

## In vitro antimicrobial and antibiofilm-forming activities of *Origanum munzurense* plant extract on some foodborne gram-negative bacteria

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**Abstract:** Plants are an important source for the discovery of antimicrobial formulations due to the many biologically active components they contain. In this study, in vitro antimicrobial and antibiofilm forming activities of ethanolic extract of *Origanum munzurense* were investigated against some strains of foodborne pathogenic bacteria. To investigate the antimicrobial activity, the minimal inhibitory concentration values against the selected bacteria and antibiofilm-forming activity were tested with the liquid microdilution method and microplate method, respectively. MICs of *Origanum munzurense* extract against the bacteria tested are 32 mg/mL for *Escherichia coli* O157: H7 ATCC 35150, 32 mg/mL for *Escherichia coli* O157: H7 ATCC 43895, 16 mg/mL for *Salmonella enteritidis* ATCC 13076, 16 mg/mL for *Salmonella typhimurium* ATCC 700408, 32 mg/mL for *S. typhimurium* ATCC 14028, and 2 mg/mL for *Pseudomonas fluorescens* ATCC 13525. *Origanum munzurense* showed the best antibacterial effect on *Pseudomonas fluorescens* ATCC 13525 strain. The least antibacterial effect was shown on *E. coli* O157:H7 ATCC 35150, *E. coli* O157:H7 ATCC 43895, and *Salmonella typhimurium* ATCC 14028. *Origanum munzurense* extract inhibited biofilm formation on *Salmonella enteritidis* ATCC 13076 strain at a concentration of 16 mg/mL. Antibiofilm formation was not observed for other bacteria. Total phenolic content of *Origanum munzurense* was 539 mg of gallic acid equivalent (GAE)/L. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of *munzurense* extract was determined to be 18 µg/L. In conclusion, *Origanum munzurense* ethanol extract has a range of antibacterial effects on the selected foodborne pathogenic bacteria and showed antibiofilm-forming activity especially for *S. enteritidis* ATCC 13076. In addition, the plant has the prominent amount of phenolic content with antioxidant and radical scavenging activity.

**Key words:** Antibiofilm, antimicrobial, minimal inhibitory concentration, *Origanum munzurense*, radical scavenging activity, total phenolic content

### 1. Introduction

Plants have been used in traditional medicine for the treatment of various diseases for centuries [1]. The important benefits of using herbal medicines are that they are safer than synthetic alternatives, have less toxic effects, have fewer side effects compared to synthetic antimicrobials, and offer affordable treatment [2]. *Origanum* (O.) species, which are also known as thyme, are aromatic perennial plants that belong to the family *Lamiaceae* [3, 4]. *Origanum* genus is represented by 22 species and 32 taxa in Türkiye. Of these, 21 are native to Türkiye [5]. Among the *Origanum* species, *O. munzurense* is an endemic species to Tunceli Province of Türkiye. It has been reported that this species is a hybrid between

two taxa named *O. acutidens* and *O. vulgare*. Although most of the distribution areas of these two species are similar, it has been reported that Tunceli Province is the only place where they form hybrids [6]. It has been reported that all species in this family contain high levels of essential oil, and the primary ingredients of this essential oil are thymol or carvacrol [7]. It is important for the pharmaceutical and cosmetic industry due to the essential oil content [8]. Studies have shown that essential oils produced from *Origanum* species have different properties such as antibacterial, antiviral, antifungal, antioxidant, antiinflammatory, and genotoxic effects [9, 10]. In addition, they are used in the treatment of diarrhea, cramps, rheumatic diseases, asthma, nausea, digestive

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problems, and infectious diseases among the people [11]. *Origanum* species are also used as herbal tea and spice in foods. *Origanum* species have different morphological and chemical components. These include linalool, thymol, carvacrol, and p-cymene which have been shown to have antimicrobial effects [3, 4]. Gram-negative bacteria were reported to be the pathogens that cause most foodborne diseases. It is known that gram-negative bacteria can be affected by heat treatment, freezing, high pressure, and essential oils of plant origin, and this is important in the food industry [12].

Biofilm is defined as a microecosystem created by various microorganisms to protect them from the environment and maintain their vital functions [13]. This structure benefits the escape of microorganisms from host defense and the development of resistance against antibiotics. It has been reported that this structure is responsible for different infectious diseases [14]. Since bacteria living in biofilms pose a health problem, various therapeutic agents are needed to prevent biofilm formation by bacteria [15].

Antimicrobials kill or stop the growth or proliferation of pathogenic microorganisms. These substances include antibiotics, preservatives, synthetic drugs, and natural products derived from animals or plants [16]. In recent years, excessive use of antibiotics and chemical antimicrobials to prevent bacterial infections seriously threatens public health and leads to resistance to drugs [17]. In addition, various synthetic preservatives are used in the food industry to extend shelf life and protect foods, which accumulate in foods and create serious public health problems. For this reason, nowadays, it has been recommended to use natural products instead of synthetic or conventional drugs [18].

*O. munzurensis* is an endemic species in Türkiye with limited studies on the antimicrobial activity and antibiofilm-forming activity against foodborne bacteria. In this study, it was aimed to determine in vitro antimicrobial and antibiofilm-forming activity of the ethanolic extract of *O. munzurensis* against some selected foodborne microorganisms known to be pathogenic to humans.

## 2. Materials and methods

### 2.1. Collection of plant specimens

Plants samples were collected from Ovacık in Tunceli in Eastern Türkiye. The identification of the plant species was performed by Ahmet Savran, a faculty member at Niğde Ömer Halisdemir University, Faculty of Science and Literature, Department of Biology-Botany.

### 2.2. Extraction of plant specimens

After the plant samples were collected, they were cleaned and the leaves and other components of the samples were separated and dried for 20 days without exposure to

sunlight. Leaf parts of the dried specimens were ground and powdered. Twenty grams of samples was taken, placed in the soxhlet device in a cellulose cartridge, and extracted with 500 mL of ethanol at 50 °C for 3 h. The ethanol in the solution was evaporated at 40 °C by a rotary evaporator (Wist, Wev 1001V). The extract obtained after condensation in the vacuum evaporator was stored at -20 °C until it was used [19, 20].

### 2.3. Preparation of stock plant extracts

The plant extract prepared with ethanol was dissolved in distilled water and 2% dimethyl sulfoxide (DMSO) with an initial concentration of 500 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.

### 2.4. Reference microorganisms used in the study

*Escherichia coli* O157:H7 ATCC 35150, *Escherichia coli* O157: H7 ATCC 43895, *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* ATCC 700408, *Salmonella typhimurium* ATCC 14028, and *Pseudomonas fluorescens* ATCC 13525 were used in the study.

### 2.5. Media used

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

### 2.6. Antibacterial and antibiofilm activity test

Pathogenic microorganisms were inoculated into TSA medium by spread plate method and incubated at optimum temperature (37 °C) overnight. Bacteria were dissolved in 10 mL of 0.9% sterile NaCl, and the bacteria concentration was adjusted to McFarland 0.5 ( $1 \times 10^8$  CFU/mL) using a densitometer (Alla, France). Cation-adjusted Mueller-Hinton Broth (CAMHB) containing various concentrations of *O. munzurensis* 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) was dispensed under aseptic conditions to 200 µL 96-well plates (Corning Costar 3599, flat bottom). Next, 20 µL of the prepared bacterial suspension was added into the wells. After the microplate was incubated at 37 °C for 24 h, absorbance values were measured at 600 nm by a microplate reader (BioTek Epoch, USA) [21].

### 2.7. Investigation of the antibiofilm property of *O. munzurensis* extract

The bacteria produced in TSA at 37 °C for 16 h were taken with a sterile swab and dissolved in 10 mL of 0.9% NaCl, and their concentration was adjusted to McFarland 0.5 with the help of a densitometer (Alla, France). Cation-adjusted Mueller-Hinton Broth (CAMHB) containing 200 µL of *O. munzurensis* extract was dispersed into 96-well microplates (Corning 3599), and 20 µL of bacteria

suspension was added into the wells and incubated at 37 °C for 24 h. At the end of 24 h, plates were washed 3 times with 200 µL of phosphate-buffered saline. The bacteria were fixed for 15 min by adding 200 µL of methanol. After the methanol was poured from the plates, the bacteria adhering to the microplate surface, which was dried at 55 °C for 1 h, were dyed with 200 µL crystal violet (Sigma-Aldrich, 109218) and the excess paint was removed by washing with tap water after 5 min. After the microplate was dried, the adhering dye was dissolved with 33% acetic acid (Merck, 100063) (200 µL), and its optical densities (OD) were measured at 590 nm (BioTek Epoch, USA) [22].

### 2.8. Measurement of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

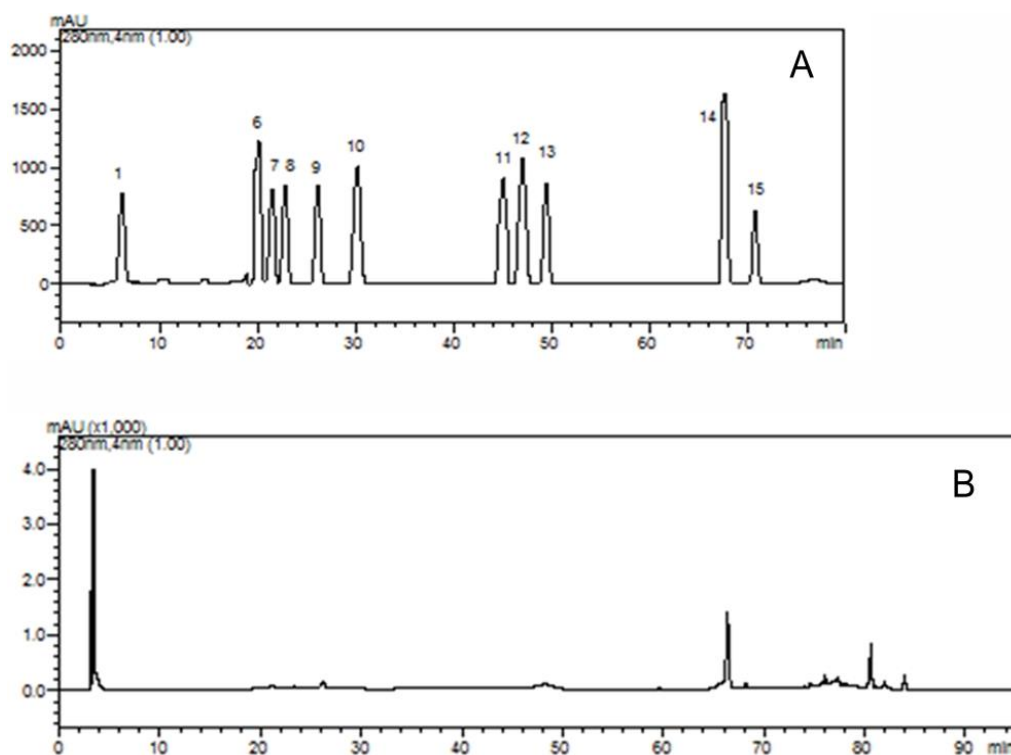
The antioxidant activity of *O. munzurense* extract was determined concerning its DPPH radical scavenging activity [23]. After taking 20, 40, 60, 80, and 100 µL of the extract into glass tubes, 600 µL of 1 mM DPPH radical was added into the tubes and the final volume was completed to 6 mL with ethyl alcohol. After 15 min of incubation at room temperature in the dark, the absorbance was read against the control solution prepared by adding pure ethyl alcohol and DPPH radical at 517 nm wavelength. The results are given as mg/L of IC<sub>50</sub> value.

### 2.9. Determination of phenolic compounds

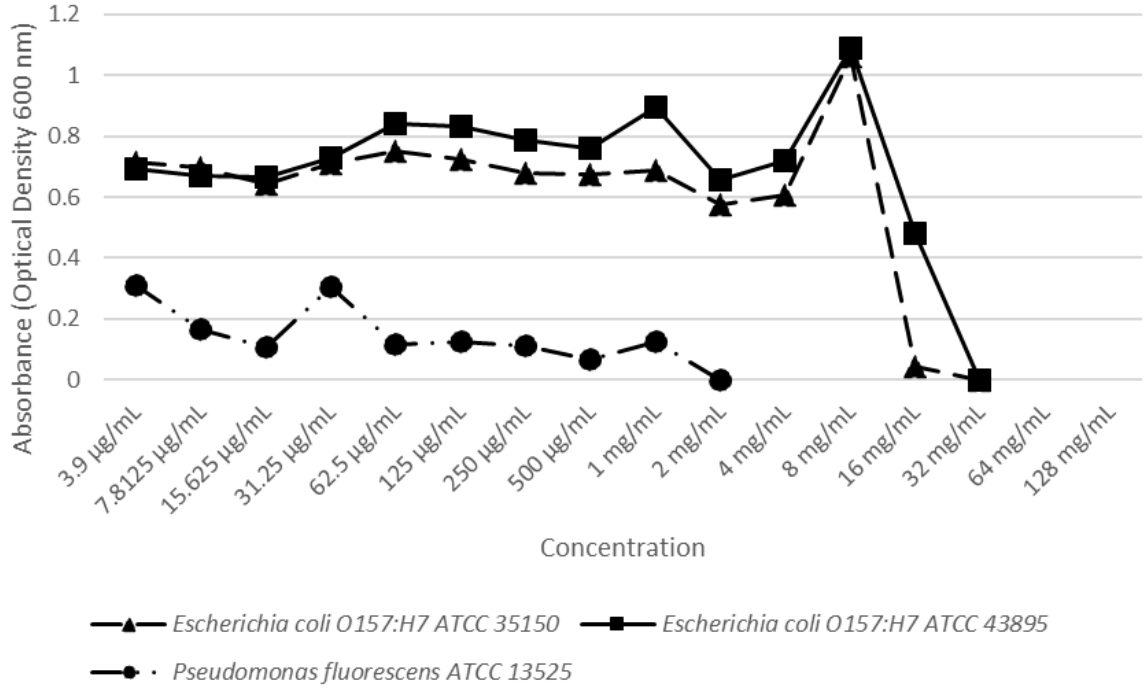
Using the Folin-Ciocalteu reagent, the total phenols, expressed as gallic acid (mg/L), were measured colorimetrically at 765 nm. Phenolic components of the extract were evaluated both qualitatively and quantitatively by HPLC [Shimadzu Prominence; Detector: DAD (SPD-M20A), Column: Zorbax C18(250\*4.6 mm, 5 microns)] using the method of Caponio et al. [24] with slight modification; 0.2 g of sample was weighed, dissolved in mobile phase, passed through a 0.45-µm filter, and injected into the HPLC system. The eluates were detected at 278 nm. In the analysis, the mobile phase was composed of A: 3% formic acid, and B: methanol. Figure 1 shows HPLC elution profile of a mixture from the standard phenolic compounds (A) and HPLC elution profile of the phenolic extract from *O. munzurense* (B).

### 3. Results

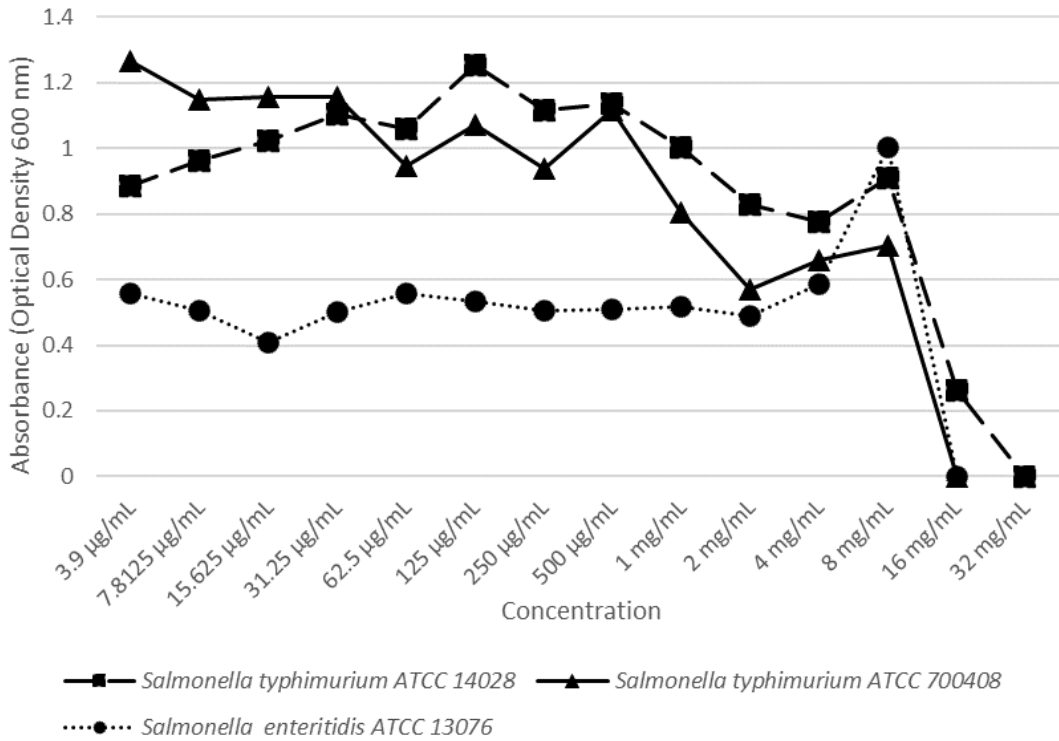
According to the liquid microdilution method, MIC values of the *O. munzurense* plant extract on selected pathogenic gram-negative bacteria strains are shown in Figures 2 and 3. Antibiofilm-forming activity of *O. munzurense* is shown in Figure 4. *O. munzurense* extract showed an inhibitory effect on *E. coli* O157:H7 ATCC 35150 and *E.*



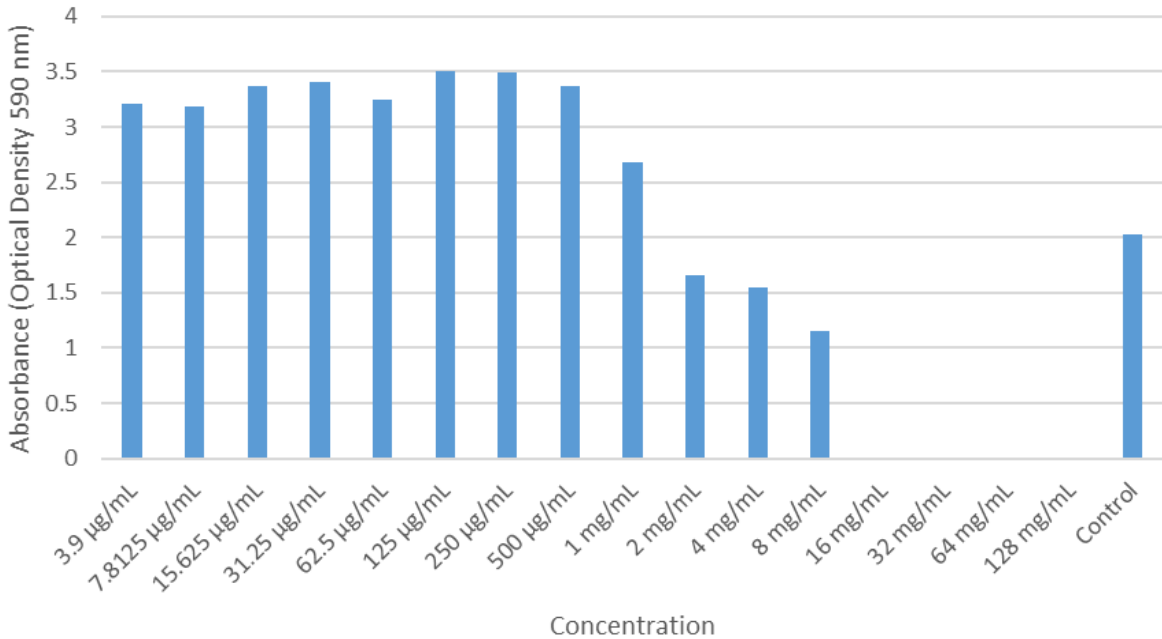
**Figure 1.** HPLC elution profile of a mixture from the standard phenolic compounds (A): gallic acid (1), vanilic acid (6), epicatechin (7), caffeic acid (8), *p*-Coumaric acid (9), ferulic acid (10), rutin (11), ellagic acid (12), naringin (13), cinnamic acid (14), quercetin (15), and HPLC elution profile of the phenolic extract from *Origanum munzurense* (B).



**Figure 2.** Minimum inhibitory concentration values of *Origanum munzurense* ethanol extract against *Escherichia coli* O157:H7 ATCC 35150, *Escherichia coli* O157:H7 ATCC 43895, and *Pseudomonas fluorescens* ATCC 13525.



**Figure 3.** Minimum inhibitory concentration values of *Origanum munzurense* ethanol extract against *Salmonella typhimurium* ATCC 14028, *Salmonella typhimurium* ATCC 700408, and *Salmonella enteritidis* ATCC 13076.



**Figure 4.** Antibiofilm-forming activity of *Origanum munzurense* ethanol extract against *Salmonella enteritidis* ATCC 13076.

*coli* O157:H7 ATCC 43895 strains at a concentration of 32 mg/mL. It showed an inhibitory effect on *S. typhimurium* ATCC 14028 strain at a concentration of 32 mg/mL, while it showed an inhibitory effect on *S. typhimurium* ATCC 700408 strain at a concentration of 16 mg/mL. It was determined that *Salmonella enteritidis* ATCC 13076 strain showed an inhibitory effect at a concentration of 16 mg/mL. The inhibitory effect was determined on *Pseudomonas fluorescens* ATCC 13525 strain at a concentration of 2 mg/mL. According to the results we obtained, the extract showed the best antibacterial effect on *Pseudomonas fluorescens* ATCC 13525 strain. The least antibacterial effect was shown on *E. coli* O157:H7 ATCC 35150, *E. coli* O157:H7 ATCC 43895, and *Salmonella typhimurium* ATCC 14028.

Ethanol extract of *O. munzurense* plant inhibited biofilm formation on *S. enteritidis* ATCC 13076 at a concentration of 16 mg/mL. *O. munzurense* ethanol extract inhibited the formation of biofilms in the MIC range of *S. enteritidis* ATCC 13076 bacteria. In addition, *O. munzurense* ethanol extract inhibited biofilm formation on *S. enteritidis* ATCC 13076 bacteria, depending on the dose. Some doses of *O. munzurense* ethanol extract forced *S. enteritidis* ATCC 13076 bacteria to form biofilms.

Total phenolic content of ethanol extract of *Origanum munzurense* was found to be 539 mg of gallic acid equivalent (GAE)/L. Individual phenolic compounds and their amounts determined in the extract are shown in

Table. DPPH radical scavenging capacity of *O. munzurense* extract was determined to be 18 mg/L.

#### 4. Discussion

*O. munzurense* plant extract showed antimicrobial activity against all bacterial strains included in the study. However, the degree of antibacterial activity varied with respect to the bacterium studied. *Origanum* species are known to contain significant amounts of thymol and carvacrol. Kokkini et al. [25] defined that carvacrol and thymol are the main monoterpene phenolic compounds, which make up about 78%–85% of the essential oil of *Origanum*. Their lipophilic properties confer the antimicrobial activity of these compounds. These compounds increase membrane permeability and fluidity and disrupt the cell's homeostasis [26]. Arfa et al. [27] reported that carvacrol with the hydrophobic property can accumulate in the cell membrane and stimulate the conformational modification of the membrane resulting in cell death due to its ability to bind hydrogen and release protons. Guarda et al. [28] reported that carvacrol and thymol can break down the outer membrane of gram-negative bacteria and release lipopolysaccharides and increase the permeability of adenosine triphosphate in the cytoplasmic membrane and change the passive permeability of the cell. Some different *Origanum* species were shown to have an antimicrobial effect; for example, *O. vulgare* had an antimicrobial effect on *S. enteritidis*. Esen et al. [29] reported that *O. vulgare*

**Table.** Phenolic compounds identified in the ethanolic extract of *Origanum munzurense*.

Phenolic content	Amount of the phenolic compound (µg/g)
Gallic acid	0.144
Vanilic acid	27.659
Caffeic acid	1731.949
Epicatechin	667.776
<i>p</i> -Coumaric acid	0.055
Ferulic acid	295.226
Naringin	152.058
Rutin	88.102
Ellagic acid	24973.473
Cinnamic acid	11.376
Quercetin	74.934

essential oil exhibited an antibacterial effect against all microorganisms used in their study (*E. coli*, *S. aureus*, *E. aerogenes*, *P. aeruginosa*, *C. albicans*, *P. vulgaris*). In another study, Sari et al. [30] investigated the minimum inhibitory concentration (MIC) of essential oils obtained from 23 samples of *O. glandulosum* Desf against 6 standard bacteria strains (*Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus hirae*, *Staphylococcus aureus*, *Candida tropicalis*, *Candida albicans*). The authors reported that the essential oils inhibit the growth of the microorganisms. All microbial strains used (gram-positive and gram-negative bacteria and yeast) were reported to show a fairly similar sensitivity to the essential oils studied, although no significant difference was observed in their sensitivity. It has also been stated that gram-positive bacteria are more sensitive to essential oil samples. This condition is thought to be caused by changes in the cell wall structures of gram-positive bacteria. Gram-positive bacteria have higher sensitivity and a simple single-layered cell wall structure, while the cell wall of gram-negative bacteria is multilayered and complex [31]. The outer membrane of gram-negative bacteria generally consists of lipopolysaccharide molecules and forms a hydrophilic permeability barrier that provides protection against the effects of large amounts of hydrophobic compounds [32]. Therefore, it can be expected that the plant extract will have a better antimicrobial effect on gram-positive bacteria. In another study, Badia et al. [33] reported that adding *O. vulgare* essential oils to Tuscan sausage extended the shelf life and slowed the growth of lactic acid bacteria, which cause spoiling of most meat products. Aliğani et al. [34] investigated the effects of essential oils obtained from *O. scabrum* and *O. microphyllum* on gram-negative, gram-positive bacteria and 3 fungi. It was shown that *O. scabrum* oil contains 74.86% carvacrol, exhibiting a strong

activity against all microorganisms tested. Yabalak et al. [9] reported that *O. munzurense* extract and its essential oils, which they obtained using different solvents and different extraction methods, showed antibacterial activity against *K. pneumoniae*, *E. coli*, *P. aeruginosa*, and *S. aureus*.

In the current study, *O. munzurense* extract inhibited biofilm formation on *S. enteritidis* ATCC 13076 bacterium. It has been reported that essential oils from thyme have the potential to inhibit the biofilm formation from the bacteria. Rossi et al. [35] reported that thyme essential oil inhibited the biofilm formed by *P. fluorescens* strains and changed its mobility. They stated that *O. vulgare* essential oil helps the separation of cells in preformed biofilms, decreases the thickness of the biofilm, and disrupts its structure. Oral et al. [36] reported that the use of essential oil of *O. onites* at MIC level prevented biofilm formation and eliminated previously formed biofilm. They also stated that at concentrations below the MIC level, microorganisms decrease the level of biofilm formation. Inhibition of biofilm formation at MIC value by *Origanum* supports our results. In our study, *S. enteritidis* strain prevented biofilm formation at MIC (16 mg/mL) value. Cabarkapa et al. [37] stated that the bioactive compounds from *O. vulgare*, *O. heracleoticum*, *Thymus vulgaris*, and *Thymus serpyllum* resulted in disruption of metabolic activity and inhibition of biofilm formation against *S. enteritidis* in a dose-dependent manner. Similarly, biofilm formation on *S. enteritidis* was prevented in our study depending on the dose. Burt et al. [38] reported that carvacrol, one of the important antimicrobial components of thyme oil, prevented the formation of biofilms on *S. typhimurium*. Lira et al. [39] reported that oregano essential oil reduced the number of *S. enteritidis* 86 bacteria over time in biofilms previously formed by *S. enteritidis* 86 on stainless steel. In another study, carvacrol (0.5%, 2%, and 5%) and

4 different pathogenic bacteria (*E. coli* O157:H7, *B. cereus*, *S. enterica*, and *S. aureus*) were added to a dough made from corn flour. It has been reported that all strains are completely inactivated within 24 h [40]. Kerekes et al. [41] investigated the efficacy of *Thymus vulgaris* essential oils and their main components on biofilms formed by *E. coli*, *Pseudomonas putida*, *Listeria monocytogenes*, and *Staphylococcus aureus*. The MIC for bacteria ranged from 0.25 to 20 mg/mL. EOs and EO components showed a good antibacterial and antibiofilm-forming effect on the bacteria tested.

Phenolic compounds in plants are the main antioxidant elements that play an important role in the prevention of the harmful effect of free radicals and reactive species [10, 11]. In our study, the total phenolic content of ethanol extract of *O. munzurense* was found to be 539 mg GAE/L. Yabalak et al. [9] found that the total phenolic content of *O. munzurense* was highest in the ethanol extract among the other solvents including methanol, water, acetonitrile, and water extract. The authors reported that the ethanol extract of the plant has a very rich phenolic content. The highest amount of individual phenolic compound determined in the extract was ellagic acid (24973.47 µg/g), while the least amount of individual phenolic compound determined in the extract was *p*-Coumaric acid (0.055 µg/g). Total polyphenol content we obtained is higher when compared

to the results reported in the literature, and *O. munzurense* is rich in antioxidant content.

Since DPPH is a convenient and reliable method used in the determination of radical scavenging activity of a particular compound [9]. The DPPH method was performed in the antioxidant activity analysis of *O. munzurense*. DPPH radical scavenging activity was found to be 18 mg/L. The findings of the present study indicate that ethanol extract of *O. munzurense* has a powerful radical scavenging activity. Similarly, previous studies on the radical scavenging activity of *Origanum* species showed high radical scavenging activity [9, 10, 42, 43, 44].

As a result, it can be concluded that the *Origanum munzurense* plant, as a natural antimicrobial agent, could be an effective alternative in controlling the microorganisms tested and could be used as a natural antimicrobial agent for the quality, biocontrol strategies, and safety of foods. In addition, findings indicate that the plant has a prominent amount of phenolic content with antioxidant and radical scavenging activity.

#### Conflict of interest

The authors declare that there are no conflicts of interest.

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