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Evaluation of the Antibacterial Effect of Hydroalcoholic Pomegranate Peel Extract on Lactobacillus Acidophilus

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ABSTRACT

Introduction: Dental caries occurs at a specific site when the lactobacilli of that site reach 8% of the total bacteria in that site. The aim of this study was to investigate the antibacterial effect of pomegranate peel extract.

Materials & Methods: This experimental in vitro study was performed with 6 standard bacterial groups, including a group with 0.2% chlorhexidine and 4 groups with pomegranate extract concentrations of 10, 25, 50 and 100 mg/mL, as well as a blank plate group for determination of the zone of inhibition and a group of standard bacterial samples for determination of the minimum inhibitory concentration (MIC) on Lactobacillus acidophilus. The data were statistically analyzed using One-way ANOVA and Tukey Honestly Significant Difference (HSD) to measure the diameter of the growth inhibition zone. A value of $P<0.05$ was considered significant.

Results: The results of the inhibition zone diameter evaluation showed that different concentrations of the extract (10, 25, 50 and 100 mg/mL) had a positive inhibitory effect on Lactobacillus acidophilus. The results of the diameter of the inhibition zone using the disc plate method indicated that as the extract concentration increased, the antimicrobial effect on the bacteria increased and a significant difference was found between the diameters of the inhibition zone of four concentrations (16.5, 23.5, 26, 29.8 mm) ($P<0.05$). Examination of the MIC using the broth dilution method demonstrated that the effective amount of hydroalcoholic extract of pomegranate peel to prevent bacterial growth ranged from 12.5 to 25 (mg/ml). Moreover, chlorhexidine showed the highest growth inhibition zone (30 mm) in all studied groups.

Conclusion: According to the results of the current study, the hydroalcoholic extract from the pomegranate peel may have antimicrobial properties and it seems that the pomegranate fruit can be used as an antibacterial agent against Lactobacillus acidophilus, which can reduce the use of antibacterial chemical compounds with lower costs and side effects.

Keywords: Pomegranate, Anti-Bacterial agents, Lactobacillus acidophilus

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Introduction

Over the last hundred years, extensive research has been conducted to identify oral bacteria. The results of these studies have led to knowledge of oral bacteria, their mutual effects on the body and general health, and their protective or beneficial effects on tooth decay.[1] The research results show that the bacterium Lactobacillus acidophilus plays a special role in plaque formation and tooth decay. Tooth decay occurs at a particular site when the Lactobacillus in that area reaches 8% of the total population of bacteria. [2] Effective methods of caries prevention include the mechanical reduction of pathogenic microbes or the use of antibacterial substances in the form of mouthwash or toothpaste. In recent years, numerous chemicals have been reported to be effective in reducing bacterial metabolism, such as chlorhexidine. Like most chemicals, this substance has some side effects. [3] These side effects of long-term use include discoloration and stains on the teeth, oral fillings and mucous membranes, stimulation of tartar formation, burning, exfoliation of mucous membranes, creation of bacterial resistance, allergic reactions and unpleasant taste are side effects of long-term use of chlorhexidine. The use of herbal and natural mouthwashes, which have the same effect as chlorhexidine, but have fewer side effects, can be a good solution to prevent the aforementioned problems. [4, 5]

In traditional medicine, different parts of the pomegranate (from the Punica granatum tree, which belongs to the Punicaceae family), including its skin, are used as a natural supplement to treat various diseases such as cancer, heart disease, digestive disorders, osteoarthritis, diabetes, anemia and tooth decay. [6, 7] A study has suggested that this plant has pain-relieving properties, which is due to the presence of phenolic compounds. [8] Pomegranate also has antibacterial, antiviral, antifungal, antioxidant and anti-inflammatory properties. [9] Numerous studies on the therapeutic effects of pomegranate peel have demonstrated that the phenolic compounds of this part of the pomegranate are three times higher than those of the core of its seeds. [7, 10]

These compounds produce antimicrobial properties through various mechanisms that destroy the microbial cell or cause the denaturation of some microbial enzymes, such as glycosyltransferase, which plays an important role in the firm adhesion of bacteria to the tooth surface. In this way, the adhesion mechanism of bacteria to the tooth surface is prevented. [11] In addition, phenolic compounds form complexes with some nutrients from the environment such as carbohydrates, proteins, vitamins and minerals and remove them from the reach of microorganisms. [12] Polyphenols also have a significant effect on the bacterial population by lowering the pH of the environment. [13] Different mechanisms describe the effect of tannin against bacterial species. [14]

It is possible that the gallic acid and punicalagin contained in pomegranate peel extract, through the mechanism of reducing penetration, justify the effect of increasing the antibiotic sensitivity of pomegranate peel. [15] Antibacterial or antimicrobial are substances that indicate selective toxicity against microorganisms. The effect of these substances depends on the concentration of the drug, the type of target pathogen and the severity of the infection. [16] Lactobacilli include 180 Gram-positive species of long and short rods without spores that are an important part of the normal flora of the mouth, digestive system and urinary and reproductive tract of women. [17, 18]

Lactobacilli are one of the most important etiologic factors in the development of dental caries and also play a role in the development of gum disease. [19] Lactobacillus acidophilus is one of the best-known species of this bacterium from the homolactic group. Studies have

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illustrated the presence of this species in the saliva of patients, in dental plaque, on the surface of the tongue, in the mucosa and in caries in children and adults. [20] Based on the known properties of pomegranate and in order to find and introduce a natural substance without side effects that is effective on the Lactobacillus involved in caries. [4] The effect of the hydroalcoholic extract of pomegranate peel on the growth rate of the standard strain of Lactobacillus acidophilus was investigated in a laboratory environment and by disk diffusion in the current study.

Materials & Methods

This study was approved by the Ethics Committee of Islamic Azad University of Medical Sciences, Tehran Branch (IR.IAU.TMU.REC .1400.030). The present study was carried out using an experimental method (in vitro study). Then, referring to the microbiology department of Shahid Beheshti University, the standard strain of Lactobacillus acidophilus bacteria was prepared from the Scientific and Industrial Research Organization of Iran with the code PTCC 1643.[21] The remaining steps were performed in the Microbiology and Pharmacognosy Laboratory of Islamic Azad University of Medical Sciences, Faculty of Pharmacy.

The bacteria were divided into 6 groups, including four groups with pomegranate extract at concentrations of 10, 25, 50 and 100 mg/ml [12, 22](based on previous studies and reports on the antimicrobial effect of pomegranate peel extract at the mentioned concentrations, these concentrations were tested . [12, 22] Each group contained six bacterial samples, a group with 0.2% chlorhexidine (Najo Company) (with one bacterial sample) as a positive control group, and a blank disc group (with one bacterial sample) as a negative control group. To determine the minimum inhibitory concentration (MIC), the number of 3 bacterial samples was taken into account (total sample size = 29).

To prepare the bacterial suspension, half McFarland concentration (standard 0.05 McFarland Ingen model 5cc) and Mueller Hinton Agar culture medium (inzhen Environment plate ready Mueller Hinton Agar 10cm from Farazmed company) were used (figure2) , and at the end, some of the bacterial suspension was inoculated onto the culture medium with a sterile swab and then a well was created on the plate (figure3). [23] To prepare the hydroalcoholic pomegranate extract, 840 grams of pomegranate peels were purchased online from panjnoosh.com. Using an industrial grinder, this amount of peel was converted into 775 grams of pomegranate powder.

To prepare the extract using the maceration method in the next step, 775 grams of the dried peel powder was soaked in 1000 ml of 70% alcohol in a decanter. Since a high absorption of the solvent of the plant was observed, 875 ml of 70% alcohol (alcohol 70% Zakaria glass 1litr) was added to the decanter after 4 hours and stirred. After 72 hours, the prepared extract was removed from the decanter and transferred to an open Pyrex container. To dry the extract, these containers were placed in the sun for 4 days. In the meanwhile, after the first 48 hours, 500 ml of 70% alcohol was added to the extract and stirred. On the fourth day, the resulting extract was collected with a spatula and placed in a rotary machine (laboratory rotary evaporator) under vacuum and at a temperature of 40 degrees to separate the alcohol remaining in the extract. It was then transferred to an opaque and closed container and stored in the refrigerator. The final amount of extract obtained was 298 grams.

To prepare the extract at different concentrations, 1 gram of the extract powder was dissolved in ten milliliters of double distilled water and filtered using a Millipore 0.45 micron filter (VLP04700

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Millipore Durapore membrane filter, 0.45 µm pore size). The calculation of the relative dilution for the preparation of the extract solution with concentrations of 10, 25, 50 and 100 mg/ml was done with double distilled water. The advantage of choosing hydroalcoholic extract over aqueous extract was that in an aqueous extract normally only polar substances can be extracted from the plant, whereas in a hydroalcoholic extract, which here means hydroethanolic, both polar and some non-polar components can be removed from the plant, allowing the properties of the plant to be more fully recognized. Based on the above, the possible antibacterial response of the hydroalcoholic extract of pomegranate fruit peel on Lactobacillus acidophilus was stronger. It also showed a better result in inhibiting the growth of bacteria. [11]

Examination of the diameter of the inhibition zone:

After preparing the hydroalcoholic extract of pomegranate, a certain amount of it was dissolved in two percent DMSO (dimethyl sulfoxide) to obtain a strain. An amount of 30 microliters of different concentrations of pomegranate extract was added to the wells. [23] The wells of the positive control groups also contained 30 microliters of chlorhexidine 0.2% (Najo) and the empty negative control. The plates were stored in glass for 24 hours at a temperature of 37 degrees. The diameter of the inhibition zone of the microorganisms was then measured in millimeters using a vernier caliper (figure4).

The lowest inhibitory concentration or MIC is the lowest concentration of the antimicrobial agent that completely inhibits the growth of the microorganism in the microdilution tube or well. One of the most suitable methods for determining the MIC value is the serial dilution method, which is expressed on the basis of mg/ml.

The final concentrations of the extract were obtained by preparing 4 different dilutions of the pomegranate extract in liquid culture and in small-volume tubes (96-well microtitration plate). Then, after dilution of the standardized microbial suspension with half the McFarland scale, each tube was prepared with an appropriate volume of microbial inoculum. After incubating the tubes for 72 hours, the liquid cultures were read using an ELISA reader at a wavelength of 630.

The data obtained in the study environment were analyzed using SPSS 26. After determining the MIC and the diameter of the inhibition zone, the one-way ANOVA test was used to compare the inhibition zone in four different concentrations of the pomegranate extract due to the normal distribution of the data. The Tukey HSD addition test was also used to verify the presence or absence of significant differences between groups in the diameter of the growth halo. The mean MIC was also reported together with the standard deviation and the calculation of the coefficient of variation (CV).

Results

Among the concentrations tested for the diameter of the inhibitory zone, pomegranate peel extract at a concentration of 100 mg/ml had the largest diameter at 29.8 ± 3.1, and at a concentration of 10 mg, it had the smallest at 16.5 ± 2.8 per milliliter of growth-free aura. Of course, chlorhexidine had the largest diameter of the inhibition zone (30 mm) in all groups studied (Table 1). Diagram 1 shows that the higher the concentration of pomegranate extract, the stronger the antibacterial effect.

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According to the Tukey HSD test, the diameter of the inhibition zone at concentrations of 25 and 50 mg/ml was not significantly different from each other (P>0.05), but the diameter at other concentrations such as 50 and 100 mg/ml was significantly different from each other (P<0.05) (Table 2). Evaluation of MIC by broth microdilution suggested that the lowest effective amount of hydroalcoholic extract of pomegranate peel to prevent bacterial growth was 12.5 mg/ml (the average±standard deviation= 16.66 mg/ml±7.2 – CV=43.2 and minimum/maximum= 12.5-25 mg/ml).

Table1. The diameter of the growth inhibition zone in the case and control groups

Groups	Average ± standard deviateon	CV	Minimum - maximum
Pomegranate extract 10 mg/ml	16.5±2.8	16.9	20-12
Pomegranate extract 25 mg/ml	23.5±2.8	11.9	27-20
Pomegranate extract 50 mg/ml	26.0±2.3	8.8	29-23
Pomegranate extract 100 mg/ml	29. 8±3.1	10.4	34-25
Chlorhexidine 2%	30	0	30-30
Blanc disc	0	0	0

test results: One-way ANOVA test indicated a significant difference between the diameter of the growth-free halo of the four concentrations (P< 0.001).

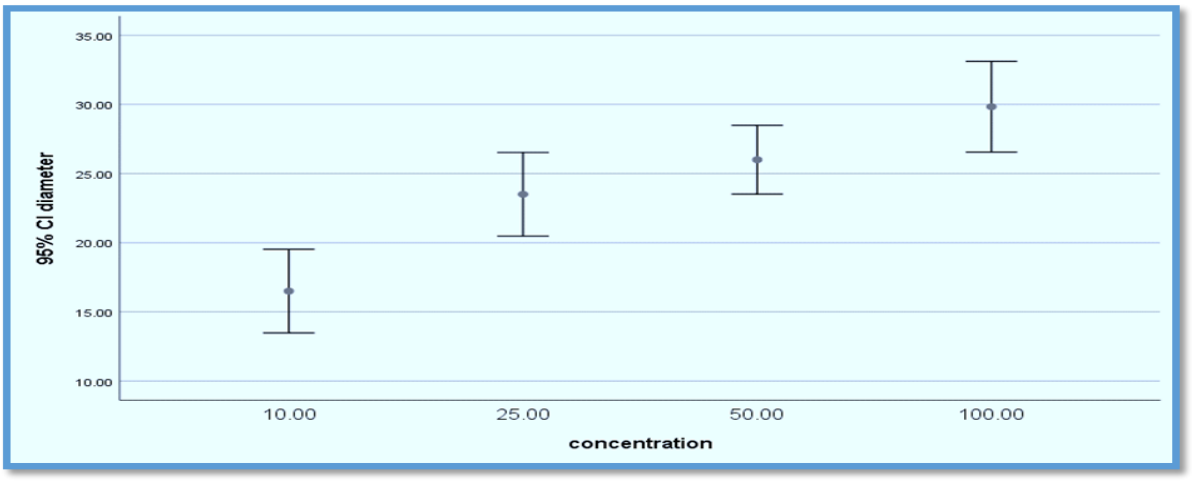

Figure 1. Comparison of the mean diameter of the growth inhibition zone in four concentrations

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Table 2. Comparison between the groups by Tukey HSD

Concentration		Mean Difference (I-J) ± Std. Error	Sig.
10.00	25.00	-7.00000* ± 1.63214	0.002
	50.00	-9.50000* ± 1.63214	0.000
	100.00	-13.33333* ± 1.63214	0.000
25.00	10.00	7.00000* ± 1.63214	0.002
	50.00	-2.50000 ± 1.63214	0.438
	100.00	-6.33333*± 1.63214	0.005
50.00	10.00	9.50000* ± 1.63214	0.000
	25.00	2.50000 ± 1.63214	0.438
	100.00	-3.83333 ± 1.63214	0.120
100.00	10.00	13.33333* ± 1.63214	0.000
	25.00	6.33333*± 1.63214	0.005
	50.00	3.83333 ± 1.63214	0.120

**** The mean difference is significant at the 0.05 level.***

Figure2: muller hinton agar culture envirnment

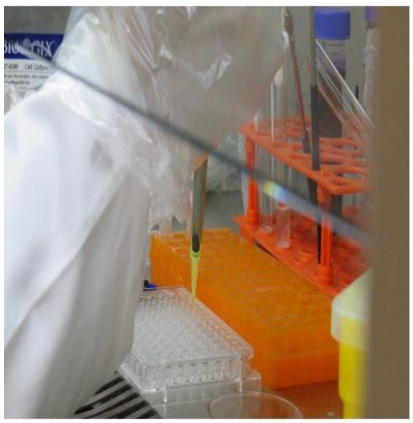

Figure3: bacterial sample preparation

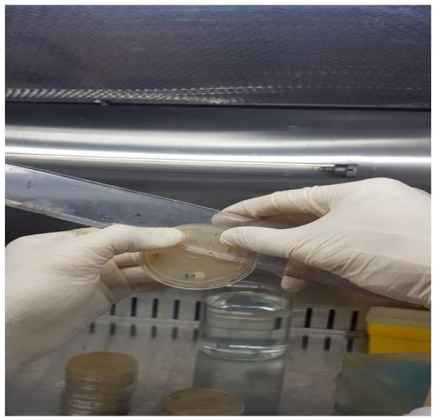

Figure 4: measurement of the inhibitory zone

Discussion

The results of the diameter of the inhibition zone by the disc plate method in the current study demonstrated that the hydroalcoholic extract of pomegranate peel had an antimicrobial effect on Lactobacillus bacteria and that the antibacterial effect increased with the increase of the concentration of the extract. When the MIC was investigated by the microbroth dilution method, it was concluded that the effective amount of hydroalcoholic extract of pomegranate peel to prevent bacterial growth is in the range of 12.5 mg/ml to 25 mg/ml. Different studies have shown that various plants such as pomegranate have antibacterial properties, that pomegranate peel extract mouthwash has statistically the greatest reduction effect on mutans and that the hydroalcoholic extract of pomegranate peel and juice has the ability to fight the main caries-causing bacteria. [24,25,26,27] Another in vitro study was conducted by Umar et al. [28] in Saudi Arabia in 2016. Pomegranate peel extract at concentrations of

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600 and 300 mg/ml showed a significant lack of growth, and their result suggested that pomegranate extract mouthwash significantly reduced the number of S.mutans bacteria. The pomegranate is one of the oldest known fruits that can be used to treat mouth ulcers and fight caries-causing bacteria such as Lactobacillus due to its compounds such as phenol, tannin, and strong antioxidant properties. [29]. The antimicrobial property of pomegranate peel extract is due to the presence of 63 heavy phenolic molecules, ellagic acid tannin, proanthocyanidin, flavonoid, gallic acid and ellagic acid, and punicalagin A and B. [30]

Studies have shown that extraction with ethanol (65-85%) results in tannins, polyphenols and procyanidins remaining stable and effective against Gram-positive and Gram-negative bacteria and fungi; therefore, water-ethanol solvent (30-70%) was used in the current study. [31] Moreover, ethanolic pomegranate peel extract contains large amounts of phenolic compounds and flavonoids compared to acetone, methanolic and aqueous extracts. Consequently, ethanol is a good solvent for the extraction of polyphenols, and the proanthocyanin content of ethanol and acetone extracts is higher than that of methanol. [32] As already mentioned, the availability of pomegranates and import restrictions were one of the motivations for conducting the present study. Moreover, pomegranate mouthwash has the potential to inhibit the development of periodontopathogenic bacteria as an alternative therapy for halitosis through several mechanisms. The researchers hope that in the near future, pomegranate mouthwash can be produced and used by dentists and the public to treat or reduce bad breath with minimal side effects. [33]

The advantages of conducting this study include the use of two techniques and criteria to determine the antibacterial effect of the hydroalcoholic extract of pomegranate peel, the control of the test conditions, the accuracy of the techniques used, and the competence of the experimenters. In addition, the Lactobacillus acidophilus species used is similar to the species found in the mouth and involved in caries, so it is possible to generalize the results to the oral cavity. The following limitations can be mentioned for the ongoing study: Coordination with the pharmacognosy group to prepare the extract and the microbiology group to perform the tests was very time-consuming, mainly due to corona conditions, and conducting the study required the preparation of various and expensive equipment. Different ways of dissolving the extract so that the solvent itself had no antimicrobial effect were considered and tested in the present study. The production of the desired bacterial strain of Lactobacillus acidophilus was not readily possible.

Conclusion

According to the results of the current study, the hydro alcoholic pomegranate peel extract has antimicrobial properties and it seems that the pomegranate fruit, which is a native fruit, can be used as an antimicrobial agent against Lactobacillus acidophilus (in various products such as mouthwash, toothpaste, candies, gels, and herbal chewing gums) and in this way the consumption of chemical compounds is reduced with more complications and costs.

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Conflicts of Interest

There is no conflict of interest to declare.

Author's Contribution

Ghazal Hassanzadehganroudsari developed the original idea and protocol, summarized the data, drafted the manuscript and edited the article. The microbiological part of the experiment was conducted under the guidance of Seyed Reza Hoseinidoost. Shadi Sarahrodi led the pharmacognostic part. The study and all its steps were supervised by Mandana Khatibi.

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