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Antibacterial Efficacy of Walnut Green Husk (WGH) Extract with Zinc Oxide Nanoparticles on *Streptococcus Mutans*

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Abstract

Background: Chemical agents, such as Chlorhexidine are used as one of dental plaque control strategy. Researchers are looking for a natural and economic substitute with same antibacterial efficacy and less complications. The aim of this study was to evaluate the antimicrobial efficacy of the Khorasan Razavi walnut green husk (WGH) extract with and without adding ZnO nanoparticles (nZnO) on *Streptococcus mutans (S. mutans).*

Methods: In this *in vitro* study, antimicrobial effect of the Hydro-ethanolic extract of WGH, was evaluated against *S. mutans*. Broth Dilution and Agar diffusion methods were used with 90 tubes containing different dilutions of WGH extract (100 to 0.006 mg/ml). ZnO nanoparticles (nZnO) were added to 45 tubes. *Streptococcus mutans* was exposed to 15 different serial concentrations of study extracts, from 100 mg/ml to 0.006 mg/ml. Minimum inhibitory concentration (MIC) of the study extracts were determined and zone of inhibition diameter was compared to positive controls (chlorhexidine 0.2%, nZnO), and negative control (sterile distilled water). The differences between the mean diameters, were analyzed by independent sample T- teS.

Results: Minimum inhibitory concentration (MIC) of study extract was found to be 50 mg/mL, with adding nZnO, MIC was reduced to 3.12 mg/mL. Mean diameter of inhibition zone at 3.12 mg/ml with and without adding ZnO nanoparticles were 17.67±0.57 mm and 8±0.001 mm, respectively, (p-value< 0.001).

Conclusions: Adding nZnO could be enhanced antimicrobial efficacy of the WGH extract against *S. mutants*, while it was still less effective than chlorhexidine.

Keywords: Dental decay, Nanoparticles, *Streptococcus mutans*, Walnut green husk, Zinc oxide.

Introduction

Tooth decay is the most common chronic disease worldwide. More than 50% of Iranian children and adults have experienced dental caries (1). Several strategies have been employed to control dental caries. Antimicrobial agents such as chlorohexidine have been recommended by evidence in high-risk individuals for dental caries (2). Recently, natural products have gained increasing popularity in health industries (3, 4). Iran is the third producer of walnuts in the world (5).Walnut green husk (WGH), as an agricultural waste, is considered a low-cost source for preparing Juglone (5 hydroxy-1,4 Naphthoquinone) (6). Antimicrobial activity of Persian walnut have been proven (7). The

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amount of phenolic compounds in green husk extracts have ranged from 32.61 mg/g of GAE (cv. Mellanaise) to 74.08 mg/g of GAE t (cv. Franquette). (8). Gram positive and negative bacteria are sensitive to phenolic compounds (۹) ,which inhibit bacterial enzyme activities (10). Also, phenolic compounds stimulate saliva secretion, which can protect teeth from decay (11).

Metal nanoparticles have been utilized in medicine and dentistry for a long time due to their bactericidal and bacteriostatic properties. Zinc oxide nanoparticles (nZnO) recognized by the Food and Drug Administration (FDA) as a safe substance and have shown effective antibacterial properties against a wide range of bacteria (12). Reaction between zinc ions with cell membrane increases H2O2 production and leads to bacterial cell membrane disintegration (13).

This study was aimed to determine minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) of the hydroethanolic WGH extract with and without adding nZnO, against *Streptococcus mutans* (*S. mutans*).

Materials and Methods

WGH extract preparation

The WGH powder (*Juglans regia* 146-1918-1) were obtained from the herbarium of Ferdowsi University of Mashhad, Iran. Ethanol and ddH2O mixture (1: 3) v/v were added to the WGH powder (10:1 w/v) and mixed well. The suspension was loaded to Soxhlet extractor for 72 hours and then concentrated in vacuum at 40 °C using of a Rotary Evaporator. The obtained extract was stored in freezer for further use. Two separate sets of WGH in Different concentrations (100 mg/ml to 0.006) were made in double-distilled water (ddH2O) and 500 ppm of Colloidal nZnO(US NANO, 18) nm) in the water was added to each concentration of one set. Then were mixed in shaker incubator at 50 °C for 48 hours and shaking speed was 40 to 50 rpm. Chlorhexidine (0.2%) and nZnO were considered as positive controls and ddH_2O was considered as negative control.

Evaluation of antibacterial activity by agar diffusion method

The antibacterial activity was evaluated by the determination of minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) using Broth dilution method (14). Briefly, one ml of 0.5 McFarland of (*S. mutans*). (ATCC 25175, Pasteur Institute, Iran) suspension was added to 1 ml of each reaction tube and mixed well? Mueller Hinton culture medium with 5% defibrillated blood of sheep was used for culturing. First, 100 µL of 0.5 McFarland suspension was cultivated on agar under sterile conditions. A volume of 100 µL of the study extracts were poured into the holes experiments plates. The plates were incubated for 48 h under anaerobic and then continued for 24 h under aerobic conditions at 37 °C. Zone of inhibition diameter was measured by the CLSI standard ruler and recorded in mm. Each test was performed three times Using 3 plates.

Statistical analysis

Independent sample T-test was used to compare the means of inhibition zone using SPSS software version 18.

Results

The Hydroethanolic extract of WGH, with and without nZnO demonstrated antibacterial properties against *S.s mutans*.

Minimum inhibitory concentration (MIC) of study extract without adding nZnO was found to be 50 *m*g/mL for *S.s mutans*, while MIC of 3.12 mg/ml was observed with adding nZnO to the study extract (Table 1, Fig. 1).

The results of MIC and MBC of WGH on *S. mutans* has been shown in Table 2. The results showed that the highest mean inhibition zone diameter (13.67±0.59 mm) was related to the concentration of 50 mg/ ml of the hydroethanolic extract of WGH.

The highest mean inhibition zone diameter

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(17.0±0.57 mm) was observed in the hydroethanolic extract of WGH with nZnO at 3.12 mg / ml concentration.

WGH with nZnO at lower concentration, showed larger inhibition zone diameter compared to WGH (p-value< 0.001).

Table 1. Antimicrobial Activity (Inhibition Zone, mm) of *Hydro-ethanolic extract of WGH without and with nZnO* on *Streptococcus mutans.*

Concentration (mg/ml)	without nZnO (Mean diameter±SD)	with nZnO (Mean diameter±SD)
100	15.33±0.08	16.42±0.06
50	13.67±0.59	15.67±1.52
25	12±1.7	8.67±1.15
12.5	9.33±1.15	14.33±0.57
6.25	11.67±0.57	17±1
3.12	8±0.001	17.67±0.57
1.56	9.67±1.15	14±1
0.78	10.33±0.57	15±0.001
0.39	0	14.33±0.57
0.19	0	9±0.001
0.09	0	9.33±0.57
0.04	0	10.66±0.57
0.02	0	11.33±0.57
0.01	0	9.6±0.57
0.006	0	9.33±0.57

Table 2. MICs and MBCs (mg/ml) of Hydro-ethanolic extract of WGH without and with nZnO on *Streptococcus mutans.*

Microorganism	MIC without nZnO	MIC with nZno	MBC without nZnO	MBC with nZnO
Streptococcus mutans **(**mg/ml)	0.78	0.39	0.39	0.19
P-value	**0.148**		**0.146**	

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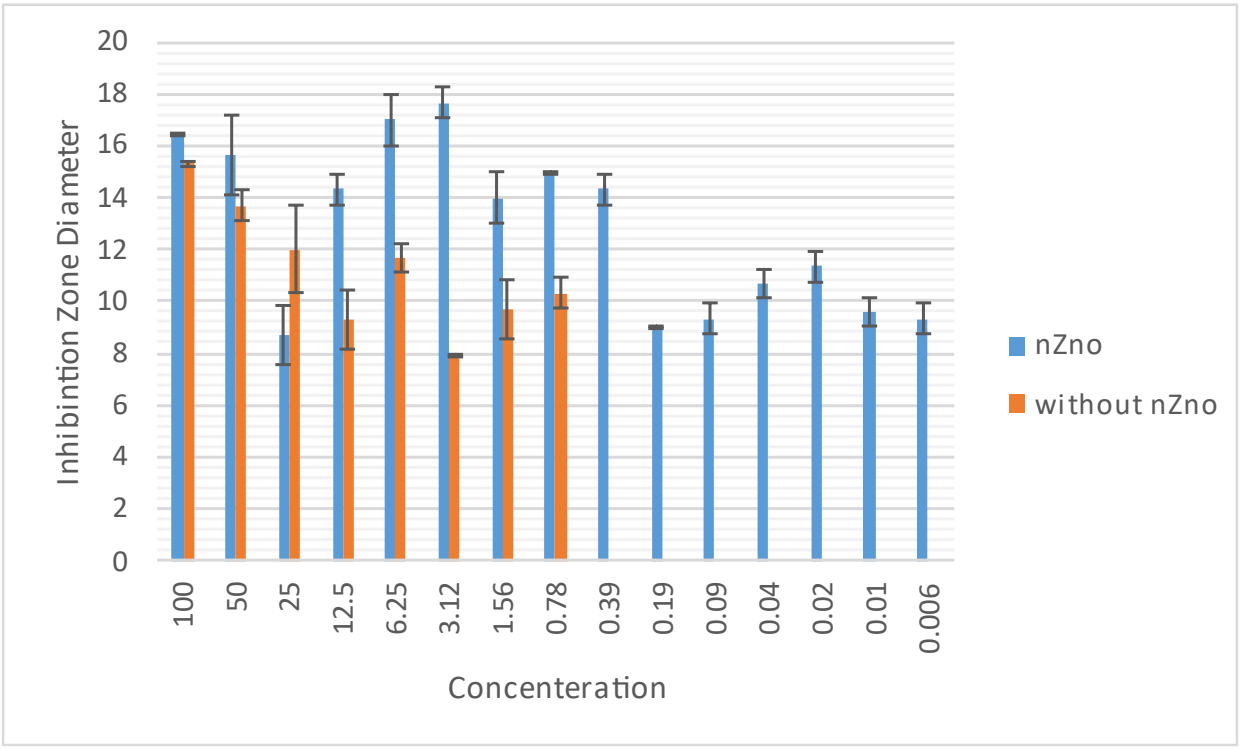

Fig. 1. Antimicrobial Activity (Inhibition Zone, mm) of Hydro-ethanolic extract of WGH without and with nZnO on *Streptococcus mutans.*

Discussion

The hydroethanolic extract of Khorasan Razavi WGH at a concentration of 50 mg/ ml inhibited bacterial growth. By adding zinc oxide nanoparticles to the green husk extract, the antibacterial efficacy was enhanced. This may be due to the antimicrobial properties of zinc oxide nanoparticles. However, it should be noted that some studies have shown that zinc nanoparticles have a toxic effect on blood and serum factors (15), which needs further investigation in future studies.

Limited studies have investigated the antimicrobial effect of green husk extract on gram-positive bacteria and fungus, and no similar study was found on the effect of this extract on *S. mutans*.

Sharafati et al. investigated the antimicrobial effect of ethanolic extract of walnut leaves on *Propionibacterium acnes in vitro*. The ethanolic extract of the plant at a concentration of 150 mg/ ml showed the largest diameter of bacterial inhibition zone (16).

Arji et al. studied the antifungal effects of methanolic and aqueous extracts of walnut husk on four Candida species. Amounts of MIC and MBC of methanolic extract of walnut husk was 20 mg/ ml for *Candida albicans* (17).

Sharafati et al. examined the antimicrobial effect of the ethanolic extract of Iranian walnut leaves on *S. mutans in vitro*. They reported 250 mg/ ml and 125 mg/ ml as MBC and MIC for this extract, respectively (18).

Zakavi et al. evaluated the effect of aqueous and ethanolic *Juglans regia* Bark extract on *S. mutans*. The highest sensitivity to ethanolic extract at a dilution of 5 mg/ ml was found (19).

In our study, Addition of nZnO enhanced the inhibitory effect of the extract such that it inhibited bacterial proliferation at 3.12 mg/ml. Darmani et al. studied the effect of aqueous extract of derum (Juglans regia; walnut tree) on several types of bacteria, including *S. mutans.* In this study, derum extract had an inhibitory effect on the growth of *S. mutans* (20).

Deshpande et al. showed that acetone extract of Juglans regia L, had growth inhibitory effect on oral microflora (21).

Nam et al study, evaluated nano-silver antimicrobial properties on *S. mutans*. They found no bacterial growth at concentrations of 1% and

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above (22). The difference between their results and our study, may be related to the using of different solvents and the type of nanoparticles.

In the study of Jung et al. the antimicrobial effect of nano-silver solution against *S. mutans* was evaluated and the sensitivity of the bacterium to nano-silver was confirmed (23).

Antimicrobial compounds mechanisms are different, the growth inhibitory effect of WGH against *S. mutans* may be related to the phenolic compounds such as tannins.

The results of present study showed that the hydroethanolic extract of WGH with nZnO could be effective in reducing the count of *S. mutans*. Additional studies such as the use of other solvents, different sizes of nanoparticles and even green synthesis of WGH nanoparticles, and the mechanism of antimicrobial activity enhancement by extract on nanoparticles should be considered.

Walnut green husk extract with nZnO showed higher inhibiting activity against the *S. mutans*.

The results of the present study can be useful in industries related to oral health products.

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