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Antimicrobial effect of Viola odorata extract with or without zinc oxide nanoparticles on *Streptococcus mutans*

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Abstract

Objective: This study aimed to evaluate the antimicrobial efficacy of Viola odorata extract, with or without the addition of zinc oxide nanoparticles (ZnO NPs), against *Streptococcus mutans*.

Methods: Two series of V. odorata hydroalcoholic extracts were prepared at concentrations of 25, 50, 100, and 200 mg/ml. Five hundred ppm ZnO NPs were added to 500 ml of the V. odorata extracts in half of the samples. The antibacterial activity of the extracts was then tested using the agar well diffusion method against *S. mutans,* and the inhibition zones were determined. The control groups were 0.2% chlorhexidine and Salvadora persica mouthwashes. Statistical analysis was performed using an independent-sample t-test, one-way ANOVA, and Duncan's post-hoc test (α=0.05).

Results: Adding ZnO NPs significantly improved the inhibition zone of V. odorata extract at all concentrations ($P < 0.05$). The largest inhibition zone was observed in the 0.2% chlorhexidine mouthwash, significantly greater than all other groups ($P < 0.001$). The inhibition diameter for S. persica mouthwash was significantly greater than that of all the V. odorata extracts ($P < 0.001$), except for the group containing 200 mg/ml extract with ZnO NPs ($P > 0.05$).

Conclusions: Adding 500 ppm ZnO NPs enhanced the antibacterial activity of the V. odorata extract. The antibacterial effect of the 200 mg/ml V. odorata extract combined with ZnO NPs was comparable to that of S. persica mouthwash *against S. mutans*. Given the anti-inflammatory and antibacterial properties of the V. odorata extract, this formulated mouthwash shows potential for improving patients' oral health.

Keywords: Tooth decay, *Streptococcus mutans*, Herbal extract, Zinc oxide, Nanoparticles, Viola odorata

Introduction

The primary bacteria responsible for the development and progression of dental caries are *Streptococcus mutans* and *Lactobacillus acidophilus*. These bacteria metabolize simple carbohydrates like sucrose and produce organic acids such as lactic acid, which lower the pH of dental plaque and thus lead to enamel demineralization (1-3). Dental caries is a dynamic process governed by cycles of demineralization and remineralization (4). Remineralizing agents such as hydroxyapatite, flourohydroxy apatite, fluoride, bioactive glass, and calcium-phosphate products could reverse or stop the dental caries process (5-7). Decreasing cariogenic biofilm formation using antimicrobial mouthwashes like chlorhexidine (CHX) is a preventive option (8). CHX is considered the gold standard among antibacterial mouthwashes. However, it has some drawbacks, including tooth discoloration, mucosal irritation, and an unpleasant bitter taste (9).

Herbal extracts have gained attention as potential alternatives to CHX, offering antibacterial and anti-cariogenic benefits without the associated side effects.

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S. persica, commonly known as Miswak, is a native plant in the Middle East, parts of Asia, and Africa. It has both medicinal and preventive properties, particularly for periodontal diseases. Research suggests that the mouthwash containing S. persica extract reduces plaque and gingival inflammation, although it is slightly less effective than the CHX mouthwash (10). Therefore, the search for herbal medicine-based mouthwashes continues.

Viola odorata, a medicinal plant traditionally used in various cultures (11), has been shown to possess antibacterial properties. Its petroleum ether, dichloromethane, ethyl acetate, acetone, methanol, and aqueous extracts have demonstrated efficacy against *Klebsiella pneumonia*, *Escherichia coli*, *Haemophilus influenzae*, *Staphylococcus aureus*, *S. pyogenes*, *Streptococcus pneumonia*, and *Pseudomonas aeruginosa* at varying intensities (12). Additionally, the hydroalcoholic extract of the V. odorata flowers has demonstrated antibacterial activity against *S. mutans* in a concentration-dependent manner (13). With its promising anti-inflammatory properties (14), V. odorata may be an ideal candidate for use in mouthwash formulations for treating inflammatory gingival diseases.

Zinc oxide nanoparticles (ZnO NPs) have been reported to have significant antibacterial properties in various studies against Gram-positive and Gram-negative bacteria (15, 16). ZnO NPs inhibit *S. mutans* growth and reduce plaque formation (17). They show enhanced surface activity, low toxicity, cost-effectiveness, and biocompatibility (15, 18, 19). Research suggests that incorporating ZnO NPs into dental materials such as conventional glass ionomers and composite resins improves their ability to combat cariogenic bacteria (20). Combining ZnO NPs with herbal extracts, such as apple, cinnamon, clove, and ginger, has also demonstrated synergistic antibacterial effects against *S. mutans* (21-23).

Despite the promising antibacterial properties of V. odorata extract, no studies have compared its efficacy with CHX or other herbal mouthwashes against oral bacteria. Furthermore, adding ZnO NPs could enhance the antibacterial effects of V. odorata against *S. mutans*. This *in vitro* study aimed to evaluate the impact of different concentrations of V. odorata hydroalcoholic extract with or without ZnO NPs against *S. mutans* and compare them with CHX and S. persica mouthwashes.

Materials and methods

Preparing the hydroethanolic extracts of V. odorata

Dried V. odorata flowers were obtained from the Ferdowsi University Herbarium in Mashhad, Iran (Voucher sp. number: FUMH - E1010). The flowers were washed with sterile distilled water, air-dried at room temperature, and ground into powder using a grinding machine. Five hundred grams of V. odorata powder were placed in a flask containing a solvent mixture of ethanol and water in a 1:3 v/v ratio. The flask was then placed in an oven and shaken for 72 hours. Afterward, the mixture was filtered using Whatman filter paper (Sigma-Aldrich, Missouri, United States), and the solvent was obliterated under a vacuum at 40°C to concentrate the extract. The concentrated extract was stored at -20°C until further use. Two grams of the extract were dissolved in 10 mL of distilled water and placed in a shaker incubator for 24 hours. After incubation, the solution was filtered through Whatman filter paper and stored at room temperature in a sterile laboratory.

Serial dilutions of the extract with 200, 100, 50, and 25 mg/mL concentrations were prepared. Four 1.5-mL tubes (Eppendorf, USA) were selected, and in the first one, 1 mL (1000 µL) of the stock solution with a 200 mg/mL concentration was added. Then, 500 µL of solution was aspirated from the first tube and transferred to the next tube containing 500 µL of sterile distilled water. The process was repeated for all subsequent tubes.

Preparing extracts containing ZnO nanoparticles

A 500 µL ZnO nanocolloid solution (BYK Chemie, Germany) with a particle size of 0.4 nm and a concentration of 500 ppm was added to 500 mL of V. odorata extract at concentrations of 200, 100, 50, and 25 mg/mL. The mixtures were placed in a shaker incubator at 50°C for 48 hours, with a 40-50 rpm shaking speed.

Antimicrobial activity

The *S. mutans* ATCC 10682 strain was obtained from the Iranian Biological Resources Center, Tehran, Iran. Colonies were transferred to sterile brain-heart infusion (BHI) broth and incubated anaerobically for 48 hours. Subsequently, the cultures were incubated aerobically at 35°C for 24 hours, and a bacterial suspension was prepared with turbidity adjusted to match a 1 McFarland standard.

The antibacterial activity of the hydroalcoholic extract of V. odorata, both with and without ZnO nanoparticles, was tested using the agar well diffusion method (21).

Table 1. Mean ± standard deviation (SD) of inhibition zone (mm) of the V. odorata extract with or without ZnO NPs at different concentrations

Concentration of V. odorata	Without ZnO NPs	With ZnO NPs	P-value
	Mean ± SD	Mean ± SD	
25 mg/ml	0.93 ± 0.21	12.33 ± 0.58	<0.001*
50 mg/ml	0.86 ± 0.12	13.00 ±1.00	0.001*
100 mg/ml	12.33 ± 0.58	14.00 ± 0.58	0.003*
200 mg/ml	13.67 ± 0.58	15.00 ± 0.01	0.016*

*Values less than 0.05 represent significant differences between the extracts in each concentration according to the t-test.

Approximately 100 µL of the microbial suspension was spread on the agar surface under sterile conditions. Holes were punched into the agar using a sterile 5 mm tube, and 100 µL of the hydroalcoholic extract, with or without ZnO NPs, was added to each well. The tests were repeated three times for each group. Control groups included 0.2% CHX and S. persica mouthwashes. The plates were incubated in an anaerobic jar with CO2 gas packs at 35°C for 24 hours. The diameters of the inhibition zones were measured with a standard ruler according to Clinical & Laboratory Standards Institute (CLSI) guidelines.

Statistical analysis

Data analysis was performed using SPSS 21.0 software (IBM Inc., New York, USA). The mean inhibition zones at each concentration of V. odorata extract, with or without ZnO NPs, were compared using the independent-sample t-test. The inhibition zones of V. odorata extracts, CHX, and S. persica mouthwashes were compared using ANOVA and Duncan's post-hoc test.

Results

Table 1 shows the inhibition zones for different concentrations of V. odorata extract, with or without ZnO NPs. According to the t-test, the inhibition zones of the samples with ZnO NPs were significantly larger than those of the plain extracts at all concentrations ($P < 0.05$; Table 1).

ANOVA indicated a significant difference in inhibition zones among the study groups ($P < 0.001$; Table 2). Pairwise comparisons using the Duncan post-hoc test showed that the inhibition diameter for the 0.2% CHX group (20.14 ± 0.92 mm) was significantly larger than that of all other groups ($P < 0.05$; Table 2). Additionally, the inhibition zone diameter for S. persica (16.41 ± 0.32 mm) was significantly more extensive than all groups ($P < 0.05$; Table 2), except for the 200 mg/ml extract with ZnO NPs (15.00 ± 0.00 mm).

The lowest inhibition zone was associated with 50 and 25 mg/ml V. odorata extracts ($P < 0.05$; Table 2). The inhibition zone of the 100 mg/ml was comparable to that of the 25 or 50 mg/ml V. odorata extracts with ZnO NPs ($P > 0.05$; Table 2). The inhibition zone of the 200 mg/ml extract was comparable to that of the 50 or 100 mg/ml V. odorata extracts with ZnO NPs ($P > 0.05$; Table 2).

Table 2. Mean ± standard deviation of inhibition zone (mm) of the V. odorata extract at different concentrations with or without ZnO NPs and the control groups

Group	Inhibition zone diameter (Mean ± SD)
200 mg/ml V. odorata extract	13.67 ± 0.57 [c]
100 mg/ml V. odorata extract	12.33± 0.57 [b]
50 mg/ml V. odorata extract	0.86 ± 0.12 [a]
25 mg/ml V. odorata extract	0.93 ± 0.21 [a]
200 mg/ml V. odorata extract + ZnO NPs	15.00 ± 0.00 [d]
100 mg/ml V. odorata extract + ZnO NPs	14.33 ± 0.57 [c]
50 mg/ml V. odorata extract + ZnO NPs	13.00 ± 1.00 [b, c]
25 mg/ml V. odorata extract + ZnO NPs	12.33 ± 0.57 [b]
0.2% chlorhexidine	20.14 ± 0.92 [e]
Salvadora persica	16.41 ± 0.32 [d]
P value	<0.001*

*Values less than 0.05 represent a significant difference between groups According to ANOVA.
Different lowercase letters represent a significant difference based on the Duncan's post-hoc.

Discussion

The present study evaluated the antibacterial effect of V. odorata extract with or without ZnONPs against *S. mutans.* At 200 mg/ml and 100 mg/ml concentrations, the inhibition zones of V. odorata extract were 13.67 ± 0.57 mm and 12.33 ± 0.57 mm, respectively. This finding is consistent with Tiwari et al.'s study (13), which demonstrated the antimicrobial potential of V. odorata against various microorganisms, including *S. mutans*, with a mean inhibition zone of 12 mm.

In this study, V. odorata extracts with ZnO NPs demonstrated enhanced antibacterial activity compared to those without NPs. The antibacterial effects of ZnO NPs are attributed to bacterial cell wall destruction and cell disruption (24-26). ZnO NPs are widely used in dentistry as antibacterial agents without compromising the mechanical properties of dental materials (27). Safari et al. (28) showed that ZnO NPs significantly improved the antibacterial efficacy of Plantago primary extract against *S. mutans*.

CHX and S. persica mouthwashes were used as positive controls in this study. The inhibition zone for 0.2% CHX was larger than for the other groups. CHX is the gold standard for reducing *S. mutans* counts and oral biofilm formation. At low concentrations, CHX disrupts bacterial cell wall structure, and at higher concentrations (>0.1%), it causes leakage of intracellular components, leading to bactericidal effects (29). While S. persica has been shown to reduce plaque scores and cariogenic bacterial counts, its efficacy is generally lower than CHX's (8). The inhibition zone diameter for CHX in this study was 20.14 ± 0.92, which is consistent with other studies (30, 31). The inhibition zone diameter for S. persica mouthwash was 16 mm, which agrees with previous studies using aqueous, acetone, and chloroform extracts of S. persica (32, 33).

The antibacterial activity of V. odorata extracts without ZnO NPs was lower than that of 0.2% CHX and S. persica mouthwashes. However, when ZnO NPs were added, the V. odorata extract exhibited an inhibition zone comparable to that of S. persica mouthwash. Moreover, V. odorata contains a variety of beneficial secondary metabolites, including flavonoids, tannins, alkaloids, and phenolic compounds (13). It also contains gallic acid (GA), a phenolic antioxidant with antimicrobial, anti-inflammatory, antimutagenic, and anticarcinogenic properties (34), as well as remineralizing potential (35). Additionally, V. odorata contains disulfide-rich peptides (DSRs) that have demonstrated antibacterial efficacy against various bacteria (36). It should be noted that antimicrobial mouthwashes are not recommended for children under six due to the risk of swallowing (37), making herbal mouthwashes and gels safer alternatives (38). ZnO is also widely considered a safe compound with low toxicity (20). Overall, the anti-inflammatory and antibacterial properties and the relative safety of the formulated mouthwash containing V. odorata and ZnO NPs suggest that it may improve patients' oral health.

This study has some limitations. Environmental factors, such as soil composition, temperature, and the plant's growth stage, can influence the concentration of active compounds in the plants. For example, cyclotides in V. odorata are more abundant during early growth stages (39). Additionally, the extraction method, incubation time, and temperature can affect the biological properties of herbal extracts (40, 41). Future studies should assess the cytotoxicity of V. odorata extract with ZnO NPs. Clinical trials are also necessary to evaluate the anticaries and remineralizing effects of mouthwashes containing V. odorata extract and ZnO NPs.

Conclusions

The antibacterial activity of the hydroalcoholic extract of V. odorata was enhanced by adding 500 ppm ZnO NPs. The antibacterial effect of the 200 mg/ml V. odorata extract combined with ZnO NPs was similar to that of S. persica mouthwash against *S. Mutans,* although lower than 0.2% CHX. Given the anti-inflammatory and antibacterial properties and the relative safety of the formulated mouthwash, it can potentially improve patients' oral health.

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Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

MM and TM contributed to the manuscript's study management, supervision, and editing. MG contributed to the manuscript's data collection and editing. PT, SSH, and HB contributed labaratoriy analysis, interpretation, and manuscript editing. SD contributed to data gathering and writing the manuscript. All the authors read and approved the final manuscript.

Ethical approval

This study was approved by the Research Ethics Committee of Mashhad University of Medical Sciences with the code: IR.MUMS.DENTISTRY.REC.1397.034).

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