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Antimicrobial Effects of Ethanol and Acetone Extracts of *Plantago major* Seed on *Streptococcus mutans*

Sadighe Mozafar [1], Elham Majidi [2], Ronak Bakhtiari [3], Mahdi Norouzi [4], Somayeh Kameli [1]

[1] Department of Pediatric Dentistry, Faculty of Dentistry, Shahed University, Tehran, Iran
[2] Department of Restorative Dentistry, Zahedan University of Medical Sciences, Zahedan, Iran
[3] Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[4] Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

Corresponding author:
Somayeh Kameli, Department of Pediatric Dentistry, Faculty of Dentistry, Shahed University, Tehran, Iran

so.kameli@yahoo.com

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Abstract

Background and Aim: This study aimed to compare the effects of acetone and ethanolic extracts of *Plantago major* (*P. major*) on *S. mutans*.

Materials and Methods: In this *in vitro* study, the antibacterial effects of eight different concentrations of the acetone and ethanolic extracts of *P. major* seed were examined on *S. mutans*, and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts and the inhibition zone diameter were assessed.

Results: The acetone extract of *P. major* seed showed a superior antibacterial effect compared with its ethanolic extract, demonstrating antibacterial effect on *S. mutans* at 1 mg/mL concentration by the cup plate method and a mean growth inhibition zone diameter of 6±0.2 mm. the MIC and MBC of this extract were 0.5 mg/mL and 1 mg/mL, respectively. The ethanolic extract of *P. major* seed had an antibacterial effect on *S. mutans* with a concentration of 0.5 mg/mL by the cup plate method with a mean inhibition zone diameter of 8.3±0.1 mm. The MIC and MBC of this extract were 2 mg/mL and 8 mg/mL, respectively. An increase in the diameter of inhibition zone was seen at higher concentrations of both extracts. The mean diameter of growth inhibition zone of chlorhexidine (CHX) was 24 mm.

Conclusion: The present results demonstrated the antibacterial activity of ethanolic and acetone extracts of *P. major* seed on *S. mutans*. The acetone extract was more effective than ethanol extract on *S. mutans*.

Key Words: Anti-Bacterial Agents; Ethanol; Acetone; *Plantago major; Streptococcus mutans*

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Introduction

Dental caries is a highly common microbial infection in humans, which imposes a high burden on the healthcare systems worldwide, especially in developing countries [1]. *Streptococcus mutans* (*S. mutans*) and *Lactobacillus* are the most common bacteria that cause dental caries. Therefore, reducing the number of these bacteria in the oral cavity can help prevent dental caries [2]. Chlorhexidine (CHX) mouthwash is an antimicrobial agent that affects a wide range of bacteria, including *S. mutans*, but its application for caries prevention has always been

controversial due to its adverse effects such as tooth and tongue discoloration, and bacterial resistance [3,4]. Thus, use of alternative antimicrobial agents such as medicinal plant extracts may be useful [4, 5]. Recently, there has been a growing interest in human societies in use of herbal medications. Low cost and fewer side effects are among the most important characteristics of medicinal plants [4, 6].

Plantago major (*P. major*) is a plant that grows in many parts of the world, including Iran, and has long been used to treat various diseases [6, 7]. Yet, research is ongoing to investigate the antimicrobial and antioxidant effects of *P. major*. However; no studies were found on the effect of this herbal extract on the oral flora and *S. mutans*.

Considering the results of studies [6, 7] indicating the antimicrobial effect of *P. major* extract on various bacteria, including Gram-positive bacteria, the present study aimed to investigate the *in vitro* antimicrobial effects of ethanol and acetone extracts of *P. major* seed extract on *S. mutans*.

Materials and Methods

During different stages of the experiment, we ensured the sterility of the working environment, the microbial culture medium, and all the materials and equipment. The present study was ethically approved by the Research Council, Dental Faculty of Shahed University.

Crude propolis sample was collected during the honey harvesting season in the Spring of 2019 from Taleghan in Alborz province (36.17307N,50.76946E) by common scraping of the frames of Apis mellifera beehives. The *P. major* seeds were collected from the National Botanical Garden of Iran (1659).

Preparation of extracts:

The extract of the plant was obtained by the maceration technique. For this purpose, 40 g of seed powder was transferred to a Falcon tube.

The tubes were capped with a cotton pellet to prevent evaporation of the solvent. Then, 350 mL of ethanol and acetone (Merck Co. Inc., Germany) were added to the ethanol and acetone samples, respectively. The solvent remained in contact with the plant for 24 hours. The solvent was added several times to the solution containing the plant from one end and exited from the other end in order to fully obtain the herbal extract. The obtained extract was placed in a rotary evaporator to evaporate the solvent. The extracts were then placed under a biological hood to allow the remaining solvent to evaporate and to ensure that the antimicrobial effects are only due to the compounds present in the extract itself. Finally, the extracts were stored in capped sterile containers away from direct light in a refrigerator [7, 8].

Inoculation of the culture media with the reference strain:

Pure culture of *S. mutans* in nutrient broth was obtained from the Iranian National Center for Genetic and Biologic Resources. Lyophilized *S. mutans* was added to trypticase soy broth and incubated at 37°C for 24 hours. Then, *S. mutans* suspension was transferred to a blood agar culture medium. One well-isolated colony of this strain was selected from the agar plate and aseptically transferred to 4 mL of sterile nutrient broth medium.

For antimicrobial susceptibility testing, the turbidity of bacterial suspension must be adjusted equivalent to 0.5 McFarland standard concentration. A 0.5 McFarland standard concentration equals a bacterial suspension containing 1.5×10^8 colony forming units (CFUs)/mL [9].

Assessment of the antimicrobial effects of ethanol and acetone extracts of P. major:

A 96-well plate was used to evaluate the antibacterial activity of the extracts [10, 11]. For this purpose, plates containing blood agar medium impregnated with the desired microorganisms were used. Using a sterile

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punch device, wells with a diameter of 6±0.1 mm were created in the blood agar culture medium. Next, 100 µL of the extract (10% dilution) was poured into each well. The media were incubated for 24 hours and then the diameter of the zones formed around each well was measured. Ethanol and acetone were used as the negative control and 2% CHX was used as the positive control.

Determination of minimum inhibitory concentration (MIC):

The MIC of P. *major* against *S. mutans* was determined by the microdilution method using a 96-well microplate. P. *major* seed extracts were used with 125, 250, 500, 1000, 2000, 4000, and 8000 µg/mL concentrations [12]. In the first row, 100 µL of various dilutions of the extract was poured into each microplate well.

Initially, all wells were supplemented with 100 µL of Mueller Hinton broth, except one, which was supplemented with 200 µL of Mueller Hinton broth and used for sterility control. Then, 100 µL of bacterial suspension was added to each well. The suspension concentration was such that each well contained 1.5×10^5 CFUs/mL of the bacteria. The same process was repeated for other extracts in separate rows.

The positive control well was inoculated with the bacterial suspension only, while the negative control well was left blank without inoculation. The microdilution plate was incubated at 35±2°C for 18-24 hours [13].

The microplate was capped and incubated at 37°C for 24 hours. After 24 hours, the microplate was removed and all wells were carefully examined. If the bacteria grow in presence of the extract, the well becomes turbid. The bacteria begin to deposit slowly, forming a white deposit at the bottom of the well. The well examination started with the one with the lowest concentration of the extract, moving towards the well with the highest concentration. The wells were examined by observation. The first well without turbidity was considered as the MIC of the extract [14, 15].

Determination of minimum bactericidal concentration (MBC) of the extracts:

In order to determine the MBC, the MIC well and two higher concentrations were considered (obviously, there was no turbidity in these two wells either). A total of 100 µL of the contents of each well was separately collected, cultured on blood agar, and incubated at 37°C for 24 hours. The lowest concentration of extract that eliminated all the bacteria was considered as the MBC [13].

Data were collected from antimicrobial tests including agar well diffusion test and MIC.

The experiments were repeated six times to ensure accuracy of the results.

Statistical analysis:

Data were analyzed by the Kruskal-Wallis test due to their non-normal distribution. The level of significance was set at $P \leq 0.05$. Multiple comparisons were carried out by the Mann–Whitney test. Bonferroni adjustment was done to control type 1 error at 0.05 level.

Results

Results of agar well diffusion test:

The acetone extract at 1 to 8 mg/mL concentrations and the ethanol extract at 0.5 to 8 mg/mL concentrations had antimicrobial effects on *S. mutans*. The minimum and maximum diameters of growth inhibition zones caused by different extracts are presented in Table 1.

As shown in Table 1, no growth inhibition zone was observed around 0.125, 0.25 and 0.5 mg/mL concentrations of the acetone extract. This extract was effective on *S. mutans* from 1 mg/mL concentration with a mean growth inhibition zone diameter of 6 mm, to 8 mg/mL concentration with a mean growth inhibition zone diameter of 17.33 mm.

The ethanolic extract was also effective on *S. mutans* from 0.5 mg/mL concentration with a mean growth inhibition zone diameter of 8.33 mm, to 8 mg/mL concentration with a mean growth inhibition zone diameter of 14 mm.

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Table 1. Growth inhibition zone diameter around certain concentrations of ethanol and acetone extracts

Type of material	Concentration (mg/mL)	Minimum zone diameter (mm)	Maximum zone diameter (mm)	Mean zone diameter (mm)
Acetone extract	0.125	0	0	0
	0.25	0	0	0
	0.5	0	0	0
	1	5±0.1	7±0.1	6±0.1
	2	9±0.3	10±0.1	9.1667±0.3
	4	10±0.1	11±0.1	10.1667±0.2
	8	17±0.1	18±0.1	17.3333±0.1
Ethanol extract	0.125	0	0	0
	0.25	0	0	0
	0.5	7±0.2	9±0.1	8.3333±0.2
	1	9±0.1	10±0.2	9.8333±0.1
	2	11±0.2	12±0.2	11.6666±0.1
	4	12±0.1	13±0.2	12.8333±0.2
	8	14±0.2	14±0.3	14±0.2
Chlorhexidine	All concentrations	23±0.1	24±0.1	23.8333±0.1

The growth inhibition zone diameter of CHX was similar in all replicates (23-24 mm).

The growth inhibition zone diameter around the 8 mg/mL concentration of acetone extract was significantly different from that around 4, 2, 1, 0.5, 0.25 and 0.125 mg/mL concentrations of the acetone extract and all concentrations of the ethanolic extract. Altogether, CHX was significantly more effective than the ethanolic and acetone extracts. In the present study, first CHX and then 8 mg/mL concentration of the acetone extract were more effective than all other extracts.

Comparison of different concentrations of acetone and ethanol extracts by a post-hoc test is presented in Table 2. As shown in Table 2, after CHX, the 8 mg/mL concentration of the acetone extract caused the largest growth inhibition zone diameter, followed by ethanolic extract at 8 mg/mL concentration, acetone extract at 4 mg/mL concentration, and acetone extract at 2 mg/mL concentration. Ethanol extract at 4 mg/mL and acetone extract at 2 mg/mL concentration had similar effects and ranked next, followed by 2 and 1 mg/mL concentrations of acetone extract and 0.5 mg/ml concentration of ethanol extract. The remaining concentrations were not effective.

Minimum inhibitory concentration (*MIC):*

-Acetone extract: Microplate examination revealed bacterial growth in wells containing 0.125 and 0.25 mg/mL concentrations,
indicating that the extract did not have any
inhibitory effect in these concentrations. No bacterial growth was observed at 0.5 mg/mL concentration; hence, the concentration of 0.5 mg/mL was considered as the MIC of this extract.

-Ethanol extract: Bacterial growth was noted in wells containing 0.125, 0.25, 0.5 and 1 mg/mL concentrations; hence, 2000 µg/mL concentration was considered as the MIC.

-*CHX:* No growth was observed in any of the wells.

Minimum bactericidal concentration (MBC):

The contents of wells #4, 5 and 6 containing 0.5, 1 and 2 mg/mL concentrations of the acetone extract were cultured. There was no colony growth in well #4 and, accordingly, 2 mg/mL concentration of the extract was considered as the MBC.

In order to determine the MBC of the ethanolic

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extract, the contents of wells #4, 3 and 2 with 2, 4 and 8 mg/µL concentrations, respectively were cultured and no bacterial growth was observed at 8 mg/µL concentration. Accordingly, 8 mg/mL concentration of the ethanolic extract was considered as its MBC.

Table 2. Growth inhibition zone diameter around different concentrations of the extracts in one replicate

Extract concentration (mg/mL)	Growth inhibition zone diameter of the acetone extract (mm)	Growth inhibition zone diameter of the ethanol extract (mm)
8	17±0.1	14±0.2
4	10±0.2	13±0.3
2	9±0.1	11±0.1
1	6±0.3	10±0.2
0.5	0	8±0.1
0.25	0	0
0.125	0	0

As shown in Table 1, the MBC was the same for all replicates and equal to 2 mg/mL for the acetone extract, and 8 mg/mL for the ethanolic extract.

Discussion

In ethnopharmacological research, antimicrobial susceptibility tests are carried out to determine how effective potential antimicrobial agents from biological extracts could be against different pathogenic microorganisms. Dental caries is an irreversible chronic disease initiated by *S. mutans*, a Gram-positive, facultative anaerobic microorganism. Preventing and controlling dental caries have been a great challenge for decades [16].

Many plants possess significant antimicrobial properties [6]. The therapeutic effects of plants depend on concentrations of their phytochemical and bioactive contents and the synergistic or antagonistic effects of these compounds [17].

Over the past 20 years, the antimicrobial effects of polyphenols have been approved on a wide range of bacteria. These compounds are now increasingly used for production of new medications due to the increased resistance of bacteria to common antibiotics [18].

P. major also contains polyphenol antimicrobial compounds such as flavonoids, tannins, and phenols. There are significant amounts of tannin in seeds and leaves of *P. major*, which affect various bacteria such as *S. mutans* [19].

In a study to investigate the antibacterial effect of 13 medicinal plants in Iran, Koohsari et al. found that *P. major* extract was more effective than all other plants on *Staphylococcus aureus, Salmonella typhimurium, Staphylococcus epidermidis, Shigella dysenteriae,* and *Enterococcus faecalis* [20]. Zubair [21] in their study confirmed the presence of phenolic compounds in *P. major* seeds, which can be the reason why the seed extract of *P. major* showed significant antibacterial activity in the present study. Consistent with the present findings, Sharifa et al. [22] found that the aqueous extract of *P. major* had no antibacterial properties on any of the tested bacteria (*Staphylococcus aureus, Escherichia coli*, and *Bacillus subtilis*), while its ethanolic and methanolic extracts had antibacterial properties. Karima et al. [23] studied the effect of ethyl acetate extract of *P. major* on Gram-negative and Gram-positive bacteria using the disc diffusion method. They found that both extracts were more effective on Gram-positive bacteria than Gram-negative bacteria and the ethyl acetate extract was more effective than the aqueous extract.

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Metiner et al. [11] compared the effects of ethanol and acetone extracts of *P. major* on different bacterial species except *S. mutans* and found that the ethanol extract was effective on all bacterial species, while the acetone extract was only effective on *Bacillus cereus* and *Escherichia coli*.

It has been shown that the solvent type can have a great effect on the final amount of polyphenol compounds and the antimicrobial and antioxidant effects of the plant extract. The type of solvent affects the solubility of the phenolic compounds and the final amount of phenol, flavonoid and tannin. According to a study by Tatiya et al., [24] acetone is the best solvent to maintain the polyphenols.

Considering the effectiveness of organic solvents as confirmed by the above-mentioned study [24], we decided to use ethanol and acetone as solvents in the present study. The results showed that both ethanol and acetone extracts were effective on *S. mutans*, but the acetone extract was more effective with a lower MIC and MBC. Additionally, it was found that higher concentrations of *P. major* extracts had greater antimicrobial effects.

Further studies are recommended to investigate the effect of ethanol and acetone extracts of other parts of P. *major* plant, such as its flowers and leaves, and also assess the effect of these extracts on *S. mutans* clinical isolates.

Conclusion

Ethanol and acetone extracts of *P. major* showed antibacterial properties against *S. mutans*. The effect of *P. major* acetone extract was significantly higher than that of its ethanol extract. However, the studied extracts had lower antibacterial effect on *S. mutans* than 2% CHX.

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