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Synergistic antibacterial effects of methanolic extract of *Melissa officinalis* L. and Mouthwash Vi-one on *Streptococcus mutant* and *Streptococcus sanguinis*

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Ali Asghar Faraji[1], Khosro Issazadeh[2], Samaneh Rouhi[3], Fatemeh Zaboli[1]*, Bita Khasi[4], Jalileh Ebn Abbas[5]

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[1]Department of Biology, Faculty of Agriculture and Food Industry, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran; [2]Department of Microbiology, Faculty of Basic Sciences, Islamic Azad University, Lahijan Branch, Lahijan, Iran; [3] Medical Microbiology Research Center, Qazvin University of Medical Sciences, Qazvin, Iran [4]Social Development and Health Promotion Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran; [5]Lung Diseases and Allergy Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Sanandaj, Iran.
Correspondence:
Fatemeh Zaboli. Department of of Biology, College of Agriculture and Food Science, Islamic Azad University, Ayatollah Amoli Branch, Amol, Iran
microbiol_sci@yahoo.com Email:

ABSTRACT

Introduction: The use of chemical and vegetable compounds reduces the microbial plaque of the tooth. The aim of this study was to investigate the synergistic antibacterial effects of methanolic extract of *Melissa officinalis* L. (*Lemon balm*) and mouthwash Vi-one on *Streptococcus mutant* and *Streptococcus sanguinis*.

Methods: Methanolic extract of lemon balm was prepared by Soxhlet method. The concentrations of 250, 125, 62.5, 31.25, 15.26, 7.81, 3.9, 1.95, 0.97 and 0.48 mg/ml of methanol extract and mouthwash prepared and mixed in the same proportion. Agar well diffusion, minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were used to determine the antibacterial effect.

Results: The highest non-growth zones were 21 mm for *Streptococcus mutant* and 22 mm for *Streptococcus sanguinis* which observed at a concentration of 250 mg/ml. The best value of MIC and the MBC for both bacteria were 7.81 and 62.5 mg/ml, respectively. As the concentration increased, antibacterial activity increased as well ($P \leq 0.05$).

Conclusion: The results of this study showed the synergistic antibacterial effects of *lemon balm* and mouthwash on bacteria. More *in vivo* researches are needed to confirm and use the above combination.

Introduction

The use of chemical and herbal mouthwashes along with mechanical methods such as brushing to cleaning the teeth, enhance the effect of mechanical methods and reduce the microbial plaque on the teeth. Microbial plaque is a damaging agent of enamel, decay and gum disease (1). Both *Streptococcus mutants* and *Streptococcus sanguinis* are one of the most important bacterial pathogens in the mouth and teeth (2). *Streptococcus mutant* with carbohydrate metabolism and high acid production, including lactic acid, acetic acid, and formic acid, are the most common cause of dental caries (4, 3). *Streptococcus Sanguinis* is the most bacterial species isolated from the dental plaque. This bacterium provides the energy necessary to grow in the absence of carbohydrate fermentation through arginine hydrolysis. Therefore, bacteria can remain in the plaque in the absence of carbohydrate fermentation and continue to grow (3).

Chemical mouthwashes have very good antimicrobial effects, but along with complications such as unpleasant color changes in the teeth and fillings, taste changes, dryness and burning sensation in the oral mucosa. Nowadays, herbal mouthwashes are available to patients and dentists due to their minimal clinical complications (3, 1). Various studies have shown the antimicrobial effects of chemical and herbal mouthwashes alone or a mixture of these two. In a study by Decker et al. using fluorescence microscopy and bacterial cell count, it was found that chlorhexidine mouthwash has an antibacterial effect on the biofilms formed by *Streptococcus sanguinis* (5). Also, Safarabadi et al. by studying 70 patients with oral tracheal intubation, showed the effect of Echinacea based herbal mouthwash on oral hygiene as same as the chlorhexidine mouthwashes (6). In another study, Haghighi et al. demonstrated that, silver nanoparticles in combination with aqueous extract of millet showed antibacterial effect on *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *E. coli*, *Serratia marcescens* and *Pseudomonas aeruginosa* at minimum inhibitory concentration (MIC) of 8 µg/ml (7).

According to the above mentioned and the importance of using herbal and chemical mouthwash, the aim of the current study was to investigate the synergistic antibacterial effects of methanolic extract of *Melissa officinalis* L. (*Lemon balm)* and mouthwash Vi-one on *Streptococcus mutant* and *Streptococcus sanguinis*.

Methods

Plant collection and preparation

The lemon balm leaves were collected from different regions of Guilan province and mouthwash Vi-one (containing Cetylpyridinium Chloride) was obtained from Tehran pharmacies. The plant specimens were collected, identified and confirmed at Herbarium Botanical Garden of Islamic Azad University of Lahijan. After washing with water, it was kept in the open air for a week and dried completely in the shade. Then, they turned into a powder with an electric mill to have a higher contact surface area with the solvent.

Plant extraction

Soxhlet method was used for extraction. In order to obtain an extract of the plant, the leaves were dried and, after separating the waste, it has been changed into the powder by the electric grinder in order to make more surface contact area with the solvent. In this study, 70% methanol was used for extraction. At first, 200 gr of the herbal sample which was turned into powder was poured into the decanter, and then, step by step, methanol was added separately to it (first warm the alcohol and then added to the decanter). The addition of alcohol continued until the entire volume of the plant in the decanter was soaked in alcohol and the alcohol was completely absorbed by the specimen. After one hour, the mixture was placed inside the decanter, and the tap was opened until the solution containing the extract and alcohol was dropped out of the decanter. After leaving the solution, the tap was closed and the solution was returned to the decanter again. This process was repeated every hour. The filter paper NO.1 was used to filter the extracts. After extraction, the specimens were placed in an evaporator to completely remove the excess solvent and dry the extract.

Preparation of herbal extract stock

Initially, 2 gr (2000 mg) of dried extracts were dissolved in a final concentration of 5 ml of dimethyl sulfoxide (DMSO) to prepare the stock solution. Vi-one mouthwash diluted with deionized water. Concentrations of 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.95, 0.97 and 0.48 mg/ml of methanol extract and mouthwash were prepared and they were mixed in the same proportion.

Supply and preparation of bacterial sample

Streptococcus mutant PTCC1449 and Streptococcus sanguinis PTCC168 (collections of fungi and bacteria from the Scientific and Industrial Research Organization, Iran) were purchased as lipophilic glass ampoules. A glass of lipophilic bacteria was opened in accordance with the instructions of the PTCC manufacturer. The ampoules were scraped out of the mass of the cotton and were completely disinfected around 70 % alcohol. The ampoules were broken down from the scratch site and under the microbiological hood near the flame, a cotton swab was removed with sterile pence. Then, 2 ml of the Muller Hinton broth medium (Merck, Germany) autoclaved with insulin syringe was added to the dry matter in the ampules and, after uniformity, was cultured linearly in the Müller Hinton agar (Merck, Germany) medium for 24 hours at 37 ° C. several colonies from each of the bacteria used in the study were taken by loop sterilization and inoculated into a test tube containing 5 ml of Muller Hilton broth medium. The medium was then placed in an incubator at 37 ° C for 2-4 hours to grow the bacteria. Then the opacity of the tubes was measured using a spectrophotometer. These opacities were matched with 0.5 McFarland turbidity tube (1.5×10^8) via physiologic serum and then diluted to 1.5×10^6.

Determination of antibacterial effect using the diffusion method from wells

In order to determine the antibacterial effect of the diffusion method from the wells, half-McFarland bacterial suspension was prepared on a Muller Hinton agar medium in a flattened manner. Then in a large plate, 10 wells of 5 mm were created at intervals equal to 2 cm. Each of these wells was poured 100 µl of sample by sampler from prepared concentrations of extract-mouthwash (50 µl mouthwash + 50 µl of methanolic extract) then the plates were incubated for 24 hours at 37 ° C (Gentamicin antibiotic with a concentration of 1.25 was used as a positive control and 100% dimethyl sulfoxide (DMSO) solution was used as a negative control (8).

Determine the minimum concentration of antibacterial inhibition by tube method

Determination of the minimum concentration of synergism antimicrobial inhibitor of methanolic extract of *Lemon balm* and mouthwash Vi-one was performed by tube method. In the method for determining the minimum inhibitory concentration, a bacterial concentration was prepared equivalent to half of McFarland. 12 tubes were taken and each of them added 1 ml of Muller Hinton broth (Merck, Germany) and 1 ml of antibacterial agent at concentrations of 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.95, 0.97 and 0.48 mg/ml (0.5 ml of the prepared concentrations of methanol extract + 0.5 ml of prepared concentrations of mouthwash) were added to each of the tubes, respectively (each separate tube was added to a separate concentration). In the next step, 1 ml of bacterial suspension was added to the tubes and the tubes were incubated at 37 ° C for 24 hours. Tube No. 11 was considered as the positive control (1 mL of Muller Hinton broth + 1 mL of bacterial suspension) and tube 12 as the negative control (1 mL of Muller Hinton broth). After incubation, the minimum inhibitory concentration was determined based on turbidity or lack of turbidity in the tubes.

Determination of the minimum bactericidal concentration

In Minimum Bactericidal Concentration (MBC) method, tubes that were free from turbidity were cultured on a medium of agar and incubated at 37 ° C for 24 hours. Then, the minimum bactericidal concentration was read. In this method, we considered the first tube that has turbidity or the first plate of the bacterium which was grown and then considered a minimum of inhibitory concentrations and a minimum bactericidal concentration before it was clear and free from opacity or a plate prior to it, respectively. All methods were performed separately for both bacteria and with three replications (9, 8).

Statistical method

A comparison of differences in effects and significant differences between concentrations were performed by one-way ANOVA and using SPSS 16 software ($P \leq 0.05$).

Table 1. Growth inhibition zone size (cm) by synergistic application of a methanolic extract of lemon balm and mouthwash Vi-one by diffusion in the well

Concentration (mg/ml)	250	125	62.5	31.25	15.62	7.81	3.9	1.95	0.79	0.48	Gentamicin 1.25 mg/ml	DMSO
Streptococcus mutans	21	20	18	16	15	12	11	10	9	9	21	-
Streptococcus sanguinis	22	21	19	16	15	15	13	12	10	10	23	-

* (-) indicates a lack of growth

Table 2. Minimum concentrations of inhibitory and bactericidal synergism of methanolic extract of lemon balm and mouthwash Vi-one by dilution in tube

Concentration (mg/ml)	250	125	62.5	31.25	15.62	7.81	3.9	1.95	0.79	0.48	MIC	MBC
Streptococcus mutans	-	-	-	-	-	-	+	+	+	+	7.81	62.5
Streptococcus sanguinis	-	-	-	-	+	-	+	+	+	+	7.81	62.5

* (+) indicates a growth

Results

The highest non-growth zone diameter was observed on Streptococcus mutant of 21 mm, at a concentration of 250 mg/ml. Also, the highest non-growth zone diameter for Streptococcus sanguinis was 22 mm and also at a concentration of 250 mg/ml (Table 1). The best rates for the minimum inhibitory concentration and the minimum bactericidal concentration for both bacteria were at the concentration of 7.81 and 62.5 mg/ml, respectively (Table 2). The statistical results showed that there were meaningful differences between the concentrations and the growth inhibitory zone and increased with the increase in the concentration of the size of the growth inhibitory zone ($P < 0.05$).

Discussion

The effects of plants on infectious agents have long been of interest to researchers and ordinary people in many parts of the world, and many of them have been confirmed in experiments (11, 10). The results of this study also showed the antibacterial Synergism effects of methanolic extract of *lemon balm* and mouthwash Vi-one on Streptococcus mutant and Streptococcus sanguinis. In 2015, Rezaei et al. showed that mouthwashes containing a toothbrush wood/Aloe Vera compound had a more effective therapeutic effect than chlorhexidine mouthwash on gum index in patients with endotracheal intubation in intensive care units (12). Khaledi et al., in a study in 2016 in Iran, showed that MIC of hydro alcoholic extract of Teucrium herb on Streptococcus mutant was 128 µg/ml and MBC was 256 µg/ml (13). Medicinal plants and their extracts and essences are used for the treatment of various infectious and noninfectious diseases due to chemical compounds and biological active ingredients, because the compounds of some of these extracts and essential oil have antimicrobial effects and are used as antimicrobial agents in the treatment of infections (14). Kaim et al. investigated the antimicrobial effects of a mixture of herbal and gum mouthwashes on Streptococcus sanguinis and mutant and achieved positive results. These mouthwashes showed superior effects in comparison with chlorhexidine and Listerine (15). In a recent study, Abdi-Ali et al. in Iran in 2015 showed synergism effects of the butanol extract of the Cyclamen coum and Ciprofloxacin on the biofilm of Pseudomonas aeruginosa (16). When two or more elements, process, or agent synergy and interact, there are usually making effects. If this effect is maximized from the sum of the works that each of these separate elements could produce, then a synergism would occur (17). In our study, the antimicrobial synergism effect of methanolic extract of lemon balm and mouthwash Vi-one on Streptococcus mutant and Streptococcus sanguinis was confirmed. A comparison of this effect with the effects of these substances alone should also be investigated in future studies. Chegini et al. in Iran in 2018 showed that MIC Medicago sativa was influenced by Streptococcus pneumonia, Haemophilus influenzae and Moraxella catarrhalis 125 mg/ml, and the inhibition of growth in the diffusion method from the disc for each of these bacteria were 13, 10 and 16 mm, respectively (18). Lee et al, in Korea in 2007 showed that Lemon balm oil was effective against methicillin-resistant Staphylococcus aureus, with a minimum inhibitory concentration of 25-26 µg/ml (19). In our study, the highest diameter of the growth inhibitory zone was due to the use of a mixture of methanolic extract of lemon balm and mouthwash Vi-one the Streptococcus mutant 21 and 22 mm for Streptococcus Sanguinis. Both of these bacteria had the highest growth inhibitory zone at the highest concentration of 250 mg/ml. Minimum inhibitory concentration and minimum bactericidal concentration for both bacteria were 7.81 and 62.15 mg/ml, respectively, which was lower than the Lee and Chegini studies (19, 18). Herbal extracts are new sources of antibacterial compounds against pathogenic bacteria. Studies have shown that there is a correlation between polyphenolic compounds and antimicrobial effects of herbs, and these compounds may have a better synergism effect with other compounds. The number and position of phenol are different in phenolic compounds, which in turn affects the amount and strength of microbial contamination and is one of the reasons for different effects in different studies. On the other hand, the hydrophobicity of herbal extracts leads to bind of the lipid layer of the cell membrane of the bacteria and, mitochondria causes the membrane to rupture and ultimately lysis and bacterial death (21, 20). Studies have also shown that the presence of Caryophyllin oxide and Germacrene-D as the main combination in the essential oil of the Melissa officinalis, and the antibacterial properties of the essential oil have also been proven (21). Considering the significant antibacterial effect of methanolic extract of Melissa officinalis, mixed with mouthwash Vi-one, the mixture of these two substances can be considered as a medicinal product and given the antibiotic resistance, can be used instead of antimicrobial drugs (22, 18). However, further

and comprehensive studies on the application of each of these substances alone and the reduction or increase of its positive and negative effects, either *in vitro* or on animal models are necessary in order to achieve comprehensive researches with a widespread collaboration between the relevant centers in this field.

Conclusion

The antimicrobial effect of plant extract and mouthwash was demonstrated in this study. Recognizing the best species of medicinal plants and extracting their pure active ingredients, simultaneously using and counting plant and chemical substances as complementary to chemical drugs can have better effects.

Ethical disclosure

Before performing the research, it was explained to the participants. An informed consent was obtained from all participants included in the study.

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Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Conflict of interest

The authors declare that they have no conflict of interest.

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