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Antimicrobial effect of hydro-alcoholic extract of apple with and without zinc oxide nanoparticles on *Streptococcus Mutans*

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Abstract

This study aimed to evaluate the antimicrobial effect of hydro-alcoholic extract of apple (*Malus domestica* Borkh. Vs.*golab* (with and without ZnO nanoparticles) on *Streptococcus Mutans* bacterium compared to 0.2% Chlorhexidine, Persica and suspension of ZnO nanoparticles. Study samples were examined in the groups of apple hydro-alcoholic extract with and without addition of ZnO nanoparticles, a positive control group (Chlorhexidine 0.2%, Persica and suspension of ZnO nanoparticles), and a negative control group (distilled water). In this experiment, a concentration of 500 PPM of ZnO nanoparticles with a diameter of 0.4 nm was used. Agar diffusion method was used to determine the Minimum Inhibitory Concentration (MIC) of apple hydro-alcoholic extract with and without adding ZnO nanoparticles. The concentrations used were 200, 100, 50 and 25 mg/ml. ANOVA statistical test was used to compare the average in the study groups. According to our results, hydro-alcoholic extract of apples alone had no effect on the target bacteria in any of the concentrations. In the group of apple hydro-alcoholic extract with ZnO nanoparticles, the mean inhibition zone was 13 mm at a concentration of 25 mg/ml. 0.2% Chlorhexidine, Persica and suspension of ZnO nanoparticles was observed with the mean inhibition zone of 20 mm, 16 mm and 15 mm, respectively. Hydro-alcoholic extract of apple with addition of ZnO nanoparticles in concentration of 25mg/ml, had growth inhibitory effect on *Streptococcus Mutans*, but it was not remarkably efficient in comparison with Chlorhexidine.

Key Words: tooth decay; *Streptococcus Mutans*; hydro-alcoholic extract, ZnO nanoparticles, Chlorhexidine.

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Nowadays, tooth decay is the most common chronic bacterial infection in humans.[1-3] In Iran, the overall prevalence of tooth decay in children is 78.6%,[4] the average in adults is 12.5±13.5.[5] The costs of treatment caused by dental decay are very high because it mostly includes the treatment of symptomatic patients and less includes preventive works.[3,6] Different approaches are used to control caries, one of the methods of caries control is using anti-microbial in people with a high risk of decay.[7] Antimicrobial toothpastes, gels and mouthwashes available in the market, despite being useful, may have side effects.[8] Therefore, there is a need to replace chemical substances with safe, effective and affordable natural products to prevent tooth decay. Considering the antimicrobial substances in their structure, plants can be considered as an alternative to chemicals because in many cases they are cheaper compared to chemical drugs and have less side effects.[9] Meanwhile, due to having phenolic compounds (the second most abundant metabolite in plants), apples can have anti-microbial, anti-viral, anti-bacterial and anti-fungal effects.[7,10] Gram-positive and Gram-negative bacteria are sensitive to phenolic compounds. Phenols in high concentration can cause rupture of cell membrane and coagulation of bacterial cell proteins, and in low concentration they can disable the basic enzymes of bacterial cells.[7] In addition, the enzymes in apples prevent the binding of mutans streptococci bacteria to the tooth surface.[1,7] The reason for the antibacterial

properties of apples is due to the presence of three phenolic compounds called quercetin, epicatechin and procyanidin, which work by disabling the protein components of the bacterial cell, and this causes apple become a strong antibiotic. Mineral nanoparticles, which have a special structure in terms of physical, chemical, biological and behavioral characteristics, have received a lot of attention. Nanomaterials, including heavy metal oxides, have a high tendency to react with biological molecules and cause them to become inactive and finally destroy viruses or bacteria. It is generally believed that nanomaterials release ions that react with proteins on the surface of bacterial cells. ZnO nanoparticles destroy the lipids and proteins of the bacterial cell membrane, and as a result, cause the cell membrane to rupture and eventually kill the bacteria. In addition, ZnO is known to be effective for *S. aureus*.[11] Mir Hosseini et al.'s study showed that the bactericidal activity of ZnO nanoparticles depends on the temperature of nano concentration and the type of microorganism, so that ZnO nanoparticles were more effective at a temperature of 25 degrees and Bacillus cereus bacteria showed more resistance.[12] According to the research done by Barreca et al., there is phloretin and its derivatives in apples and apple tree leaves, which can prevent the growth of Gram positive bacteria, especially against Staphylococcus aureus, Listeria monocytogenes and methicillin-resistant strains of Staphylococcus aureus. In addition, Phloretin also works against Gram-negative bacteria such as *Salmonella Typhimurium*.[13] In Lou et al.'s study, the antibacterial effects of apple skin and fruit phenols were compared to penicillin. MIC of penicillin against *E.coli*, *S. auerus* and *L. monocytogenes* was equivalent to 1.25 mg/ml or even less, but the polyphenols in apple fruit showed weaker results than penicillin.[14] Zhou et al. showed that apple-derived phloretin at a concentration of 16 µg/ml can protect some cells against the damage of *Staphylococcus aureus* USA300 in the culture medium.[15] Considering the role of *Streptococcus mutans* as the main microorganism in the initiation of the tooth decay process and recommending preventive guidelines in the use of antimicrobial substances in people with high caries risk, including children, the approach of controlling *Streptococcus mutans* in saliva and dental plaque in people with high caries risk can be considered as an effective preventive approach in caries control.[16] On the other hand, the use of natural substances instead of chemicals is more accepted among the consumers of products related to oral health. For this reason, in the present study, the hydro-alcoholic extract of native Khorasan apple with and without the addition of ZnO nanoparticles was evaluated to inhibit the growth of *Streptococcus mutans* bacterium.

Materials and Methods

Rose apple native to Khorasan Razavi with the scientific name *Malus domestica* Borkh. Vs.*golab*, approved by Ferdowsi University of Mashhad Herbarium (Voucher sp. No.: FUMH – E1011), was selected for the study. In this research, all parts of the apple were used except the seeds. The apple was washed, cut into pieces and dried. Then the dried powder was poured into a funnel made of filter paper and placed into the tank of the Soxile machine. The extract extractor was placed on a flask containing a solvent of ethanol and water in a ratio of 3:1. Then, it was concentrated by removing the solvent in a vacuum at a temperature of 40 degrees Celsius. The concentrated extract was kept in a cool environment away from light until use. 2 g of the extract was dissolved in 10 ml of distilled water and diluted to prepare 4 serial dilutions from 200 mg/ml to 25 mg/ml, respectively. Study extract samples without nanoparticles were allowed to dissolve completely in a shaker incubator for 24 hours. After this period, it was sterilized by Whatman 1 strainer and divided into sterile laboratory tubes for testing and kept at room temperature. A fresh solution of this substance was used for each experiment. Considering the high concentration of 3g and 4g, finally, the initial concentration of 2g in 10 ml of water was selected as 200 mg/ml. It was prepared from the initial concentration of 200 mg/ml in the form of serial dilution up to 9 tube dilutions, ie 780 µg/ml. In this way, initially, 9 tubes with a volume of 1.5 ml (Eppendroff, USA) were selected. In 8 tubes, 500 µl of sterile distilled water was added. In the first tube (tube zero), 1 ml (1000 µl) of the 200 mg/ml stock tube was poured pure from the initial stock. After that, 500 µl was taken from the stock tube and mixed well with 500 µl of sterile water in tube number one. This was repeated until the ninth tube, and the volume taken from the last tube was discarded. According to the initial tests, none of the eight concentrations had any effect on the target bacteria, so four initial dilutions, namely 200, 100, 50 and 25 mg/ml, were selected and tested. To prepare samples containing nanoparticles, the colloidal solution of ZnO nanoparticles with a diameter of 0.4 nanometers with a concentration of 500 ppm was added to 4 dilutions of apple extract, i.e. 200, 100, 50 and 25 mg/ml. In all stages of experiments, the concentration of ZnO nanoparticles was considered constant. In order to distribute the nanoparticles uniformly in the suspension, the samples containing nanoparticles were placed on the sonicator probe before consumption. By using the sonicator probe, nanoparticles do not precipitate anymore. 0.2% Chlorhexidine, persica and ZnO nanoparticles suspension were used as positive control[17] and distilled water as negative control. After preparing serial dilutions of apple hydro-alcoholic extract, 0.5 ml of 500 ppm concentration of nanoparticles were mixed with 0.5 ml of apple hydro-alcoholic extract in 1.5 ml tubes and placed in a shaker incubator to prevent of precipitation and keep nanoparticles suspended during the tests. Agar diffusion method was used to investigate the antibacterial properties of apple hydro-alcoholic extract. In this method, Muller Hinton's culture medium

Fig 1. *Müller Hinton cultivation environment*

(Hesarak Karaj Institute) was used.[18] At first, 100 microliters of studied bacteria were harvested from McFarland's turbidity and cultured on agar surface under sterile conditions using a glass hook steak. Using the end of a sterile 5 mm tube, dimples were created on the culture medium as many dilutions as studied (Figure 1). From the serial dilutions prepared from the apple hydro-alcoholic extract, only 100 µl volume was poured into the dimples. These experiments were performed three times, and in each time, sterile distilled water was used as a negative control and 0.2% Chlorhexidine mouthwash, Persica and ZnO nanoparticles suspension were used as a positive control. The plates were incubated for 48 hours in an anaerobic jar containing Co2 using gas pack and 24 hours in an incubator under aerobic conditions at a temperature of 35°C. Also, in order to investigate the antibacterial properties of the combination of ZnO nanoparticle solution and apple hydro-alcoholic extract, all the above experiments were performed exactly. The inhibition zone created by the The Clinical & Laboratory Standards Institute (CLSI) standard ruler were measured and recorded in millimeter units. The number obtained was the MIC of apple hydro-alcoholic extract alone and apple hydro-alcoholic extract with ZnO nanoparticles. Data analysis was done using one-factor analysis of variance. The level of significance in statistical tests was considered equal to 5%. Considering that in laboratory studies, the number of repetitions is usually desired and considering that we do not expect to see many changes in similar laboratory conditions, therefore three repetitions were included for the experiment. Due to the fact that two types of extracts (apple hydro-alcoholic extract without ZnO nanoparticles and apple hydro-alcoholic extract with ZnO nanoparticles) were investigated in four different dilutions, therefore the number of experiments was 24 times.

Results

Antibacterial properties of apple hydro-alcoholic extract (*Malus domestica* Borkh. Vs.*golab*) were investigated on

Table 1. *Diameter of inhibition zone of apple hydro-alcoholic extract (Malus domestica) with the addition of ZnO nanoparticles by concentration on Streptococcus mutans.*

Diameter of the inhibition zone / Concentrations (mg/ml)	Mean	Highest	SD	P value
200	10/3	11/0	0/58	0.004
100	11/0	12/0	1/0	
50	12/3	13/0	0/58	
25	13/0	13/1	1/0	

Table 2. *Comparison of the mean diameter of inhibition zone of apple hydro-alcoholic extract (Malus domestica) with the addition of ZnO nanoparticles on Streptococcus mutans with the positive control group of Chlorhexidine 0.2%.*

~~Diameter~~ of the inhibition zone / Concentrations (mg/ml)	Mean	SD	p value
200	10/3	0/58	0.001
100	11/0	1/0	0.004
50	12/3	0/58	0.002
25	13/0	1/0	<0.001

serial dilutions of apple hydro-alcoholic extract without ZnO nanoparticles and apple hydro-alcoholic extract with ZnO nanoparticles. The positive control groups included 0.2% Chlorhexidine, Persica and ZnO nanoparticles suspension, and distilled water was considered as a negative control. The bacteria used was *Streptococcus mutans* ATCC 10682. In this study, the samples were examined in terms of the diameter of the inhibition zone in terms of two variables, group and concentration. First, using two-factor analysis of variance, it was determined that both factors have an effect on each other ($p<0.001$), so each factor was investigated separately. In the comparison of concentration by study groups, apple hydro-alcoholic extract group alone did not show antimicrobial effect on *Streptococcus mutans* in any of the studied concentrations from 200 to 25 mg/ml. The group of apple hydro-alcoholic extract (*Malus domestica*) containing nanoparticles of ZnO in all the studied concentrations showed the inhibition zone of *Streptococcus mutans*, the diameter of the inhibition zone by concentration is shown in Table 1.

The comparison of different concentrations of apple hydro-alcoholic extract (*Malus domestica*) with ZnO nanoparticles with the control group of 0.2% Chlorhexidine (diameter of inhibition zone was 20 mm) is shown in Table 2. In all studied concentrations, from 200 to 25 mg/ml, the mean diameter of inhibition zone of apple hydro-alcoholic extract with the addition of ZnO nanoparticles was significantly less than that of Chlorhexidine. One-sample t-test was used to compare two groups.

Comparison of different concentrations of apple hydro-alcoholic extract (*Malus domestica*) with ZnO nanoparticles with the positive control group of ZnO nanoparticles suspension is presented in Table 3. In Table 3, the comparison of different concentrations of apple hydro-alcoholic extract (*Malus domestica*) with ZnO nanoparticles in the positive control group of Persica with the diameter of the inhibition zone of 16 mm can be seen using the one-sample t-test to compare the two groups. In each of the concentrations of 200, 100, 50 and 25 mg/ml, the average diameter of the inhibition zone of apple hydro-alcoholic extract with the addition of ZnO nanoparticles was significantly lower than that of Persica.

Comparison of different concentrations of apple hydro-alcoholic extract (*Malus domestica*) with ZnO nanoparticles with the positive control group of ZnO nanoparticles suspension is presented in Table 4. In Table 4, the comparison of different concentrations of apple hydro-alcoholic extract (*Malus domestica*) with ZnO nanoparticles with the positive control group of suspension of ZnO nanoparticles alone with a inhibition zone of 15 mm was used using one sample t test. In each of the concentrations of 200, 100, 50 and 25 mg/ml, the average diameter of the inhibition zone of apple hydro-alcoholic extract with the addition of ZnO nanoparticles was significantly lower than that of the ZnO nanoparticles suspension. According to the diameter of inhibition zone of apple hydro-alcoholic extract with ZnO nanoparticles, which was equal to 13.1 mm in the

Table 3. *Comparison of the results of the antimicrobial effect of the apple hydro-alcoholic extract (Malus domestica) with the addition of ZnO nanoparticles on Streptococcus mutans with the positive control group Persica.*

~~Diameter~~ of the inhibition zone / Concentrations (mg/ml)	Mean	SD	P value
200	10/3	0/58	0/003
100	11/0	1/0	0/013
50	12/3	0/58	0/002
25	13/0	1/0	<0/001

Table 4. *Comparison of the results of the antimicrobial effect of the apple hydro-alcoholic extract (Malus domestica) with the addition of ZnO nanoparticles on Streptococcus mutans with the positive control group of ZnO nanoparticles suspension alone.*

Diameter of the inhibition zone / Concentrations (mg/ml)	Mean	SD	P value
200	10/3	0/58	0/005
100	11/0	1/0	0/020
50	12/3	0/58	0/012
25	13/0	1/0	0/001

best case. It is clear that compared to the suspension of ZnO nanoparticles, these two substances did not have a synergistic effect on each other.

Discussion

The aim of this study was to find the minimum inhibitory concentration of apple hydro-alcoholic extract with and without adding ZnO nanoparticles against *Streptococcus mutans* bacterium; for this purpose, four different concentrations of the studied extracts were tested. The results showed that apple hydro-alcoholic extract alone did not inhibit the growth of bacteria at any concentration. By adding ZnO nanoparticles to apple hydro-alcoholic extract at a concentration of 25 mg/ml, the diameter of the inhibition zone was observed with an average of 13 mm. According to our results that show a relatively weak antimicrobial effect of the apple hydro-alcoholic extract with and without the addition of ZnO nanoparticles, it seems that the high amount of sugar in the apple extract and the high affinity of *Streptococcus mutans* to sugar hinder the antimicrobial effect. However, in a study by Behera et al., apple juice had a significant antimicrobial effect against gram positive *Streptococcus mutans* bacterium.[19] The difference in the results can be related to the different types of apples used in the two studies. The apple used in their study was native to India, and in the current study, apple (*Malus domestica* Borkh. Vs.*golab* native to Khorasan was used). Also, this difference can be justified by the different strains of *Streptococcus mutans* bacteria used. Behera et al. investigated the complete extract of the desired fruit juice without adding solvent.[19] In another study, Sunilson et al.[20] investigated the antibacterial effect of Malaysian Fuji apple during a study. The tested bacteria were *streptococcus mutans* and other bacteria that cause tooth decay. According to their research, apple extract in petroleum ether solvent had no antibacterial effect against *Streptococcus mutans*, but in ethanol solvent, the average diameter of inhibition zone was 16.7 and in water solvent it was 15.7 mm.[20] The difference between the results in the current study and Sunilson's study can be related to the different types of apples used. Also, the solvent in the present study was a combination of water and ethanol. The bacteria used in our research was a specific strain obtained from the Iranian Biological Reserve Center and was not isolated from patients, so it is likely that the strains used in these two studies were different.

In the study of Tahmourespour et al.,[7] apple extract reduced the number of *Streptococcus mutans* colonies in saliva by 54.2%, but the decrease in the average number of colonies in the Chlorhexidine group was significantly higher (92.8%) than the apple extract group.[7] In the present study, the apple hydro-alcoholic extract alone had no antibacterial effect on *Streptococcus mutans* and this effect was slightly improved by adding nanoparticles. This effect was probably related to ZnO nanoparticles. In Pires et al.'s study, apple hydro-methanolic extract had antibacterial effect against gram positive bacteria.[21] In our study, apple hydro-ethanolic extract was used, which had no effect against *Streptococcus mutans* alone. This difference in results can be related to the type of apple used and the apple extraction method. In the method of Pires et al., their solvent contained 80% methanol and 20% water, while in the present study, the solvent was ethanol and water in a ratio of 70:30. The higher amount of alcohol probably plays a role in destroying more microorganisms.

Also, Pires et al.[21] conducted a study on bacteria isolated from patients, while we used standard *Streptococcus mutans* with American Type Culture Collection Identity Document 10682 (ATCC ID). In the present study, it was shown that the apple hydro-alcoholic extract along with ZnO nanoparticles is effective against *Streptococcus mutans* bacterium, although this effect was less than the ZnO nanoparticles suspension alone. ZnO nanoparticles have special properties that can be used in different ways, such as breaking apart the outer membrane of bacteria and destroying enzymes inside the cell, as well as its special oxidative properties, as well as increasing the contact surface of ZnO with bacteria.[22] But in the current study, adding these particles to the apple hydro-alcoholic extract has reduced the effect of nanoparticles; the cause of this problem may be related to the high content of sugar in the apple fruit used in this study.

It is possible that the amount of total phenol as an effective antimicrobial substance is different in different apple species, and this may be one of the reasons for the difference in the results of different studies.

There were limitations in this study which are similar to the study of Kabir et al.[23] extraction at the boiling point of the solvent can decompose heat-sensitive compounds that can be important for the study. Also, for the efficient extraction of Soxhle, the sample must be in powder form, which cannot be done well in the case of apple, and due to the sticky nature of the fruit, it causes it to stick together even after much drying.

In conclusion, according to our results, the effect of apple hydro-alcoholic extract with the addition of ZnO nanoparticles on the growth of *Streptococcus mutans* bacterium was determined. The important point of this evaluation is the effectiveness of apple hydro-alcoholic extract with the addition of ZnO nanoparticles in growth inhibitory of *Streptococcus mutans*, but compared to 0.2% Chlorhexidine, Persica and ZnO nanoparticles suspension, it was significantly less effective.

List of acronyms

ATCC ID - American Type Culture Collection Identity Document
CLSI - The Clinical & Laboratory Standards Institute
MIC - Minimum Inhibitory Concentration
ZnO - Zinc Oxide

Contributions of Authors

This study was designed by TM and MM. SH prepared the hydro-alcoholic extracts of apple. MA performed the antimicrobial tests. FF collected and analyzed the data and wrote the manuscript. TM and MM reviewed the data, supervised the process and revised the manuscript. All authors read and approved the final edited manuscript.

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Conflict of Interest

The authors declare they have no financial, personal, or other conflicts of interest.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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