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RESEARCH ARTICLE

Anticariogenic Activity of Oregano against *Streptococcus Mutans* Biofilm for Investigating its Potential Use as an Oral Care Agent

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ABSTRACT

Being consumed as a spice, *Origanum* species are also utilized for alternative medical treatment and are therefore of great economic importance. This study examined the effect of *Origanum vulgare* L. subsp. *gracile* (OVG) essential oil (EO) against planktonic cell growth and biofilm formation of cariogenic *Streptococcus mutans*as well as its cytotoxic activity. The antimicrobial potential of EO was screened by broth microdilution method. Crystal violet (CV) biofilm assay method has been used to determine the antimicrobial activitiy and the biofilm formation was also displayed through scanning electron microscopy (SEM). The minimum inhibitory concentration (MIC) value of the EO was determined as 2.5±0.31 mg/mL. The EO acted as an anti-biofilm agent by preventing biofilm formation at sub-MIC concentrations (27.15 to 33.2%). The SEM imaging of biofilms treated with EO showed density and morphological changes when compared to the untreated group. To determine the cytotoxicity, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay wascarried out using the NIH-3T3 murine fibroblast cells andand exhibited IC_{50} value of 427±1.05 µg/mL.This study has shown that with strong antibacterial and antibofilm capacity, the EO of the OVG can be used as a potentialanticariogenicagent in the oral care products.

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Introduction

Origanum genus is native to Europe, America, North Africa and Central Asia widespread herb belonging to family Lamiaceae (Sözmen et al., 2012; Tepe et al, 2016). Thisplant is widely consumed as a very popular spice all over the world and are therefore of great economic importance (Taşçıoğlu et al., 2018). Turkey has largest number of oregano varieties and is a leading country in the world for oregano production (Cetingul et al., 2007).

The dried leaves of the plant have been used in medicine for centuries as a cure for various diseases (Sharifi-Rad et al., 2021). Recent findings have shown that OV has the bactericidal and fungicidal activity against almost all pathogens (Brondani et al., 2018; Khan et al., 2018).

According to the report of the World Health Organization (WHO), about 100% of adults and 60-90% of children in school age have tooth decay (WHO, 2012). It is thought that *Streptococcus mutans* is the main aetiological agent of human dental decay. This bacterium has various abilities to colonize onto the tooth surfaces (Ogawa et al., 2011). An important virulence feature of bacteria is that they can form biofilms known as dental plaque. *S.*

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*mutans*produces a variety of proteins, such as glycosyltransferase enzymes, glucagon binding proteins, surface proteins, and collagen binding protein, in a coordinated way to produce dental plaque and induce dental decay (Matsumoto-Nakano, 2018).

However, the accumulation of dental plaques is not the only mechanism that plays animportant role in tooth decay. In addition, reactive oxygen species which are produced excessively by oral bacteria could cause oxidative damage and inflammation of the gingival tissue, periodontal ligament and even alveolar bone, especially by *S. mutans* (Canakci et al. 2005; Jurczak et al., 2017). *S. mutans* which leads to dental caries is one of the most abundant bacteria present in the oral cavity. When *S. mutans* adhere to the teeth surface, it has the ability to form biofilm layer which facilitate to lead toinfections and tooth caries (Melok et al., 2018).

In fact, the daily removal of the tooth plaque is very notable for the prevention of tooth caries, gum inflammation and periodontitis (Marchetti et al., 2011). However, increasingly, clinical trials indicate that only brushing activity itselfis not adequately effective in preventing plaque formation (Van DerWeijden and Hioe, 2005). Because of their hydrophobic nature, EOs cause inhibition of bacterial enzymatic activity and bacterial cell membrane degradation (Edris, 2007). Mouthwashes that contain EOs were found to be noticeablyactive in reducing bacterial viability and the density and bulk of biofilms formed in the mouth (Quintas et al., 2015).

Although EO of OVG was reported to have good antimicrobial and antifungalactivity, its use as an antibiofilm agent in dental applications against *S. mutans*has not been studied yet. In this scope, the preventive potential of OVG EOagainst cariogenic *S. mutans* bacteria has been investigated in detail. For this purpose, MIC of the OVG EO and its antibiofilm potential have been determined against *S. mutans*. Morphological changes of the *S. mutans*biofilm layer after OVG EO treatment were also observed by SEM. Cytotoxic capacity of the OVG EO was figured out against NIH-3T3 murine fibroblast cells.

Material and Methods

Plant Collection and Isolation of the Essential Oil

The above-ground parts of OVG (Voucher No. ARB-OV02) was collected in Adana (Turkey). The plant was authenticated by a taxonomist and specimen was stored in Muğla Sıtkı Koçman University, Turkey. The essential oil (EO) was obtained withwater distillation method.

Determination of MIC

S. mutans (ATCC 25175) certified reference strain was obtained from the American Type Culture Collection (ATCC). The MIC of the EO was evaluated for antibacterial activity using broth microdilution assay (NCCLS, 1993). Two-fold serial dilutions of EO were pippetedinto microplate wells, and bacterial suspension (10^7 CFU/mL) was added to into EO. Then, the microplate was incubated at 37°C and optical density of wells were measured at 640 nm after 24 h of incubation.

Antibiofilm Activity

To assess the inhibition effect on *S. mutans* biofilm formation, bacterial suspension that contained 10^7 CFU/mL bacteria in Brain Heart Infusion Broth (BHIB) with 1% sucrose was inoculatedinto polystyrene flat-bottom plates and cultured in the presence of subMIC concentrations of the EO at 37 ºC for 72 h. A positive control, without EO and a negative control, without bacterial inoculation were used as control groups (Boran and Ugur, 2015).

Following the incubation period, non-adherent planktonic cells in the wells were removed and the wells were smoothly rinsed with phosphate buffer solution (PBS, pH 7.4). Then, the wells were stained with 1% CV solution. Lather, the plates were rinsed with steriledistilled water to remove non-absorbed stain. Finally, ethanol (95%) was added to dissolve the CV staining, and the absorbances were recordedat 590 nm using a microplate reader.

SEM

The inhibition of biofilm formation was visualized by SEM. Briefly; sterile glass coverslip pieces (20x20 mm) were placed into tubes. Bacterial suspension that contained 10^7 CFU/mL bacteria in BHIB with 1% sucrose was inoculated with subMIC concentrations of the EO. Control group was prepared without EO. After incubation at 37ºC for 72 h, the planktonic cells were removed and each tubewith coverslip was washed thoroughly with PBSto remove non-adherent cells. Prior to imaging, the coverslips containing the biofilm layer were fixed in 2.5% glutaraldehyde at 4 ºC for 2 h. Then, the coverslips were washed with PBS for 1 h andthe cells dehydrated in increasing ethanol concentrations. After that, they were air-dried, gold-coated (Emmitech K550, UK) and examined with SEM (JEOL JSM-7600F; JEOL Ltd., Tokyo, Japan).

Cytotoxicity of the Essential Oil

- ***Cell Culture***

NIH-3T3 (ATCC) cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) with high glucose, supplemented with 1% penicillin-streptomycin (10000 Units/mL Penicillin-10000 µg/mL Streptomycin) and 10% fetal bovine serum. The cell culture was cultivated in a humidified atmosphere at 37ºC in 5% CO_2.

- ***Cytotoxicity***

The cytotoxicity of the OVG EO was screened using MTT colorimetric assay (Mosmann, 1983). NIH-3T3 cells were cultured in the medium in 96-well plates as described above, at plating density of 10.000 cells per well (200 µL), and incubated at 37°C, 5% CO_2, and 100% relative humidity for 24 h. After 24 h incubation, the medium was replaced with the medium containing the OVG EO at various concentrations (125 to 2000 µg/mL). Then, the plates were incubated under the same conditions for 24 h. Sextuplicate

(6 replicates) was maintained and the medium with dimethyl sulfoxide (DMSO) (without the EO) served as control. After the incubation period, 20 µL of MTT were added to each well and were incubated at 37°C for additional 3 h. The medium containing MTT were poured off, 100 µL DMSO was added to solubilise the formed formazan crystals in each well, and the plates were put in an orbital shaker for 15 min. Finally, the absorbances were measured at 540 nm.

The % cell inhibition was calculated using the following formula and the graph between % cell inhibition and concentration were plotted, from which $IC_{50}$was calculated:

$$(\%) = [100 \times (Sample_{abs})/ (Control_{abs})]$$

Statistical Analysis

All experiments except from the cytotoxicity were performed in triplicates. Data were organized and analyzed using Microsoft Excel and one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered as statistically significance.

Results

Effect of OVG EO on Planktonic Cells

In vitro antibacterial activity against *S. mutans* was assessed by two-fold serial dilution of EO and the MIC was defined as the lowest EO concentration that restricted the visible growth of tested microorganism. Results of preliminary antibacterial testindicated that EO of OVG was active against cariogenic *S. mutans* and the MIC value were determined as 2.5±0.31 mg/mL.

Antibiofilmactivity of OVG EO

The inhibition effect of EO on *S. mutans* biofilm formation was carried out by CV biofilm assay at sub-MIC concentrations. The EO has been found to have an antibiofilm effect on *S. mutans* as dependent on the concentration of the tested compound. The EO could inhibit the biofilm formation of in the range of 27.15–33.2% at sub-MIC (MIC/8 to MIC/2) concentrations.

Antibiofilm effect of the EO was also confirmed by SEM. The photomicrographs in Figure 1A and 2A showthe biofilm formation in control tubes (without EO) with a large amount of adherent cells, and Figure 1B and 2B show the biofilm formation treated with EO at MIC/2 concentration. Observations confirmed that EO possesses a strong inhibitory effect on *S. mutans* cell density and biofilm formation (Figure 1B). In the groups that *S. mutans* biofilm treated with EO, structural changes were visible, where the shape of the bacterial cells were changed and the surfaces were ruptured/deformed (Figure 2B). However, no morphological changes were observed in control tubes (Figure 2A).

Cytotoxicity

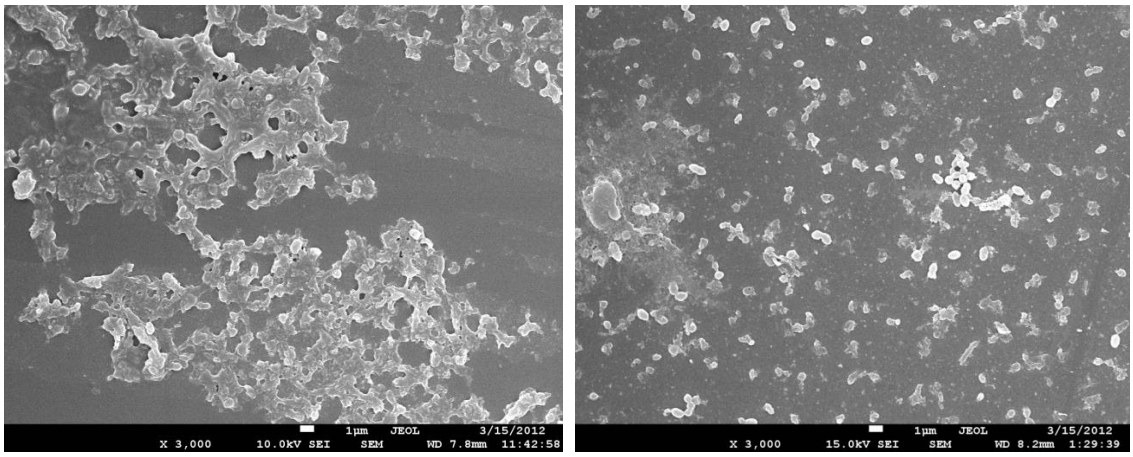
The cytotoxic effect of EO was assessed against NIH-3T3 fibroblast cells using the MTT assay. The IC_{50} value of the EO on NIH-3T3 fibroblast cells was found as 427±1.05 µg/mL (Figure 3).

Discussion

Streptococcus is an important bacterium that participates in the development of caries due to its mechanism of adhesion to a solid surfaceand its extraordinary ability to produce biofilms (Krzysciak et al., 2014). In this genus, *S. mutans* is considered one of the most important pathogens that play a role in biofilm formation on teeth surface (AbdusSalam et al., 2004). The antimicrobial agents are important approaches to prevent microbial infection on the tooth, but it's reported that the dental biofilm shows enhanced resistance to antimicrobial agents (Kouidhi et al., 2015).

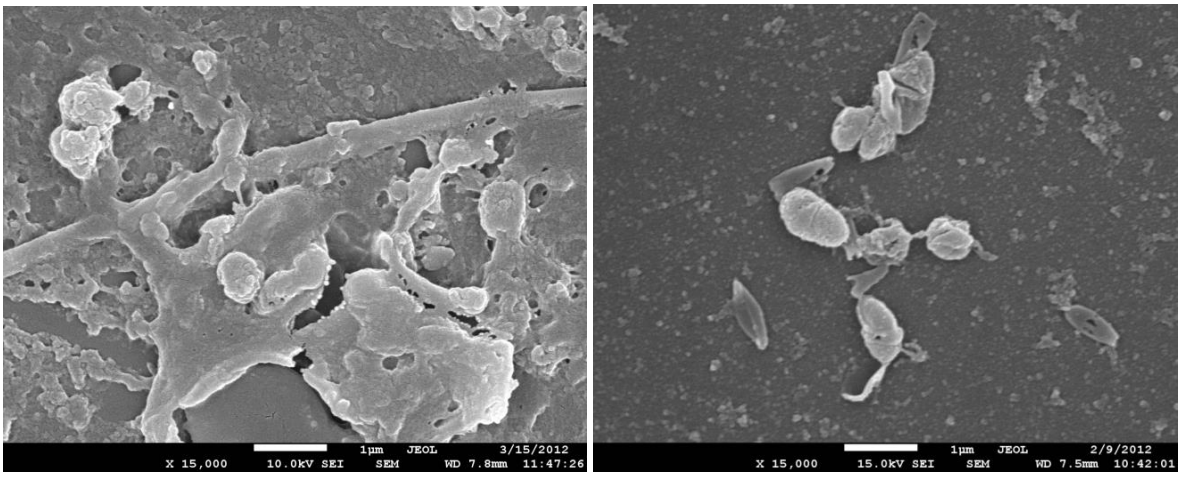
An alternative way to control biofilm formation is the prevention of microbial colonization. Thus, at a first step, we analyzed the antibacterial activity of OVG EO against *S. mutans* planktonic cells and determined its MIC value. Then, we evaluated the antibiofilm ability of different concentrations (sub-MIC) of the EO. Concentrations as low as MIC/8 resulted in a reduction of adherent cells, with 27.15% inhibition of *S. mutans* biofilm formation compared with untreated control group. Biofilm inhibition results by addition of sub-MIC concentrations of EO against *S. mutans* indicated that the obtained antibiofilm effect was dose-dependent. The highest biofilm inhibition ratio (33.2%) was observed at MIC/2 concentration. The reason for biofilm inhibition may be due to the inhibition of the biofilm formation pathway or the death of planktonic cells in the early growth stage. In another study, *S. mutans* biofilm formationwas significantly reduced at 5.00 mg ml^{-1} *O. vulgare* methanol extract, whereas the lower concentrationsdid not affected the *S. mutans* biofilms (Hickl et al., 2018).

These results were confirmed by SEM. It is observed in Figure 1A and Figure 2A that there are large layers of biofilms colonized on the surface of the control coverslips. On the other hand, the EO treated coverslips presented cellular biofilm detachment (Figure 1B). It was observed that *S. mutans* cells in the EO-treated group had serious effects on the morphology of the cell membrane (Figure 2B). This cellular biofilm detachment, morphological changes and cell lysis can cause alteration of membrane permeability and leakage of cell contents and eventually lead to cell death. This may possibly be due to the lipophilic property of EOs and their components that target the bacterial membranes. In one study, oregano EO was reported to increase *Staphylococcus aureus* membrane permeability. In the study, it was stated that EO caused leakage of potassium and phosphate ions from *S. aureus*, causing cell death (Lambert et al., 2001).



(A) Control (B) Oregano EO

Figure 1. Antibiofilm effect of EO on *S. mutans* biofilm formation. (A) Control (3000 magnification). (B) Biofilm treated with MIC/2 of EO (3000 magnification).



(A) Control (B) Oregano EO

Figure 2. Scanning electron micrographs of randomly chosen areas of *S. mutans* biofilm at 72 h. (A) Control (15000 magnification). (B) Biofilm treated with MIC/2 of EO (15000 magnification).

In addition to, an only one report on the antimicrobial activity of OVG extract has been published (Moradi et al., 2014). In fact, there are some studies about the antimicrobial activity of *O. vulgare* EO or extracts against "non-oral" bacterial pathogens (Boskovic et al., 2015; Fratini et al., 2017). As far as weknow, there is a study about the antimicrobial and antibiofilm effects of *O. vulgare* against *S. mutans* (Hickl et al., 2018), but the antimicrobial and antibiofilm effects of *O. vulgare* ssp. *gracile* against *S. mutans* have not been studied previously.

Hickl et al. (2018) reported that *O. vulgare* methanol extracts had low antibiofilm activity against *S. mutans* at low erextract concentrations (at 0.02-2.5 mg/mL) whereas it significantly reduced the biofilm formation at 5.00 mg/mL^{-1} (at minimum bactericidal concentration). However our study, OVG EO could inhibit the biofilm formation as 33.2% at MIC/2 (1.25 mg/mL). Thymol, carvacrol, ρ-cymene and γ-terpinene were identified as the main components in OVG EOs that grows in Turkey (Kilic et al., 2016). A potential explanation for this antimicrobial or antibiofilm effect of EO may be the high content of carvacrol and thymol or the presence of other phenolic compounds, such as γ-terpinene and p-cymene. It has been specified that the hydroxyl group of carvacrol and thymol play a major role in its antibacterial effect (Saad et al., 2013; Nazzaro et al., 2013). They interact with cell membrane that causes functional and structural damages and membrane potential loss (Hyldgaard et al., 2012).

The possible cytotoxic effect of EO was evaluated on NIH-3T3 fibroblast cells after 24 h of incubation with EO (ranging from 125 to 2000 µg/mL) (Figure 3). The results of cytotoxic activity test indicated that the incubation of NIH-3T3 fibroblast cells with EO ranging from 125 to 250 µg/mL did have no significant effect on cell viability. The IC_{50} value of EO was found to be 427±1.05 µg/mL.In a study, oregano EO had lower cytotoxicity against non-tumor cell line HEK293, and the value of IC_{50} is determined as 310 µg/µL (Elshafie et al., 2017). In another study, no significant effect on viability of SAF-1 cells was noted with respect to the ethanolic extract of *O. vulgare* at concentrations of 0.001 to 0.75 mg/mL^{-1} (Beltrán et al., 2018).

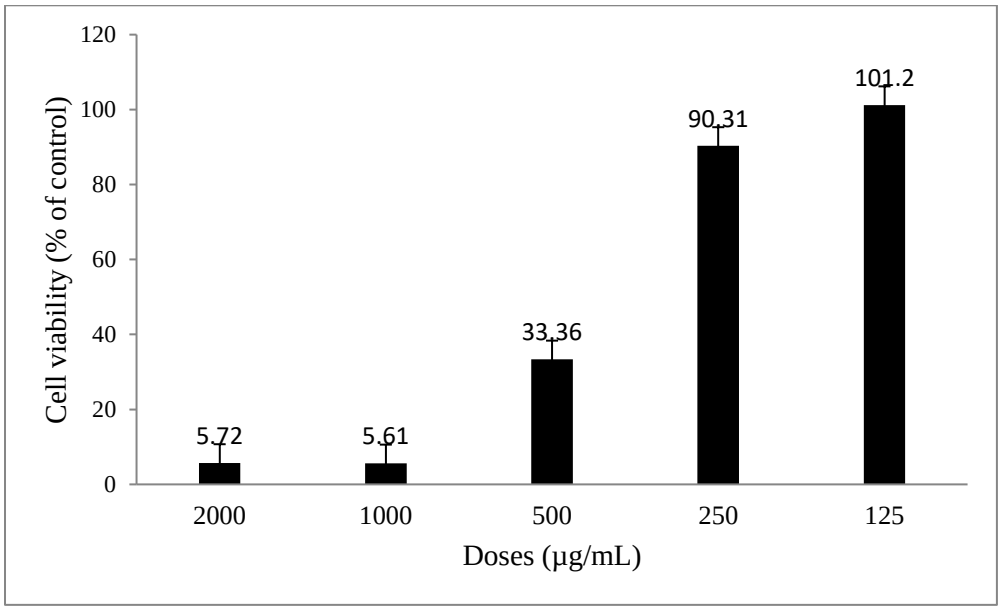


Figure 3. Cytotoxic effects of *O. vulgare* subsp. *gracile* EO on NIH-3T3 fibroblasts. Results are represented as viability ratio compared to the control group.

Conclusion

In summary, this study show that the OVG EO of has a distinctive antibacterial and antibiofilm activity against cariogenic *S. mutans*, an aetiological agent of dental caries. The use of the active concentrations can be considered as safe which may suggest a possible use of this plant as an alternative remedy to prevent dental problems. These data provide evidence that *O. vulgare* subsp. *gracile* could be potentially used as a natural additive in the oral care products for prevention of oral diseases, including dental biofilm.

Compliance with Ethical Standards

a) Authors' Contributions

RB conducted the minimal inhibitory concentration and antibiofilm activity; wrote the article with the contribution from all co-authors. NS performed the cytotoxic activity. TB performed the SEM imaging of biofilms treated with EO. AU conceived the experiments, supervised all stages of the research. All authors have approved the final version of the manuscript.

b) Conflict of Interest

The authors declare no conflict of interest

c) Statement of Human Rights

This study does not involve human participants.

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