and dental disorders in books called "Treatments" [5].

Dental caries highly increases in the presence of acidogenic bacteria like Streptococcus mutans [1,6]. S. mutans is the first and the most important microorganism in dental plaque and its cariogenicity was proven [1,7,8]. This microorganism plays the main role in initiation of caries lesion formation while Lactobacillus contributes to caries progression [7,8] and also causes root caries lesions. In addition to these two microorganisms, Actinomyces viscosus plays an important role too [7,8]. Therefore decontamination of bacterial base of caries is one of the ways to remove this widespread infection. Based on this information we have selected seven plants [Salvia officinalis, Pimpinella anisum, Satureja hortensis, Rhus coriaria, Carum copticum, Menthe longifolia, Achillea millefolium], which were used to treat oral and dental defects [5,9-13] in Iran's traditional medicine; to research about their antibacterial effects against cariogenic bacteria. Besides, the modern medical texts have also noticed the medical usages of these plants [14,15]. Although there have been some researches about antioxidant and antimicrobial effects of A. millefolium [16], P. anisum [17,18], M. longifolia [19], S. hortensis [20,21], C. coticum [22], R. coriaria [22] and also

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different medical effects of *S. officinalis* [23-29] in recent years, there hasn’t been any studies about the antibacterial effect of these plants against cariogenic bacteria except for one research which evaluated the antimicrobial effect of *S. officinalis* only against *S. mutans* [26].

In the present study *S. officinalis* which is more well-known was selected as an herbal positive control. According to the fact that these plants are native in Iran and preparation of their extraction is possible, the goal of this research is to identify the antibacterial effect of seven plants (*S. officinalis, P. anisum, S. hortensis, R. coriaria, C. copticum, M. longifolia, A. millefolium*) against *Streptococcus mutans, Lactobacillus rhamnosus* and *Actinomyces viscosus* through two experimental methods. The null hypotheses were: 1- All extracts would have antibacterial effect against three cariogenic bacteria. 2- There would be no differences between seven extracts.

**Methods**

**Plant extraction**

Extraction was done by "maceration" method. First of all 50 gr of the shoot of *S. officinalis, S. hortensis, M. longifolia* and *A. millefolium*, the seed of *P. anisum* and *C. copticum* and the fruit of *R. coriaria* in the dried form were weighted with a digital balance (LibROR AEU – 210 Japan). After that, they were mashed and then added to 1500cc of a solvent (half ethanol and half water) and were shaken (Heidolph, Unimax 2010 Germany) by 90 cycle/min for 48 hours until they got homogenous. After that the solutions were filtered with strainer (watmann 0/5mm USA) and put on rotary evaporator (Heidolph WD 2000) to evaporate the solvent. Finally the pure extracts were kept in sterile vials in refrigerator to be used in microbial tests.

**Antibacterial susceptibility tests**

First of all, to prepare the bacteria [*Streptococcus mutans* (ATCC: 35668), *Lactobacillus rhamnosus* (ATCC: 7469), *Actinomyces viscosus* (ATCC: 15987)] the lyophilized samples were cultured in broth medium overnight (24h in 30-35°C). After turbidity occurring in broth medium, the samples were cultured on solid environment (BHI agar (Spain CONDA) for *A. viscosus, BHI agar* (8 CONDA) + 5% blood sheep for *S. mutans, MRS broth* (Germany,Merck) for *L. Rhamnosus*) to ensure about their purity. After that the antibacterial effect of every extract was evaluated against 3 bacterial species by two methods.

**Broth macrodilution method**

This method was introduced according to CLSI (Clinical Laboratory Standard Institute 2006 A4 USA) protocol. First, 800 μg/ml stock solutions were prepared from each extract and sterilized with 0/22 μm Millipore filters. Then dilutions of extracts were done with sterile water in the range of 0/78 – 400 μg/ml. After preparing the suitable culture, pouring them to plates of 4mm thickness, and making the bacterial suspension in the amount of 1/5 × 10^6 cfu/ml (like the previous method); the bacterial suspension was then spread on the plate surfaces with a swap. Then some wells were punched on agar surfaces with 6mm diameter and 30mm distance between each. After that 500 μl of different dilution of extracts were poured to the wells. Finally they were incubated in 37°C for 20h. Zone of inhibition around the wells were evaluated and measured by millimeter. Distilled water in one well was negative control, and chlorhexidine in another one was positive control.

Statistical analysis was done by Kruskal – Wallis between groups, and if they were significant, each pair group was then compared with another by Dunn – Procedure test (P-value < 0.05).

**Results**

The results of broth macrodilution method

The MIC and MBC of extracts against 3 under study bacteria are given in Table 1.

All extracts inhibited the growth of all bacteria. *S. officinalis* had the highest inhibitory effect against all three bacteria. Beside the bacteriostatic effect of all extracts, they have also had bactericidal effects against the three bacteria in this evaluated range; except *C. copticum* against *Lactobacillus* in which it’s MBC was more than 200 μg/ml. [lower amount of MIC and MBC means the higher antibacterial effects].

The results of agar well diffusion

For *S. mutans*, only *S. officinalis, P. anisum* and *R. coriaria* had growth inhibitory effect and the effect of *S. officinalis* and *R. coriaria* were higher and significantly more than *P. anisum* in the concentration of 100, 200 and 400 μg/ml (P < 0.001). In the concentration of 25 and 50 μg/ml, *R. coriaria* had the highest antibacterial effect which was significantly higher than *P. anisum* (p=0.002) and *S. officinalis* (P=0.001). Also in lower concentrations there weren’t any zone of inhibition (Figure 1).
For *L. rhamnosus*, only *P. anisum*, *C. copticum* and *R. coriaria* made zone of inhibition that their diameter in 100, 200 and 400 μg/ml for *R. coriaria* were significantly more than *P. anisum* (P<0.001) and *P. anisum* was more than *C. copticum* (P=0.038). *C. copticum* made zone of inhibition only in 400 μg/ml. In 50 and 25 μg/ml, diameter of zone of inhibition for *P. anisum* was significantly more than *R. coriaria* (P=0.002) and *C. copticum* (P=0.001). Lower concentrations didn't make any zone of inhibition (Figure 2).

For *A. viscosus* only the extracts of *M. longifolia*, *R. coriaria* and *C. copticum* made some zone of inhibition which their diameter in 200 and 400 μg/ml for *M. longifolia* was significantly more than *R. coriaria* (P<0.001) and *R. coriaria* was more than *C. copticum* (P=0.006). In 50 μg/ml only *M. longifolia* made zone of inhibition which was significantly more than *C. copticum* (P=0.004) and *R. coriaria* (P<0.001). Lower concentrations didn't make any zone of inhibition.

**Discussion**

In this study, two experimental methods have been used to evaluate the antibacterial effect of seven of Iran's native plants against three cariogenic microorganisms. All extracts from evaluated plants have shown antibacterial effect against these three cariogenic bacteria by broth macrodilution method, but not by agar diffusion method in all extracts. That might be because the confounding factors that influence the result of agar diffusion methods are more than broth dilution methods; e.g. diffusion ability of antimicrobial agent through the agar.
which depends on its nature [32,33]. By broth macrodilution method the inhibitory effect of *S. officinalis* was the highest which was followed by *M. longifolia*, *S. hortensis*, *C. copticum*, *R. coriaria*, *A. millefolium* and *P. anisum*. Therefore, our first hypothesis was proved to be right nevertheless, the second one was rejected.

In this method *S. officinalis* which was also herbal positive control had the best antibacterial effect against the three evaluated bacteria. *S. officinalis* is a well-known plant which its antimicrobial effects have been approved by many researches [23-29]. The phytomedicine texts have mentioned the usage of *S. officinalis* to treat anorexia, mouth and throat infections and increased perspiration [14]. There are some evidences of its antibacterial, antifungal and antiviral effect [14,15,24-29]. There are alpha and beta thujone (26-60%), 1,8 cineol (6-16%) and other flavonoids like epigenin in *S. officinalis* [14]. There are also some agents like linalool, borneol and α, β-caryophyllene in essential oil of *S. officinalis* [14]. In previous studies the effect of hydro-alcoholic extract of the leaf of *S. officinalis* against collagenolytic and the activity of *Porphyromonas gingivalis* was evaluated [15] and strong antibacterial and antifungal effects of *S. officinalis* against wide spectrum of bacteria like *Pseudomonas*, *Aspergillums* and *Candida* were reported [15,34] which was in agreement with the results of our research.

*A. millefolium* is one of the plants that has been used to treat the wounds, infected and gastrointestinal disorders, and even to control the blood cholesterol for many years now [11,14]. There are some agents like chamazulene, caryophyllene, 1,8 cineol and some flavonoids like epigenin and rutin that are found in *A. millefolium* [14]. This plant had inhibitory and cidal effect against these evaluated microorganisms in our study which is similar to the results of Candon’s research about antibacterial effect of essential oil of *A. millefolium* [16].

*M. longifolia* is a medicinal plant that is also used as food. It has been shown that the essential oil of different types of Mentha had strong antibacterial effects [35]. Metanolic extract of *M. longifolia* had also antibacterial and antifungal effects against wide spectrum of gram negative and gram positive bacteria and fungus [19]. In hydro-alcoholic extract of *M. longifolia* there are piperitone (60-80%), β – caryophyllene (5-15%), 1,8 Cineol (2-7%) and some flavonoids like hesperidin and quercitrin [14]. By broth macrodilution, the bactericidal effect of hydro-alcoholic extract of *M. longifolia* against *Lactobacillus* was significantly more than the well-known plant, *S. officinalis*; which shows the probable effect of the main effective agent – piperitone – against *Lactobacillus*. In addition, the effect of *M. longifolia* against *S. mutans* was
good and after \textit{S. officinalis}, had the most inhibitory and cidal effect against that bacterium. On the other hand, it has been reported that \textit{M. longifolia} didn't have any inhibitory effect against some \textit{Streptococcus} like \textit{S. pyogenes} [35]; therefore this effect seems important and needs more researches. The effects of \textit{M. longifolia} against \textit{A. viscosus} weren't the same as the two other cariogenic bacteria. It's probably because of the character of \textit{A. viscosus} which is an anaerobic bacterium and produces some yellow colonies named “Sulfur Granule” in both tissue and aqueous environment. The higher resistance of this bacterium might be the result of being in these granules. In addition this bacterium needs blood and serum environment and doesn't grow very well in simple environments [36]. In addition, the effect of flavonoids in the plant should be concerned, especially the antibacterial effect of hesperidin which had been evaluated separately [37].

\textit{P. anisum} is also a useful plant in traditional medicine of Iran. The common usage of this plant is for gastrointestinal disorders, but it is used for superior respiratory organ disorders, fever and mouth and throat infections, as well [14]. Acetonic extract of \textit{P. anisum} is effective for growth inhibition of some bacteria like \textit{Escherichia coli} and \textit{Staphylococcus aureus} and its essential oil has good effect against different spectrum of bacteria like \textit{Salmonella typhi} and \textit{E. coli} [34]. The main agent of this plant is transe–anethole (94%) and there are β-caryophyllene and some flavonoids like epigenin and isovitexin in \textit{P. anisum} [14,15]. In the present study \textit{P. anisum} had good inhibitory effect against \textit{S. mutans} and in higher concentrations against \textit{Lactobacillus} and \textit{A. viscosus} which are probably the result of the presence of anethol, β-caryophyllene and flavonoids.

In our research the effects of \textit{P. anisum} is similar to bactericidal effect of aqueous extract of \textit{P. anisum} against oral flora by disc diffusion method [38]. Probably, the hydrophilic agents in \textit{P. anisum} play an important role in antibacterial effects.

\textit{S. hortensis} is also a medicinal plant that is used as food and has antibacterial and antifungal effect against a wide spectrum of bacteria and fungus [34,39]. The essential oil of \textit{S. hortensis} has mild antiseptic effect and also as it has Cymol and carvacrol in it, its aqueous extract has antiviral effect [14,34]. The main agents of \textit{S. hortensis} are carvacrol and P- cyeme [14,34]. The phenolic type of carvacrol has a well-known antimicrobial effect [34,40,41], and doesn’t dissolve in water. As the hydrophobic agents like carvacrol influence on cellular membrane of microorganisms and inhibit their growth [42]; and because of the hydrophilic nature of \textit{S. hortensis} that prevents the diffusion in aqueous environment; therefore \textit{S. hortensis} had moderate antibacterial effect in this study.

\textit{R. coriaria} is a well-known plant in traditional medicine of Iran [10,13]. As long as we know, the antimicrobial effect of the fruit of \textit{R. coriaria} hasn’t been evaluated against cariogenic bacteria yet. The results of our study are similar to the results of another study about the effect of methanolic extract of the leaf of \textit{R. coriaria} against gram positive and gram negative bacteria and \textit{Candida} [43]. But, it should be considered that the agents in the leaf and fruit of \textit{R. coriaria} like β-caryophyllene, caryophyllene oxide and triponoid agents are different. \textit{R. coriaria} had good inhibitory and cidal effect against all three cariogenic bacteria with both experimental tests. Probably, the antibacterial effect may be from tanens like tannic acid (13-27%) and gallic acid. The presence of some agents like β-caryophyllene and α-terpineol in \textit{R. coriaria} and \textit{M. longifolia} is an important issue; especially according to the results of its bactericidal effect against \textit{Lactobacillus}, which probably shows the special effect of these agents against \textit{Lactobacillus}. This effect is very important, because the MBC of \textit{M. longifolia} and \textit{R. coriaria} was the same. On the other hand, presence of acidic agents may limit the usage of this plant to prevent dental biofilm plaque formation; but we can use it after excluding the acidic agents by some solvents like ether – 2 – petrol. The main agents of \textit{R. coriaria} are β-caryophyllene, terpenoid agents, \textit{monosesqui} and diterpenes [16].

\textit{C. coticum} is a plant which is used for gastrointestinal disorders in Iran’s traditional medicine [10,13]. It is the only member of Apiaceae family which has thymol and carvacrol and from this perspective it is similar to \textit{S. hortensis}. The effect of hydro-alcoholic extract of \textit{C. coticum} against three cariogenic bacteria is probably reported for the first time in this study; but the effect of essential oil of \textit{C. coticum} against different spectrum of gram positive and gram negative bacteria and dermatitis was evaluated in the past [34]. According to the presence of the same agents like carvacrol and γ-cymene in essential oil and hydro-alcoholic extract, probably these two agents can be the antibacterial agents.

The other important point is the presence of similar agents -caryophyllene- in \textit{P. anisum}, \textit{M. longifolia}, \textit{R. coriaria} and \textit{A. millefolium} [14,15]. In addition there are 1,8 cineal in \textit{M. longifolia}, \textit{A. millefolium} and \textit{S. officinalis} [14,15] and they can probably be the effective antibacterial agents.

The differences between two methods -broth macro dilution and agar well diffusion- is probably because of the presence of polar agents which are better released in the aqueous environment of broth dilution method and show the antibacterial effect. This difference can help us to identify the group of effective agents according to their polarity.

Based on the fact that all of the extracts showed antibacterial effect by broth macrodilution method; the plant didn't make zone of inhibition by agar diffusion method, may probably show antibacterial effect in higher concentration because of more proper concentration gradients in solid environment.

Some of the plants used in this study such as \textit{S. hortensis}, \textit{R. coriaria}, \textit{M. longifolia} can also be used as food; therefore their consumption in high risk patients like the patients who have xerostomia or those who suffer from severe decay due to excessive numbers of radiotherapy, can be useful and without any harm. Meanwhile, the world’s attraction to traditional treatment and the necessity of having medicines derived from plants, and microbial resistance to chemical drugs; preparing some products like antimicrobial mouthwashes from these plants, after animal and human studies to determine the therapeutic dose, seem to be beneficial and necessary.

**Conclusion**

Despite all limitations of our study, the final results show
that all of the plants had growth inhibitory effect against cariogenic bacteria in broth macrodilution method and S. officinalis had the greatest growth inhibitory effect against all three cariogenic bacteria. All of the extracts, except C. coticum, also had bactericidal effect in the evaluated range of concentration. By agar diffusion method R. coriaria had antibacterial effect against all three cariogenic bacteria.

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