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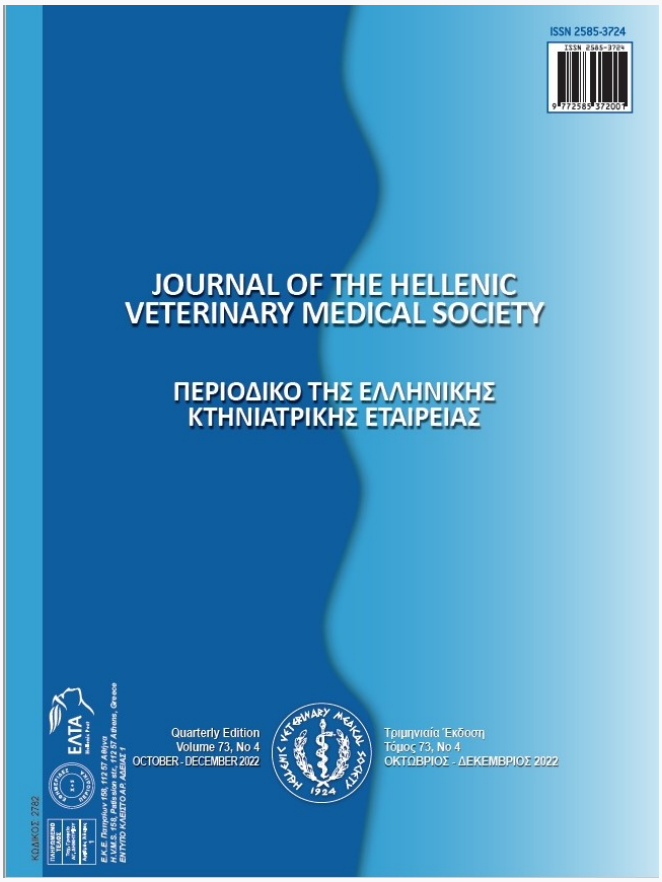


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# Antimicrobial and antibiofilm forming activity of *Origanum munzurense* against some Gram-Positive bacteria and yeast

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**ABSTRACT:** *In vitro* antimicrobial and antibiofilm forming activities of *Origanum munzurense* extract were investigated against some gram-positive bacteria and yeast strains. Liquid microdilution method and microplate method were used for the assessment of antimicrobial and antibiofilm forming activities, respectively. Minimal inhibitory concentration values were found to be 2 mg/ml for *Enterococcus faecalis* ATCC 29212, 64 mg/ml for *Listeria monocytogenes* RSKK 02028, 2 mg/ml for *L. monocytogenes* RSKK 472, 2 mg/ml for *Bacillus cereus* NRRL 569 , 128 mg/ml for *S. aureus* ATCC 25923, 32 mg/ml for *S. aureus* (MRSA) ATCC 43300 strain, 64 mg/ml for *Candida tropicalis* ATCC 13803, 2 mg/ml for *C.albicans* ATCC90028, 1 mg/ml for *S.aureus* FRI 918 and 1 mg/ml for *Staphylococcus epidermidis* ATCC 12228 strain. The extract prevented the biofilm formations of *E. faecalis* ATCC 29212, *S.aureus* FRI 918 and *S.epidermidis* ATCC 12228 at concentrations of 2 mg/ml, 500µg/ml and 250µg/ml, respectively. No biofilm formation was observed for the other bacteria tested. As a result, *Origanum munzurense* extract has antibacterial and antibiofilm activity on the selected pathogenic microorganisms tested. Due to these properties of *O.munzurense*, it could be considered as a plant-based antimicrobial agent to be used especially in the food industry.

**Keywords:** *Origanum munzurense,* Antimicrobial, Antibiofilm, MIC

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## INTRODUCTION

*Origanum* (O.) species are fragrant aromatic perennial herbaceous shrubs belonging to the *Lamiaceae* family (Baser and Kirimer, 2006; Yılmaz et al., 2017). Among these species, *O. munzurense* is an endemic species that grows in Tunceli province in eastern Turkey. This species has been described as a hybrid between *O. acutidens* and *O. vulgare.* Although the majorityof the distribution area of these two species is similar, it has been reported that these species overlap and form hybrids only in Tunceli province (Dirmenci et al.,2019). In studies with *Origanum* species, researchers have reported that the obtained essential oils have multifaceted effects such as antibacterial, anti-inflammatory, antiviral, antioxidant, antifungal and anti-genotoxic (Yabalak et al., 2020). All species in this family contain large amounts of essential oil and its main components are carvacrol and thymol (Baser, 2001), while also other components, such as linalool, p-cymene etc exist (Baser and Kirimer, 2006; Yılmaz et al., 2017). The essential oil of the members of this family is therefore used in the pharmaceutical and cosmetic industry (Hayta and Arabacı, 2011). It has also been known that *Origanum* species are used as spice and herbal tea, but also in the treatment of diarrhea, nausea, asthma, cramps, rheumatic diseases, digestive problems and infectious diseases (Dorman et al., 2004).

Due to the fact that an increased number of bacteria has developed resistance to antibiotics, the treatment of infections that threaten public health is nowadays very complicated (Hızel, 2011). Yeasts can colonize in the human body and cause serious health problems ranging from simple infections to life-threatening conditions. The pathogenicity of *Candida* is attributed to its attachment to the host cell and biofilm formation (Polke et al.,2015). Biofilm has been defined as a micro-ecosystem formed by different microorganisms to protect against harmful agents from the environment and to maintain their vital functions (Temel and Eraç, 2018). As a structured community of bacterial cells enclosed in a self-generated polymeric matrix, adhered to a living or non-living surface, it has some advantages that contribute to the formation of resistance (Kumar and Anand, 1998). It has been reported that this structure helps microorganisms to escape from host defense, is responsible for various infectious diseases, and contributes to the development of resistance of microorganisms to antibiotics (Marcinkiewicz et al., 2013). The most common resistance-related mechanism is that antimicrobial agents cannot cross the biofilm barrier and cannot affect microorganisms in the biofilm environment as easily as they affect planktonic cells. In the biofilm community, resistance genes are more easily transferred from one microorganism to another (Amorena et al., 1999). Since microorganisms living in biofilms pose health problems, various therapeutic agents are needed to prevent the formation of this micro-ecosystem (Camps et al., 2011).

Antimicrobials are substances that prevent the growth and reproduction of bacteria and yeasts or eliminate them. These include synthetic drugs, antibiotics, preservatives and natural products derived from animals or plants (Romulo et al., 2018). Recently, excessive use of synthetic antimicrobials to prevent bacterial infections has seriously threatened public health, and it also leads to the formation of drug resistance (Pimchan et al.,2018). In addition, various synthetic food additives are used in the food industry to protect food and extend its shelf life, which causes public health problems due to their accumulation in food. For this reason, it has been recommended to use natural products instead of other antimicrobials in recent years (Mostafa et al., 2018).

The antimicrobial and antibiofilm activities of *Origanum munzurense*, an endemic species in Turkey, have not yet been documented very well. In this study, the *in vitro* antimicrobial and antibiofilm forming activity of the ethanolic extract of *Origanummunzurense* plant against microorganisms known to be pathogenic for humans was examined.

## MATERIAL AND METHODS

### Collection of Plant Specimens

The plant sample was collected from Tunceli-Ovacık in eastern Turkey. Species identification was carried out by a botanist from Niğde Ömer Halis Demir University, Faculty of Arts and Sciences, Department of Biology-Botany.

### Extraction of plant samples

After the plant samples were collected, they were cleaned, the leaves and other components of the samples were separated and dried for 20 days without sunlight. The leaf parts of the dried samples were passed through the grinder and turned into powder. 20 g of the samples were collected, placed in a soxhlet device in a cellulose cartridge, and extracted with 500 ml of ethanol at 50 °C for 3 hours. The ethanol in the solution was evaporated at 40 °C in a rotary evapora-

tor (Wist, Wev 1001V). After condensation in the vacuum evaporator, the remaining extract was stored at -20 °C until used (Aslantürk, 2010; Azwanida, 2015).

**Preparation of Stock Plant Extracts**

The plant extract prepared with ethanol was dissolved in distilled water and 5% Dimethyl sulfoxide (DMSO) (Merck, Germany) at an initial concentration of 50 mg/mL. Stock plant extracts were sterilized with a 0.22 µm pore diameter syringe filter (Millipore A762933) and stored at -20 °C until use.

**Standard Microorganism Strains:**

Standard microorganisms were obtained from Department of Food Hygiene and Technology, Burdur Mehmet Akif Ersoy University. *Listeria monocytogenes* RSKK 02028, *L. monocytogenes* RSKK 472, *Enterococcus faecalis* ATCC 29212, *S. aureus* ATCC 25923, *S. aureus* (MRSA) ATCC 43300, *Bacillus cereus* NRRL 569, *Candida tropicalis* ATCC 13803, *C. albicans* 90028, *S. aureus* (enterotoxin E) FRI 918, *Staphylococcus epidermidis* ATCC 12228 strains were used.

**Media**

Tryptic Soy Agar (TSA, Merck M105458) was used for amplification of reference bacterial strains, and cation-adjusted Mueller Hinton Broth (CAMHB, Merck M110293) was used for antibacterial susceptibility testing.

**Antibacterial and Antibiofilm Activity test**

**Determination of MIC Value of *Origanum munzurense* Extract**

Pathogenic microorganisms were inoculated into TSA medium by the scratch plate method and incubated overnight at the optimum temperature (37 °C). Bacteria were dissolved in 10 ml of 0.9% sterile NaCl and after the bacterial concentration was adjusted to McFarland 0.5 (≈1x$10^8$ KOB/ml) using a densitometer (Alla, France), various concentrations of *Origanum munzurense* extract (3.9 µg/ml, 7.8 µg/ml, 15.63 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml, 8 mg/ml, 16 mg/ml, 32 mg/ml, 64 mg/ml, 128 mg/ml) cation-adjusted Mueller-Hinton Broth (CAMHB) was aseptically dispensed into 200 µl 96-well plates (Corning Costar 3599, flat bottom). 20 µl of the prepared bacterial suspension was added to them. After the prepared microplate was incubated for 24 hours at 37 °C, absorbance values were measured at 600 nm by a microplate reader (Sudagidan and Yemenicioğlu, 2012).

**Investigation of the antibiofilm properties of *Origanum munzurense* Extract**

For this test, bacteria grown in TSA for 16 hours at 37 °C were collected with a sterile swab and dissolved in 10 ml of 0.9% NaCl, and its concentration was adjusted to McFarland 0.5 with the help of a densitometer (Alla, France). 200 ml of cation adjusted Mueller-Hinton Broth (CAMHB) containing different concentration of *Origanum munzurense* extract was distributed on 96-well microplates (Corning 3599) and incubated at 37 °C for 24 hours by adding 20 ml of bacterial suspension. After 24 hours, the plates were washed 3 times with 200 ml of PBS (Phosphate Buffered Saline). Bacteria were fixed for 15 minutes by adding 200 ml of methanol. After the methanol was poured from the plates, the bacteria attached to the microplate surface, which were dried for 1 hour at 55 °C, were stained with 200 ml of crystal violet (Sigma-Aldrich, 109218), and after 5 minutes, the excess dye was removed by washing with tap water. After drying the microplate, the adsorbed dye was dissolved with 33% acetic acid (Merck, 100063) (200 ml) and its optical densities (OD) absorbance values were measured at 590 nm (BioTek Epoch, USA) (Sudagidan and Aydin, 2010).

**RESULTS**

*In vitro* antibacterial activity of *Origanum munzurense* extract plant was determined against the bacterial and yeast strains by liquid microdilution method. *In vitro* antibiofilm effect of the extract against the bacterial and yeast strains was determined by microplate method. MIC values on the tested bacterial and yeast strains are shown in Figures 1, 2, 3, and the antibiofilm effect is shown in Figure 4. The ethanolic extract of *Origanum munzurense* plant showed an inhibitory effect at concentrations of 2 mg/ml for *E. faecalis* ATCC 29212, 64 mg/ml for *L. monocytogenes* RSKK 02028, 2 mg/ml for *L. monocytogenes* RSKK 472, 2 mg/ml for *B. cereus* NRRL 569 , 128 mg/ml for *S. aureus* ATCC 25923, 32 mg/ml for *S. aureus* (MRSA) ATCC 43300 strain, 64 mg/ml for *C.tropicalis* ATCC 13803, 2 mg/ml for *C.albicans* ATCC90028, 1 mg/ml for *S. aureus* FRI 918 and 1 mg/ml for *S. epidermidis* ATCC 12228 strain.According to the results,*Origanum munzurense* extract showed the best antibacterial activity on *S. aureus* (enterotoxin E) FRI 918 and *S. epidermidis* ATCC 12228 strains at 1 mg/ml concentrations. It showed the least antibacterial effect on *S. aureus* ATCC 25923 bacteria at 128 mg/ml concentration.

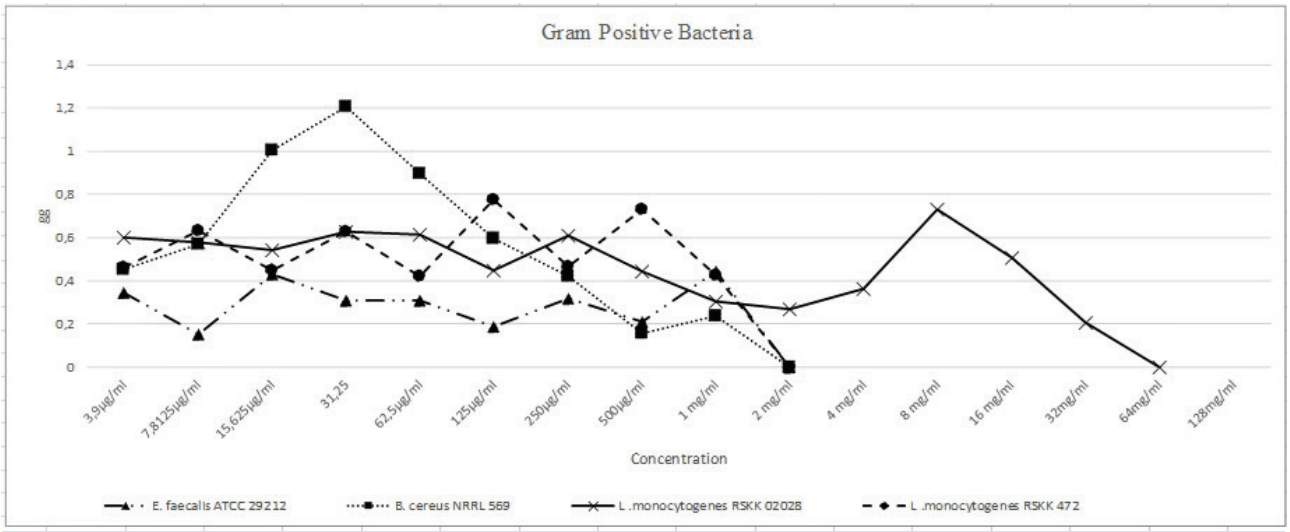


**Figure 1.** Minimal inhibitory concentration (MIC) values of *Origanum munzurense* ethanol extract against *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* RSKK 02028, *L. monocytogenes* RSKK 472, *Bacillus cereus* NRRL 569

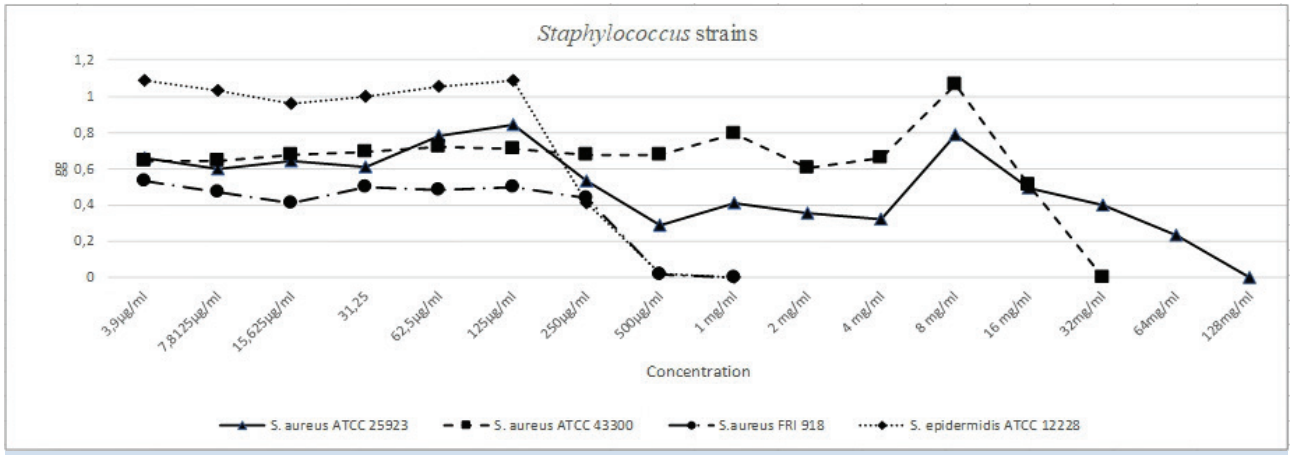


**Figure2.** Minimal inhibitory concentration (MIC) values of *Origanum munzurense* ethanol extract against *S. aureus* ATCC 43300, *S. aureus* ATCC 25923, *S. aureus* FRI 918 and *Staphylococcus epidermidis* ATCC 12228

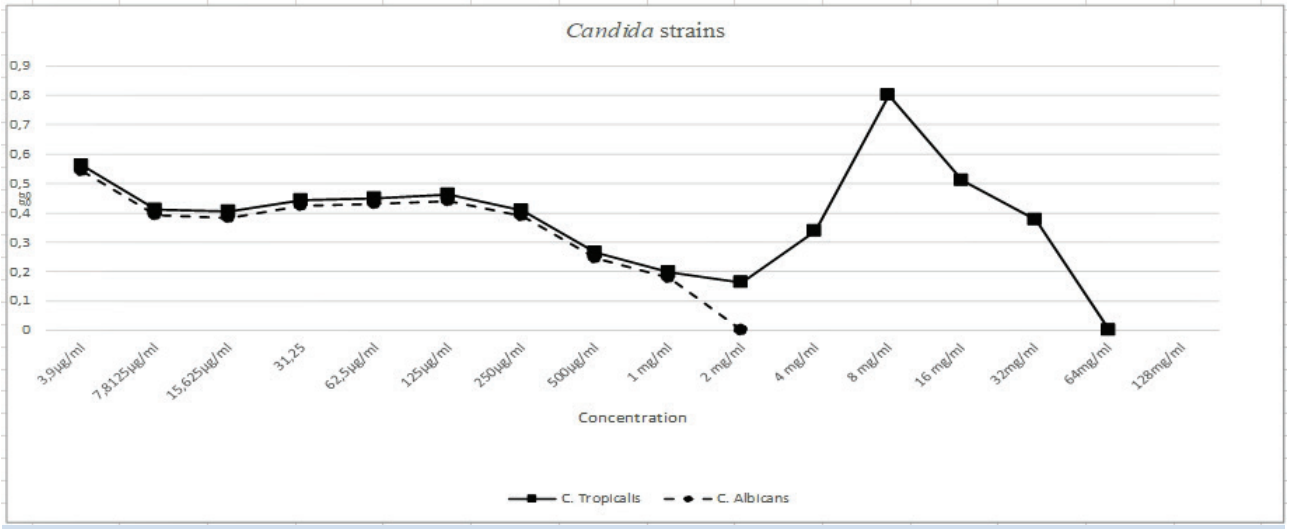


**Figure3.** Minimal inhibitory concentration (MIC) values of *Origanum munzurense* ethanol extract against *Candida tropicalis* ATCC 13803 and *C.albicans* 90028

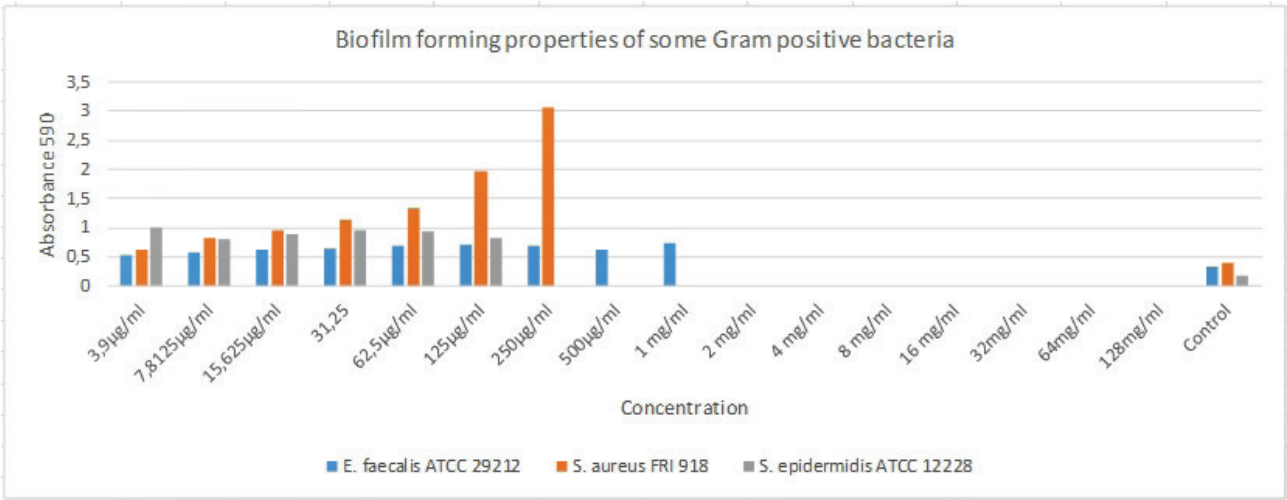


**Figure 4.** Antibiofilm forming activity of *Origanum munzurense* ethanol extract against *Enterococcus faecalis* ATCC 29212, *S. aureus* FRI 918 and *Staphylococcus epidermidis* ATCC 12228

The ethanol extract of *Origanum munzurense* plant inhibited biofilm formation in *E. faecalis* ATCC 29212 at a concentration of 2 mg/ml which falls into the range of MIC values. Morever, it prevented biofilm formations by *S. aureus* FRI 918 and*S. epidermidis* ATCC 12228 at concentrations of 500µg/ml and 250µg/ml, respectively. According to the results, *O.munzurense* extract showed the best inhibitory activity on biofilm formation by *S. epidermidis* ATCC 12228 strain with 250µg/ml concentration. It showed the least inhibitory effect on *E. faecalis* ATCC 29212 at a concentration of 2 mg/ml. For the other bacteria tested, no biofilm formation was observed under the conditions we studied.

## DISCUSSION

*Origanum munzurense* plant extract showed antimicrobial activity against all bacterial strains included in the present study. *Origanum* plant is known to contain large amounts of carvacrol and thymol. Kokkini et al. (1997) reported that carvacrol and thymol are the main monoterpene phenolic compounds, constituting approximately 78-85% of *Origanum* essential oil. The antimicrobial activity of these compounds is attributed to their lipophilic properties. These compounds disrupt cell homeostasis by increasing membrane fluidity, permeability and the balance of inorganic ions (Lambert et al., 2001). Due to the hydrophobic property of carvacrol, it can be accumulated in the cell membrane and stimulate conformational modification of the membrane resulting in cell death due to its hydrogen bonding and proton-releasing ability (Ben Arfa et al., 2006). In a study investigating antibacterial activity of *O. munzurense* extract and essential oils obtained by using different organic solvents and different extraction methods, antibacterial activity against *Klebsiella pneumoniae*, *E. coli, P. aeruginosa* and *S. aureus* was reported (Yabalak et al., 2020). Rodriguez-Garcia et al. (2016) reported that carvacrol and thymol make the cell membrane permeable, and this effect is greater against gram-positive bacteria. Badia et al. (2020) showed that adding *O. vulgare* essential oils to Tuscan sausage slows the growth of lactic acid bacteria that cause spoilage of most meat products and prolongs shelf life. *Origanum* essential oil was also shown to have strong antifungal activity against *C. albicans* and *C.parapsilosis* in the present study.

In this current study, *Origanum munzurense* extract inhibited biofilm formation of *E. faecalis* ATCC 29212, *S. aureus* FRI 918 and *S. epidermidis* ATCC 12228 bacteria. Kerekes et al. (2019) investigated the effectiveness of *O. majorana* (marjoram) and *Thymus vulgaris* (thyme) essential oils and their main components on the biofilm formed by *E. coli*, *L. monocytogenes*, *P. putida* and *S. aureus*. The MIC values for the bacteria ranged from 0.25mg/ml to 20 mg/ml. The investigated essential oils and their components were reported to have good antibacterial and antibiofilm-forming effects on the bacteria tested. These results are parallel to the results found in our study. Although MIC values ranged from 1-128 mg/ml for the bacteria tested, most of these MIC values were within 1-2 mg/ml in our study. In another study, Rossi et al. (2018) reported that *O.vulgare* essential oil prevented the formation of biofilms formed by *P. fluorescens* strains and changes its motility. It was suggested that

*O.vulgare* essential oil contributes to cell separation in preformed biofilm, reduces the thickness of the biofilm and disrupts its structure. Oral et al. (2010) determined the minimum inhibitory concentrations of *O. onites* essential oil on some microorganisms using broth dilution method, and MIC values for *Staphylococci* were within 0.05-0.8% (v / v).They reported that the use of essential oil at MIC levels inhibited biofilm formation and eliminated the preformed biofilm. The authors indicated that at concentrations below the MIC level, the biofilm formation level of microorganisms decreased. These results are in accordance with the present study in that biofilm inhibitory effect is at MIC values for the tested bacteria. In our study, *E. faecalis* ATCC 29212 strain prevented biofilm formation at MIC (2 mg/ml) determined. Moreover, biofilm formations were prevented for *S. aureus* FRI 918 and *S. epidermidis* ATCC 12228 at concentrations of 500 µg/ml and 250 µg/ml, respectively. These values are below MIC concentrations for *S. aureus* FRI 918, *S. epidermidis* ATCC 12228 strains. Čabarkapa et al. (2019) stated that the bioactive compounds in four essential oils obtained from *O. vulgare*, *O. heracleoticum*, *Thymus serpyllum* and *Thymus vulgaris* have inhibitory effects on biofilm formation and metabolic activity deterioration against *S.* enteritidis. They also reported that the investigated essential oil and essential oil components showed a concentration-dependent biofilm formation inhibitory activity. In our study, concentration of the extract inhibiting biofilm formation ranged from 250 µg/ml to 2 mg/ml for *E. faecalis* ATCC 29212, *S.aureus* FRI918, *S. epidermidis* ATCC 12228 strains.

## CONCLUSIONS

As a result, ethanolic extract of *O. munzurense* plant as a natural antimicrobial agent can be an effective alternative for controlling the tested microorganisms, bio-control strategies and maintenance of biofilm-free systems. The bacteria studied in this study are mainly found foodborne products. Therefore, *O. munzurense* can be an alternative plant-based agent to chemical preservatives and can be used as a natural antimicrobial agent for the preservation and safety of foods.

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