



The Effects of the Grape Seed Extract on *Streptococcus mutans* and *Candida albicans*: An In Vitro Study

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Abstract

Background: Oral pathogens can affect a large population and the general health of individuals. Based on the evidence, the grape seed extract (GSE) contains herbal compounds that have the ability to suppress oral pathogens associated with caries and fungal diseases. In this regard, the current study aimed at evaluating the effect of GSE on *Streptococcus mutans* and *Candida albicans*.

Methods: The grape (*Vitis vinifera* L.) seed was used in this in-vitro experimental study. After the preparation of methanolic GSE, its effect on *S. mutans* and *C. albicans* was assessed at 0.25-256 mg/mL concentrations. Then, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by macro broth dilution methods. Finally, data were analyzed by SPSS, version 20.

Results: The results showed that GSE inhibits the growth of *S. mutans* and *C. albicans*. In addition, the MIC and MBC of the extract against *S. mutans* and *C. albicans* were 2 and 8 mg/mL, as well as 32 and 64 mg/mL, respectively.

Conclusions: In general, GSE had a significant antimicrobial effect on *S. mutans*, while it only affected *C. albicans* at high concentrations.

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Keywords: Grape Seed Extract, *Streptococcus mutans*, *Candida albicans*

Received June 1, 2020

Accepted June 18, 2020

ePublished June 30, 2020



Citation: Aslanimehr M, Alizadeh A, Azmoudeh F. The effects of the grape seed extract on *Streptococcus mutans* and *Candida albicans*: An In Vitro Study. Avicenna J Dent Res. 2020;12(2):35-39. doi: 10.34172/ajdr.2020.08.

Background

Oral infectious diseases including dental caries, periodontal diseases, and the loss of teeth affect a large population (1). Among oral cavity diseases, dental caries is of great importance and has a high prevalence. In other words, dental caries is one of the commonest infectious diseases (2). *Streptococcus mutans* is considered as the main pathogen in the development of dental caries (3), and prevention is the most important component in the management of dental caries (4). The use of antimicrobial agents such as chlorhexidine and sodium chloride is one of the methods for preventing the formation of biofilms and the growth of bacteria in dental caries. However, the regular use of such common chemicals to prevent caries is not suggested due to the limitations attributed to these agents (5).

Although antibiotics such as penicillin and erythromycin are reported to effectively prevent dental caries in humans and animals, they are not clinically used due to various side effects (2). Therefore, there are several commercially available anti-caries agents although efforts for finding new anti-microbial agents continue due to their unwanted side effects and high costs and considering the emergence of resistance to antibiotics (2-6).

Candida albicans is the most common cause of fungal

Highlights

- ▶ GSE inhibits the growth of *S. mutans* and *C. albicans*.
- ▶ GSE is more effective against *S. mutans* than *C. albicans*.
- ▶ GSE as a natural product can be used for medical purposes due to its antifungal and antibacterial activity and availability.

diseases in humans and the fourth cause of infection (7).

Oral candidiasis, as denture stomatitis, is reported in 65% of the people who use full dentures (8). Amphotericin B and azoles are predominantly used for the treatment of *Candida* spp. infections. However, toxicity and resistance to these antifungal agents are great concerns (8).

Due to its poor permeability throughout the cell membrane, amphotericin B causes severe side effects such as renal damage. To reduce the intensity of these side effects, it is often combined with other antifungal agents such as azole. Nonetheless, recent evidence indicates the increasing resistance of *C. albicans* against azoles. Therefore, a lower dose of amphotericin B due to its combination with a new anti-fungal agent has received attention (7).

Various herbal extracts are reported to have antimicrobial and antifungal activities (8), including grape seed extract (GSE) (9,10). This extract has a wide range of therapeutic and medicinal properties, which is attributed

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to the high concentrations of proanthocyanidins (11). Different studies have varied results about the antibacterial effect of GSE. For example, Mirkarimi et al concluded that GSE had no bactericidal or bacteriostatic effects against *S. mutans* while Swadas et al found that it had an inhibitory effect against *S. mutans* (12,13). Considering the aforementioned issues and the high importance of understanding the antibacterial and antifungal activity of herbal compounds, the current study aimed to evaluate the effect of GSE on *S. mutans* and *C. albicans*.

Materials and Methods

Based on the aim of this in-vitro study, *S. mutans* ATCC1633 and *C. albicans* ATCC10231 were purchased from Razi Vaccine and Serum Research Institute, Karaj and Microbiology Department of Qazvin University of Medical Sciences, Qazvin, Iran, respectively. The grape (*Vitis vinifera* L.) seeds were collected from red grapes available in the market. After washing and drying, the seeds were ground into a powder with a mill in 2-minute intervals in order to prevent their heating. Then, a 25-g grape seed powder was added to 100 mL of methanol and stirred well, followed by placing the suspension in a shaker incubator for 48 hours for better extraction. In addition, the extracts were filtered through Whatman filter Paper No. 1, concentrated on a rotary evaporator, and finally, spread in thin layers on glass plates (14).

Next, 2 mL of the saline was added to vials containing lyophilized bacteria and shaken to prepare a bacterial suspension. For culturing the bacteria, 1 mL of the prepared suspension was spread on agar plates, which contained the brain-heart infusion (BHI) agar and Sabouraud dextrose agar for *S. mutans* and *C. albicans*, respectively. The plates were then incubated for 24 hours at 37°C.

Further, *S. mutans* plates were placed in a CO₂ jar before incubation, and then 24-hour cultures were prepared to evaluate the antimicrobial activities of the extracts. Next, a loopful of the culture was transferred to a sterile tube containing the broth medium and shaken for homogenization. To standardize the test, the prepared suspension was adjusted to the 0.5 McFarland turbidity. Furthermore, the comparison was performed both visually and by a spectrophotometer. The 0.5 McFarland turbidity represented 1.5×10^8 CFU/mL. Then, the suspension was diluted 1:150 in order to be adjusted to 10⁶ CFU/mL to prepare a standard suspension for measuring the minimum inhibitory concentration (MIC).

To evaluate the antimicrobial activity of the extracts, MIC and minimum bactericidal concentration (MBC) were measured according to the Clinical and Laboratory Standards Institute guidelines using a macro-broth dilution method.

To prepare the extract in the desired concentration in the range of 0.25-256 mg, 512 mg of the dried extract was dissolved in 1 mL of methanol. Then, the solvent was filtered through a 0.22-µm Millipore filter in order to make

it sterile. For this purpose, 11 sterile tubes containing 0.5 mL of the broth medium (except for the first tube) were provided and 0.5 mL of the prepared stock extract was added to the first and second tubes. After pipetting up and down, 0.5 mL of the second tube was added to the third tube and this process continued. Based on this method, 0.5 mL of each tube was serially added to the next one and 0.5 mL was discarded from the last tube in order to provide serial dilutions. All tubes were added 0.5 mL of the prepared microbial suspensions (Figure 1). Moreover, two tubes were provided, including the positive and negative control containing microbial suspension plus culture media and the diluted extract plus culture media, respectively. Additionally, an alcoholic control group containing the same concentration of available alcohol in different concentrations of the extract was separately studied in order to remove the effect of alcohol in the GSE solution. The tubes were then incubated for 24 hours at 37°C. In addition, 100 µL of the positive control tube non-diluted (10⁻²) and at 10⁻⁴ dilutions were passaged on agar plates in order to count the colonies after 24 hours of incubation.

Moreover, all tubes were passaged on agar plates non-diluted (10⁻²) and at 10⁻⁴ dilutions for each concentration after 24 hours of incubation.

For the colony count, 100 µL was taken from each tube with a sampler and cultured on Sabouraud dextrose agar and the BHI agar for *C. albicans* and *S. mutans*, respectively. After incubation at 37°C for 24 hours, the number of colonies was counted and then calculated based on CFU/mL by applying dilution coefficients (Figure 2). To confirm the quality control and the accuracy of the stages, the experiment was replicated three times for each microorganism and the mean measures were recorded accordingly. Eventually, the obtained data were analyzed by SPSS, version 20 using a *t* test.

Results

To report the results, (+), (-), and (0) indicated no growth, reduced growth, and the lack of bacterial or fungal colonies, respectively (Table 1).

The plate culture of the tube containing the lowest extract concentration and negative growth was considered as MIC.



Figure 1. Different Concentrations of GSE Added with 0.5 mL of Prepared Microbial Suspensions. Note. GSE: Grape seed extract.

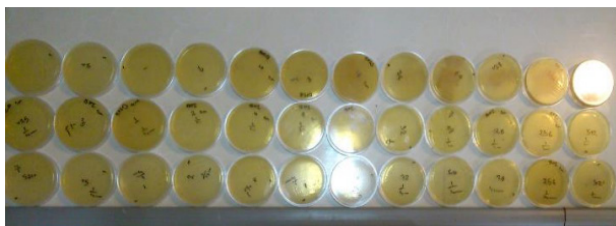


Figure 2. Samples Cultured on Sabouraud Dextrose Agar After 24 Hours.

The paired *t* test results showed a significant difference in the colony number of *S. mutans* between the positive control group and the GSE group at 0.25, 0.5, and 1 mg/mL concentrations ($P < 0.05$). According to the data in Table 1, the growth was positive at these concentrations and colony counts increased compared with those of the positive control group. Hence, GSE at the above-mentioned concentrations could not inhibit *S. mutans* growth.

The number of colonies at the concentrations of 2 and 4 mg/mL was less than that of the positive control group. Therefore, the MIC of the GSE was 2 mg/mL because, at this concentration, the *S. mutans* colony count did not increase after 24 hours while it decreased by 16%.

The growth of *C. albicans* was positive at the concentration of 0.25-16 mg/mL, and the number of colonies increased compared with the positive control group to such an extent that the colony count was dramatically high and uncountable at the 0.25-2 mg/mL concentration. Accordingly, the GSE had no control over the fungus and could not inhibit the growth of *C. albicans* at these concentrations. The results further revealed that the number of colonies at the GSE concentration of 32 mg/mL was less than that of the control group ($P = 0.02$). Therefore, the MIC of the GSE was 32 mg/mL, as the colony count did not increase after 24 hours at this concentration while it represented a 68% decrease.

Thus, the MICs of the GSE against *S. mutans* and *C. albicans* were 2 and 32 mg/mL, respectively.

As shown in Table 1, the GSE at ≥ 8 mg/mL could completely remove the bacteria and no *S. mutans* colony was detected in its culture plate. According to the definition of MBC (the minimum concentration capable of eliminating 99.9% of the microorganisms), the tube containing the lowest concentration of GSE without any grown colony on its culture plate was considered as MBC.

Therefore, the MBCs of GSE against *S. mutans* and *C.*

albicans were 8 and 64 mg/mL, respectively, because GSE could completely remove the microorganisms at this concentration. The impact of alcohol contained in GSE was considered in all the above-mentioned results.

Discussion

Due to the increased microbial resistance to chemical compounds and the significant positive effects of medicinal plants on the treatment of diseases, the use of herbs and their active ingredients is increasing. Moreover, the greater compatibility of herbal compounds with the human immune system, availability, and the public preferences of using natural resources compared with chemical compounds are other factors that have influenced the increased use of plants in health problems (15). The current in-vitro study aimed at investigating the effect of GSE, as a natural product, against two major microorganisms in oral and dental diseases.

GSE has drawn the attention of researchers because of its biological and pharmacological properties such as anti-inflammatory, anti-tumor, antimicrobial, antioxidant, and immune-stimulating features and the like (16). In addition, the grape seed is considered as a rich source of polyphenolic compounds, and the antimicrobial activity of polyphenols has been approved against a large number of pathogens (17).

Candida albicans is the most common etiologic cause of oral fungal diseases (18). On the other hand, *S. mutans* are the most important and pathogenic microorganisms in tooth decay and play a pivotal role in the onset of caries (5).

Totally, the results of the current study showed that the GSE can be effective in the growth inhibition of *S. mutans* and *C. albicans* (although lower levels of *C. albicans*).

In the current study, the MIC and MBC of the GSE against *S. mutans* were 2 and 8 mg/mL, respectively. In a study by Smullen et al, the MIC of the GSE was 2 mg/mL against *S. mutans* (3).

Further, Furiga et al reported that MIC and MBC values for *S. mutans* were 1 and 4 mg/mL, respectively (19).

The difference between MIC and MBC values might be influenced by different factors such as weather, geographical region, and plant breeding conditions, affecting the phenolic content of the grape. Due to the difference in the chemical composition of the grapes, the antimicrobial effects are also different (12).

Several studies confirmed the inhibitory effect of the GSE

Table 1. The Amount of Microorganism Growth in Different GSE Concentrations

GSE Concentration	0.25	0.5	1	2	4	8	16	32	64	128
<i>Streptococcus mutans</i>	+	+	+	-	-	0	0	0	0	0
P value	0.005	0.023	0.031	0.022	0.053	0.027	0.027	0.027	0.027	0.027
<i>Candida albicans</i>	+	+	+	+	+	+	+	-	0	0
P value	--	--	--	--	0.018	0.019	0.184	0.023	0.042	0.042

Note. GSE: grape seed extract.

on *S. mutans*. For instance, Furiga et al found a reduction in the number of micro-colonies and the thickness of the formed biofilm in the presence of the GSE (19).

In another study by Zhao et al, GSE was reported to be dose-dependent in the prevention of the enamel decay by suppressing the growth of *S. mutans* and biofilm formation (5).

Other studies indicated the antibacterial activity of the GSE against *S. mutans*. For instance, EL-Adawi reported 55.6% and 63.2% inhibition of *S. mutans* species in 10% and 15% of the GSE, respectively (2).

Contrary to the findings of the current study, Mir Karimi et al showed that the GSE had no effect on *S. mutans*. The contradiction among the results of these studies might be due to the examined microbial strain since the current study used *S. mutans* ATCC reference while Mirkarimi et al applied *S. mutans* PTCC reference (12). Accordingly, the results can be generalized only to the studied bacteria.

Furthermore, the extraction method is one of the most important stages that can significantly affect the extraction of antimicrobial compounds and thus the capability of the extract for removing different microorganisms, especially the type of applied solvents (21). In their study, Mirkarimi et al used the two-component solvent (ethanol + water) with a ratio of 70:30 while the current study utilized a one-component solvent (methanol). Therefore, the use of different solvents can be another reason for the differences in the results of the studies (20). Jayaprakasha et al demonstrated different efficacies of solvents in the extraction of various GSE agents (21).

The results of another study also showed the effect of the GSE on pathogenic fungi such as *Candida* spp. and dermatophytes (15).

In the present study, the antifungal ability of the GSE against *C. albicans* was reported as MIC = 32 mg/mL and MBC = 64 mg/mL, which is consistent with the findings of the study by Smullen et al (3). They reported a MIC of 32 mg/mL for the GSE. Additionally, Han's in-vitro study on mice confirmed the inhibitory effect of the GSE on *C. albicans* (7).

Other studies indicated the antifungal effect of resveratrol on *C. albicans*. Resveratrol is one of the ingredients that exists in grapes and other herbs containing polyphenols, which has antibacterial and antifungal effects (22-25).

In another study by Jung et al, the MIC of the GSE against *C. albicans* was reported as 20 µg/mL. A remarkable difference was expected between the results of the above-mentioned study and those of the present study. This compound mostly accumulates in the grape skin and is available in lower amounts in the grape seed. On the other hand, the findings of another study demonstrated that resveratrol has more antifungal activities compared with other polyphenolic compounds (23).

Conclusions

In general, the GSEs were proved to be active against

C. albicans and *S. mutans*. Therefore, the GSE as a natural product can be used for medical purposes due to its antifungal and antibacterial activity and availability, especially in toothpastes, gels, varnishes, and mouthwashes.

Finally, it is suggested that future studies investigate bacterial and fungal strains isolated from patients in addition to standard strains.

Conflict of Interest Disclosures

The authors declared that they have no conflict of interests.

Acknowledgement

This study was supported by the Department of Microbiology, Qazvin University of Medical Sciences.

Ethical Statement

This study was approved by the Research Ethics Committee of Qazvin University of Medical Sciences (code: IR.QUMS.REC.1394.811).

Authors' Contribution

FA, AA and MA developed the concept and design of the study. MA and AA Performed laboratory steps. FA was primarily responsible for writing the manuscript. All authors have read and approved the final.

Funding

Part of the costs of this project were supported by Qazvin University of Medical Sciences.

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