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Anti-bacterial and Anti-biofilm Effects of the Methanolic Extract of Zingiber Offcinale on *Streptococcus Mutans* Clinical Isolates

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Abstract

Background and objectives: There is an obvious need for an effective anti-bacterial and anti-biofilm agent to serve as an alternative to the currently used synthetic anti-microbial agents. This study sought to assess the anti-bacterial and anti-biofilm effects of the methanolic extract of Zingiber officinale (Z. officinale) on *Streptococcus mutans* (*S. mutans*) clinical isolates.

Materials and methods: This in vitro study evaluated 10 clinical isolates of *S. mutans* obtained from dental plaque, and its standard strain. The methanolic extract of Z. officinale was obtained by the maceration technique. The anti-bacterial activity of the extract, chlorhexidine (CHX), and chloramphenicol was evaluated by the agar diffusion technique and measurement of the diameter of the growth inhibition zones, and also by determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) by the broth micro-dilution technique. The anti-biofilm effect of the microorganisms was evaluated by the microtiter plate technique. Statistical comparisons were made by the Wilcoxon and Mann-Whitney tests (alpha = 0.05).

Results: The mean diameter of the created growth inhibition zone in *S. mutans* culture was 15 ± 1.49 mm by the Z. officinale extract, and 21.1 ± 1.59 mm by CHX and chloramphenicol, with no significant difference among them ($P > 0.05$). The MIC and MBC of the methanolic extract of Z. officinale were 57.6 ± 13.86 and 76.8 ± 27.7 mg/mL, respectively. The sub-MIC of Z. officinale extract decreased biofilm formation by the isolates by averagely 20%.

Conclusion: The methanolic extract of Z. officinale significantly decreased biofilm formation (as a one of the antibiotic-resistant mechanism) by *S. mutans* clinical isolates, which is a promising finding.

Keywords: Biofilms; Chlorhexidine; *Streptococcus Mutans*; Zingiber Officinale.

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1. Introduction

Dental caries is caused by the accumulation of cariogenic biofilm (1). *Streptococcus mutans* (*S. mutans*) is the main microorganism responsible for caries development. Dental caries is irreversible, and carious lesions can only be restored with dental restorative materials. If left untreated, progression of dental caries can affect the pulp, and necessitate root canal therapy (1).

Dental biofilm is a complex of bacteria firmly adhering to the tooth surface. In comparison with planktonic form, bacteria in dental biofilm are much more resistant to harsh environments,

antibiotics, and immune defense mechanisms, and therefore, they are hard to eliminate. Thus, prevention and treatment of biofilm-related infectious diseases are difficult (2).

S. mutans has several factors to enhance biofilm formation on the tooth surface. The ability to synthesize glucan from sugars is an important virulence factor of *S. mutans* that can lead to biofilm formation (3). In this biological process, *S. mutans* releases glucosyl transferases for the synthesis of glucan, which adheres to the bacteria-tooth interface, and results in bacterial adhesion to the surface and biofilm formation.

Antimicrobial agents that can prevent biofilm formation by *S. mutans* can be successfully used for caries control. Currently, several antimicrobial agents such as chlorhexidine (CHX) and sodium fluoride are used for clinical prevention of dental caries and oral hygiene maintenance. Nonetheless, CHX has drawbacks such as cytotoxicity and causing tooth staining (4). Furthermore, bacterial resistance to antimicrobial agents such as CHX, macrolide antibiotics, and tetracyclines is an emerging problem that highlights the need for other antimicrobial and anti-biofilm agents (5). *S. mutans* can cope with environmental stimuli, and develops resistance against bacteriocins and different antimicrobial agents to remain viable in the oral environment. Evidence shows that the clinical isolates of *S. mutans* develop resistance against fluoride as well. Thus, search is ongoing to find other effective cariostatic agents (6-8).

A growing interest has been recently observed in herbal products with optimal antimicrobial activity comparable to that of antibiotics but with fewer cytotoxicity(2). Zingiber officinale (Z. officinale) belongs to the family of Zingiberaceae, and is extensively used due to its numerous health benefits. It can improve the immune system, and has anti-inflammatory, anti-emetic, anti-cancer, and anti-oxidant properties(9). It lowers the blood glucose level and has antihyperlipidemic effects(9). Evidence shows that the aqueous, ethanolic, and methanolic extracts of Z. officinale have antimicrobial effects against *S. mutans*, *Streptococcus sanguinis*, *Lactobacilli*, and some other Gram-positive and Gram-negative microorganisms (10-12).

Considering the need for an effective antibacterial and anti-biofilm agent to serve as an alternative to the currently used synthetic antimicrobial agents, this study sought to assess the antibacterial and antibiofilm effects of the methanolic extract of Z. officinale on *S. mutans* clinical isolates.

2. Materials and methods

In order to collect specimen, 10 swab samples were randomly obtained from supragingival dental plaque and carious lesions of patients. A sterile swab was rubbed on the surface of maxillary and mandibular teeth in a posteroanterior direction for 1 minute. After 30 seconds, it was pressed on the tongue and was finally dipped in the saliva and immediately transferred to a laboratory in 1 mL of phosphate buffered saline at a pH of 7.2. Written informed consent was obtained from all patients.

2.1. Eligibility criteria for patients

The patients had to have an oral or dental infection, no antibiotic use at the time of sampling, no systemic disease, and no current disease.

2.2. Microbial culture and identification

The samples were cultured on mitis salivarius agar and blood agar at 37°C and 10% CO2 for 72 hours. The grown colonies were identified according to the standard tests (morphological assessment of the colonies, Gram-staining, catalase activity, growth in presence of 6.5% NaCl, esculin hydrolysis, hemolytic activity, breakdown of lactose, mannitol, sorbitol, raffinose, and inulin, acid production, Voges–Proskauer test, and urea hydrolysis).

2.3. Preparation of microbial suspension

The cultured colonies were inoculated on Mueller Hinton agar and after growth and proliferation, 3-4 colonies were collected and transferred into a test tube. Saline was added to create a suspension with 0.5 McFarland standard concentration containing 1.5×10^8 colony forming units/mL, which was confirmed spectrophotometrically.

2.4. Preparation of the extract

Ginger was collected and identified by the Biotechnology Institute of Shahid Beheshti Medical School. It was dried in ambient air away from sunlight. The leaves and stems were then collected and milled. Next, 500 g of the powder was immersed in a solvent composed of methanol-chloroform in 30:70 ratio for 48 hours. It was then filtered and transferred to a rotary evaporator for solvent evaporation under vacuum. The residual material was dissolved in the lowest amount of methanol possible for degreasing and was subsequently filtered. The solvent was evaporated again, and the residual material was dissolved in a small amount of dichloromethane or chloroform and dehydrated by using sodium sulfate. The solvent was evaporated under vacuum and the pure extract was collected. Z. officinale extract was then dissolved in 4% dimethyl sulfoxide for the next steps.

2.5. Assessment of anti-bacterial activity by the agar well diffusion method

In this method, 500 µL of the microbial suspension with 0.5 McFarland standard concentration was cultured in a Mueller

Hinton agar plate. Wells with 6 mm diameter were created in the agar with 2.5 cm distance from each other. Next, 100 µL of the methanolic extract of Z. officinale with 100 mg/mL concentration was added to each well. Chloramphenicol antibiotic served as the positive control. Next, the culture media were incubated at 37°C for 24 hours. The diameter of the growth inhibition zones formed around the wells was then measured in millimeters.

2.6. Determination of MIC and MBC

The MIC and MBC of the extract were determined by the microtiter plate technique using Resazurin reagent. In this method, 100 to 0.39 µg/mL concentrations of the extract were added to wells #1 to #9 of a sterile 96-well plate. The 10^{th} well served as the bacterial control, the 11^{th} well served as the medium control, and the 12^{th} well served as the extract control. Initially, 100 µL of the Mueller Hinton broth was added to each well, except for the first well. Next, 100 µL of the extract was added to the first and second wells. Accordingly, the concentration of the extract in the first well was 100%. Next, 100 µL was collected from the second well, and transferred to the third well. This process was continued until the 9^{th} well, and 100 µL of the contents of the 9^{th} well was discarded. Next, a 24-h bacterial culture was used to create a microbial suspension with 0.5 McFarland standard concentration, containing 1.5×10^8 colony forming units/mL and was diluted 1: 100 and added to all wells in an amount of 10 µL/well, except for the 11^{th} and 12^{th} wells (negative controls). The lowest concentration of the extract that inhibited bacterial growth (showing no turbidity) was recorded as the MIC. To determine the MBC, samples were taken from all wells showing no bacterial growth, and cultured on Mueller Hinton agar and incubated at 37°C for 24 hours. The plate with the lowest concentration of extract that showed no bacterial growth indicated the MBC of the extract (13-15).

2.7. Biofilm production

Biofilm production in vitro was performed by the micro-titer plate technique using tryptic soy broth (TSB). The 10 clinical isolates along with the standard-strain *S. mutans* were cultured on Mueller Hinton agar, and incubated at 37°C for 24 hours. Next, 2-3 colonies from the fresh culture were collected and cultured in TSB in a sterile 10 mL test tube and incubated at 37°C for 15-18 hours in a shaker incubator operating at 200 rpm. The optical density (OD) of the medium was adjusted to 0.1 at 600 nm (corresponding to 0.5 McFarland). Subsequently, 200 µL of the microbial suspension was cultured in polystyrene plates (Biofil, China) in triplicate and served as the control; 100 µL of the microbial suspension and 100 µL of the extract were added and incubated overnight at 37°C. A series of three wells only containing TSB served as the negative control. The culture medium of each well was removed and the contents were rinsed with 300 mL of phosphate buffered saline (pH of 7.2) three times. Bacteria that formed biofilms on the bottom and sides of the plate were fixed with methanol for 15 minutes. Then, the plate was turned upside down and left to air dry. The biofilm layer formed at the bottom and on the walls of the plate was fixed, and stained with 200 µL of 1% crystal violet for 15 minutes at room temperature. The dye was added and the contents were rinsed with 1X phosphate buffered saline three times. The microplate was allowed to dry at room temperature, and the dye attached to biofilm was released by using 200 µL of 33% acetic acid. The OD of the wells was read by an ELISA Reader at 570 nm wavelength. The results were interpreted as follows according to the OD of the negative control group:

Negative: OD of control ≥ OD of specimen

+1: 2 × OD of control ≥ OD of specimen ≥ OD of control

+2: 4 × OD of control ≥ OD of specimen ≥ 2 × OD of control

+3: 4 × OD of control ≥ OD of specimen

Use of (+1, +2, +3) or (week, medium, strong) are usually use for describe the potential of biofilm formation by crystal violet staining method (16).

The percentage of biofilm reduction by different concentrations of the extract was calculated according to the OD of untreated control and treated specimens as follows:

Percentage of inhibition = ((OD of negative control - OD of medium control) - (OD of test - OD of extract control)) / (OD of negative control - OD of medium control) × 100

2.8. Statistical analysis

Due to non-normal distribution of data shown by the Kolmogorov-Smirnov test ($P < 0.05$), comparisons were made by the Wilcoxon signed-rank test and Mann-Whitney test using SPSS version 26 (alpha = 0.05).

3. Results

3.1. Microbial culture and identification

3.1.1. Agar well diffusion

The mean diameter of the growth inhibition zone of standard-strain *S. mutans* was 15.00 ± 1.49 mm for the extract, 21.8 ± 1.87 mm for CHX, and 21.1 ± 1.59 mm for chloramphenicol. Table 1 shows the mean diameter of the growth inhibition zones of the 10 clinical isolates caused by different materials. As shown, CHX and chloramphenicol resulted in significantly larger growth inhibition zones than Z. officinale extract, indicating their higher antimicrobial activity ($P < 0.05$).

3.1.2. MIC and MBC

Table 2 presents the MIC and MBC of the extract and CHX against standard-strain *S. mutans* and its 10 clinical isolates. As shown, CHX had higher antibacterial activity than the extract.

3.2. Anti-biofilm effect

All clinical isolates and the standard strain *S. mutans* produced biofilm. The methanolic extract of Z. officinale had anti-biofilm effect on standard strain and clinical isolates of *S. mutans* in sub-MIC concentration. The reduction in biofilm formation was 20%, and this effect was significant compared to the control group ($P < 0.001$).

Table 1. Mean diameter of the growth inhibition zones (mm) of the 10 clinical isolates of *S. mutans* caused by different materials.

CHX	Chloramphenicol	Extract
23	24	16
25	23	17
19	19	13
21	20	14
20	20	14
22	22	16
24	22	17
22	18	15
20	17	13
22	20	15

Table 2. MIC and MBC (mg/mL) of the extract and CHX against standard-strain *S. Mutans* and its 10 clinical isolates.

	Parameter	Mean ± SD (mg/mL)	Minimum	Maximum
Extract	MIC	57.6 ± 13.86	32	64
	MBC	76.8 ± 27.7	64	128
CHX	MIC	0.5 ± 0	0.5	0.5
	MBC	0.5 ± 0	0.5	0.5

4. Discussion

This study evaluated the antibacterial and antibiofilm effects of the methanolic extract of Z. officinale on *S. mutans* clinical isolates. The results showed a MIC and MBC of 57.6 and 76.8 mg/mL, respectively and a diameter of growth inhibition zone of 15 mm in *S. mutans* culture caused by the methanolic extract of Z. officinale, indicating its optimal antibacterial activity against *S. mutans*. Sheikhinejad et al. (17) reported the MIC of aqueous and methanolic extracts of Z. officinale to be 12.5 mg/mL. This value was 25 mg/mL for ethyl acetate and hexane extracts of Z. officinale. The MBC of the methanolic and aqueous extracts of Z. officinale was 25 mg/mL while this value was 50 mg/mL for the ethyl acetate and hexane extracts. The methanolic extract yielded the largest growth inhibition zone followed by its aqueous extract with no significant difference. Thus, they suggested the aqueous and methanolic extracts of Z. officinale for optimal antibacterial activity against *S. mutans*. Their results were different from the present findings which may be due to the fact that they evaluated the standard-strain *S. mutans* while 10 clinical isolates of *S. mutans* were also evaluated in the present study, and it has been well confirmed that the resistance of clinical isolates to antibacterial agents is higher than that of standard strains (17). Soleimani and Jafari (18) evaluated the antibacterial effects of the extract of Z. officinale on *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *S. mutans* by the agar well diffusion technique and determination of MIC and MBC. They showed that the methanolic extract of Z. officinale prevented the growth of Gram-positive and Gram-negative bacteria at 6.25 µg/mL concentration. Its chloroform extract had a bactericidal effect on *S. mutans*, *Staphylococcus aureus*, and *Listeria monocytogenes* with a MBC of 12.5, 50, and 100 mg/mL, respectively. Its hexane

extract was only effective against Gram-positive bacteria (18). Difference between their results and the present findings can be due to differences in geographical locations from which ginger was collected, and evaluation of different microbial strains (they only evaluated the standard-strain *S. mutans*). Also, they obtained the extract right after collection of ginger; while, commercially available ginger was used in the present study. Azizi et al. (10) evaluated the effect of Z. officinale extract on *S. mutans* and *Streptococcus sanguinis* and reported a MIC of 0.02 mg/mL for S. mutans and 0.3 mg/mL for *Streptococcus sanguinis*. The MBC of the extract was reported to be 0.04 mg/mL for *S. mutans*, and 0.6 mg/mL for *Streptococcus sanguinis*. They reported significantly superior results for antibacterial activity of this extract, compared with the present findings, which may be due to evaluation of standard strains of the bacteria, and not clinical isolates. Gull et al. (19) evaluated the effect of Z. officinale extract on 8 drug-resistant bacterial species including *Salmonella typhi*, *Shigella*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidisa*, and *Klebsiella pneumonia* and showed that all of the tested microorganisms had low susceptibility to the aqueous extract of Z. officinale with a MIC of 0.05 mg/mL to 1.0 mg/mL. Variations in the reported results are due to evaluation of different microorganisms. Karuppiah and Rajaram (20) evaluated the antibacterial effects of ethanolic extract of Z. officinale against Gram-negative and Gram-positive multi-drug resistant microorganisms and showed that all of them were sensitive to the raw extract except for *Enterobacter* and *Klebsiella*. All other species were sensitive to the ethanolic extract of Z. officinale. The highest growth inhibition zone (19.45 mm) belonged to *Pseudomonas aeruginosa*, with a MIC of 67 µg/mL against it. Tajbakhsh and Soleimani (21) found that Z. officinale extract (0.125 mg/mL) had the highest effect on *Staphylococcus aureus*, *Salmonella typhimurium*, and *Staphylococcus epidermidis*. Hasan et al. (12) evaluated the effect of methanolic extract of Z. officinale on *S. mutans* virulence factors. They reported a MIC = MBC of 0.256 mg/mL for *S. mutans* and showed that the extract down-regulated the virulence genes and also, decreased biofilm formation by inhibition of synthesis and adhesion of glucan. They strongly recommended ginger as a cariostatic agent (12). Their results were in agreement with the present findings, confirming the antibiofilm effects of ginger. However, El-Sherbiny (22) reported a MIC of 1.2 mg/mL for aqueous, and 1 mg/mL for methanolic extract of Z. officinale against *S. mutans*. The MIC and MBC of the methanolic extract were 32 and 64 mg/mL, respectively. Difference in the reported values between their study and the present investigation can be due to adoption of different methods for extraction.

Future studies are recommended to use high-performance liquid chromatography for extraction to obtain more accurate results. Also, the possible side effects of the application of Z. officinale on oral mucosal cells should be investigated. Furthermore, the effects of Z. officinale extract should be evaluated against other important microorganisms. It would also be helpful to expand future research by exploring potential synergistic effects with other natural compounds or combinations with existing oral care agents. Additionally, investigating how the findings might be translated into practical applications for oral hygiene products could enhance the real-world impact of the research.

5. Limitations

Our sample size is limited and a larger sample size would increase the study's reliability. Also, higher concentrations of Zingiber officinale extract might be more effective, therefore should be investigated in further studies.

6. Conclusion

S. mutans is one of the main microorganism responsible for caries development, that can produce biofilm as an important antibiotic-resistant mechanism. So, finding a substance like herbal extract that can combat this antibiotic-resistant mechanism can be very helpful in treating patients.

The methanolic extract of Z. officinale can significantly decreased biofilm formation by S. mutans clinical isolates, which is a promising finding.

Ethics

This in vitro, experimental study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.DRC.REC.1398.185) and evaluated standard-strain S. mutans (ATCC35668) obtained from the Microbiology Department of Shahid Beheshti Medical School, and its clinical isolates obtained from patients with dental infections.

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Using artificial intelligence (AI)

None.

Author contributions

Seyed Reza Khosravani: Investigation, Data curation, Writing – Original draft

Leila Azimi: Validation, Resources, Writing – Review & editing

Mohammadreza Rahimi Nahoji: Investigation, Data curation

Narges Panahandeh: Conceptualization, Methodology, Supervision, Project administration

Conflict of interest

The authors declare no conflict of interest.

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