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Research Article

Evaluation of the Antibiofilm and Antimicrobial Properties of *Ziziphora tenuior* L. Essential Oil Against Multidrug-resistant *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii is one of the most important gram-negative microorganisms which lead to opportunistic hospital-acquired infections. A great part of the infections it causes is produced by strain resistant to all the antibiotics used. In our study, the Essential Oil (EO) of the *Ziziphora tenuior* L. antimicrobial and antibiofilm effects on multidrug resistant (MDR) *A. baumannii* isolates were researched. In addition, antioxidant, cytotoxic activity and chemical composition of EO were investigated. As a result of the gas chromatography-mass spectrometry (GC-MS) analysis of the *Z. tenuior*EO pulegone was found as the major component by 74.37%. It was observed when the antimicrobial activity of the EO was examined that it had >30 mm disc diffusion values. Minimal Inhibition Concentration (MIC) values were found between 0.6-1.25 $\mu\text{L mL}^{-1}$ and Minimal Bactericidal Concentration (MBC) values were found between 2.5-5 $\mu\text{L mL}^{-1}$. Minimal biofilm inhibition concentration (MBIC) values of the EO were found as 0.3-1.25 $\mu\text{L mL}^{-1}$ and Minimal Biofilm Eradication Concentration (MBEC) value as 5-10 $\mu\text{L mL}^{-1}$. It was seen that the MIC value damaged the biofilm formations constituted by the *A. baumannii* strains by 51-84%. At 25, 12.5 and 6.25% EO concentrations, no cytotoxic appeared for the fibroblast cells in terms of the cytotoxic activities ($p>0.05$). Findings that were obtained in our study seem promising for the development of phytotherapeutic agents that could be used in the treatment of the MDR *A. baumannii* infections.

Key words: *Acinetobacter baumannii*, *Ziziphora tenuior*, biofilm, antibiofilm activity, multidrug resistant

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Discovery of the antibiotics achieved a great breakthrough in the field of medicine, saved numerous lives and become a great turning point within the history of humanity. However, rapidly developing resistant strains accompanied this awesome discovery (Davies and Davies, 2010). *Acinetobacter baumannii* is one of the most important Gram negative microorganisms which lead to opportunistic hospital-acquired infections all around the world. In particular, the infections it causes are produced by the strains resistant to all the antibiotics used (Davies and Davies, 2010; Antunes *et al.*, 2014).

Roles of various factors in the growth of resistant strains were showed with laboratory studies. One of these factors is biofilms (Davies and Davies, 2010; Gillings *et al.*, 2009). Sessile microorganisms in the biofilms can be more resistant up to 1000 times against an agent compared to the planktonic forms of the same microorganism. Therefore, it is important to realize the relationship between the biofilm and the resistance for struggling against the bacterial infections (Penesyanyan *et al.*, 2015).

The problem of resistance against the antibiotics, which has been increasing substantially, has led to the need for searching new treatment agents. Today, natural products seem privileged with regard to this matter and the interest in herbal drugs is stirring up again. The potential activities of herbal extracts and some of their constituents in struggling with resistant bacteria are extensively researched today (Yap *et al.*, 2014).

The *Ziziphora* genus is a plant which is represented with five species and two sub-species spread in many regions of Turkey (Kaya *et al.*, 2013). It is known that *Ziziphora* species have an antimicrobial effect on some microorganisms and traditionally used with the purpose of medical treatment by scientific environments and for the treatment of various diseases within public (Dakah *et al.*, 2014; Ozturk and Ercilsi, 2007; Pirbalouti *et al.*, 2013).

In our study, we aimed at determining the antimicrobial and antibiofilm effect of the *Ziziphora tenuior* L. Essential Oil (EO), which was obtained from the Aegean region of Turkey, on the Multi-Drug Resistant (MDR) *A. baumannii* isolates and the chemical composition of this oil, its antioxidant effect and cytotoxic values. We assume that our study will contribute to the literature on struggling against the multidrug resistant microorganisms, which is very significant today.

MATERIALS AND METHODS

Plant material and extraction of essential oil: Aboveground organs of the plant, which was collected from the flora in the vicinity of Manisa in the Aegean region of Turkey, were dried in the dark in the laboratory environment. The essential oil was obtained with the hydrodistillation method using the cleverger apparatus after the milling process.

Analysis and Identification of the oil: Chemical composition of *Z. tenuior* EO was designated according to the method applied by Aksit *et al.* (2013). In split mode, 50:1, a Perkin-Elmer Clarus 500 Series GS system equipped with a Flame Ionization Detector (FID) and BPX-5 apolar capillary column (30 m×0.25 mm, 0.25 m i.d.) equipped with a mass spectrometer were used for the GC analyses. The carrier gas used was helium (1.0 mL min⁻¹). The injector temperature was set at 250°C and the FID was operated at 250°C. An initial column oven temperature of 50°C was elevated to 220°C at a rate of 8°C min⁻¹ and held for 5 min. The requirements of the mass spectrometer included 250°C transfer line temperature, 250°C ion source and 70 eV energy of ionization. Standard components existed for most of the EO constituents and the Van den Dool and Kratz equation was used in pursuant of the retention times of homolog n-alkane series to determine Kovats Retention Indices (Ri) for all the sample components. Calculation of the compounds' relative peak area percentages relied on the FID data.

Cytotoxicity of *Ziziphora tenuior* essential oil: A mouse connective tissue fibroblast cell line, L929 (ATCC cell line, NCTC clone 929) was cultured in RPMI medium (Sigma, St. Louis, MO, USA) supplemented with 10% Fetal Calf Serum (FCS) (Sigma, St. Louis, MO, USA) and 2 mM mL⁻¹ L-glutamine. The medium with the cell culture had no antibiotic addition. Cultivation of the cultures was performed in an incubator with 5% CO₂ and at 37°C until confluence was observed in the cell monolayer, which occurred after about 7-8 days. At all times, the exponential growth phase of the cells was the period when assays were applied.

The study was implemented on the basis of the method used by Polat *et al.* (2014). The number of the viable cells is analyzed by the proliferation assay through the cleavage of tetrazolium salts, which are added to the culture medium and for this, the water soluble tetrazolium (WST-1) labeling reagent (Roche, Mannheim, Germany) is used. Ninety six-well microtiter plates were used to seed the cells and the concentration was 1×10⁵ cells mL⁻¹ within a 100 µL final volume for each well. Treatment of the cells was carried out

after 24 h and different *Z. tenuior* EO concentrations were used, that is, 100, 50, 25, 12.5 and 6.25% v/v. Incubation of the cells took 24 h and performed within a humidified atmosphere (37°C, 5% CO₂). Then, 10 µL of the WST-1 labeling reagent were added to 10 µL of culture medium in each well. For the measurement of the sample absorbance at 450 nm against the control, a microtiter plate reader (Thermo Scientific Microplate Photometer, Multiskan FC, USA) was used. Measurement of the absorbance was performed 2 h following the beginning of the tetrazolium reaction. The percentage viability was obtained by comparing the Optical Densities (OD) of the samples and the negative control:

$$\text{Cell viability (\%)} = \frac{\text{OD}_{450} (\text{sample})}{\text{OD}_{450} (\text{negative control})} \times 100$$

2,2-diphenyl-1-picrylhydrazyl (DPPH) photometric assay:

Antioxidant activity of *Z. tenuior* EO was determined using the DPPH photometric assay (Golfakhrabadi *et al.*, 2015). From the EO samples at different concentrations, 15 µL were added to 5 mL of a 0.004% of DPPH in methanol. The reaction mixture was mixed in the vortex mixer and kept in the dark at the room temperature for 60 min. At the end of the duration, the absorbance of the mixture was read against a blank at 517 nm in the spectrophotometry. Ascorbic acid was used as the positive control. All the tests were conducted three times. The following formula was used for the calculation of the percentage of the radical scavenging activity that belonged to the EO:

$$\text{Inhibition of DPPH (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

A₀ = Control absorbance

A₁ = Sample absorbance

Microorganisms: *Acinetobacter baumannii* strains used in the study were isolated in the clinical microbiology laboratories of Cumhuriyet University Hospital between 2014 and 2015. Bacteria were identified in line with the working instructions of the manufacturer by using the BD Phoenix (Becton Dickinson, Sparks, MD, USA) automatized microbiology system and antimicrobial susceptibility tests were conducted. Resistance and susceptibility of the strains against the antimicrobials were assessed as per the Clinical Laboratory Standards Institute CLSI document (CLSI, 2014).

Antimicrobial and antibiofilm activity

Disc diffusion assay: After the incubation of the MDR *A. baumannii* strains in the nutrient broth at 37°C, 100 µL

from the suspensions prepared as per 0.5 McFarland was spread over the nutrient agar surface. The disc (6 mm in diameter) were impregnated with 20 µL of the EO (10 µL disc⁻¹) at the concentration of 500 µL mL⁻¹ and placed on inoculated agar. As the negative control, Tween 20, which is the solvent of the EO, was used. Incubation of the inoculated plates was performed at 37°C. The inhibition zone was measured to assess the antimicrobial activity against the *A. baumannii*. In this experiment, each assay was applied three times.

Microdilution assay: The broth microdilution method which was implemented to find out the antimicrobial activities of *Z. tenuior* EO was applied in line with the suggestions of the (CLSI, 2014). A micro-well dilution method was taken as a basis for the discovery of the *Z. tenuior* EO's minimum Inhibitory Concentration (MIC) values against the bacterial strains. Twenty four hours broth cultures of the bacteria were prepared as per the standard of 0.5 McFarland. After the dissolution of the *Z. tenuior* EO in the nutrient broth which had 0.5% (v/v) Tween 20 for the purpose of testing, serial two fold dilutions were prepared in nutrient broth-containing 96-well plates at a concentration varying between 0.03-2% (v/v). Whereas only the nutrient broth was used as the negative control, bacteria suspensions which didn't contain EO were used as the positive control. A Bio Tek microplate reader (Bio Tek Instruments, Inc., Winooski, VT, USA) was used for the measurement of the OD at 550 nm. The optical density at the 24th h of the inoculum didn't change or decrease at the MIC, which was found as the lowest EO concentration, when compared to the reading at the beginning. For Minimum Bactericidal Concentration (MBC) determination, 10 µL was taken from each well after incubation and spot inoculated onto nutrient agar and incubated for 24 h at 37°C. The concentration at which no growth was observed on subculture was determined as the MBC.

Minimum biofilm inhibitory concentration assay: The lowest concentration at which the antimicrobial agents prevent the biofilm formation is called Minimum Biofilm Inhibitory Concentration (MBIC) (Macia *et al.*, 2014). In order to determine the antibiofilm activity of the *Z. tenuior* EO on the MDR *A. baumannii* strains, the microtiter plate method was used. This method was applied with some modifications as described by Heydari and Eftekhari (2015). Twenty four hours cultures of the *A. baumannii* strains in the nutrient broth with the addition of 1% (w/v) glucose were prepared as per 0.5 McFarland (10⁸ CFU mL⁻¹) and then put into 96 wells with 100 µL per each. Hundred µL was added from the 0.03-2%

(v/v) concentrations of the EO into each well. While wells that had no EO were employed as the negative control, the cell cultures without EO addition were used as the positive control. The supernatant was washed three times using sterile distilled water and eliminated after the incubation at 37°C for 48 h. After the plates were dried with air during 30-40 min, they were stained with 0.1% (w/v) crystal violet. Distilled water was used to wash the plates three times after 30 min and then they were dried. Afterwards, 95% ethanol was used to solubilize the crystal violet and the absorbance was read through a microplate reader (Bio Tek Instruments, Inc., Winooski, VT, USA) at 550 nm. The MBIC was characterized as the EO concentration and it had OD \leq negative control. This test was performed in three times and the final reading was taken as an average of the three readings used further.

Minimum biofilm eradication concentration assay:

Minimum Biofilm Eradication Concentration (MBEC) is the lowest concentration which can damage the structure of the emerging biofilm (Macia *et al.*, 2014). The method that we applied to determine the MBEC resembles the method applied by Perumal (2013). Two hundred microliters were taken from *A. baumannii* strains (10^8 CFU mL⁻¹) and inoculated into 96-well microtiter plate wells and subjected to incubation at 37°C for 48 h. The medium was blotted out following the formation of the biofilm and Phosphate Buffered Saline (PBS) was used to wash the well three times for the purpose of eliminating non-adherent cells. Serial dilutions of the EO (from 0.03-2% (v/v)) were distributed into all the wells and applied to the emerging biofilm structures. Incubation of the plates was conducted at 37°C for 24 h and then the wells were washed with the PBS, afterwards, the crystal violet staining method was used to stain them, which is described above. The positive control was biofilm without EO. The existing biofilms were eradicated from the bottom of the treated wells at a concentration which was considered as the MBEC.

Percentage of the biofilm eradication:

The MDR *A. baumannii* strains were helped to form biofilm with the method explained in the MBEC determination study. After the biofilm formation, MIC value of EO was prepared and 200 μ L was distributed into each well. Wells with no EO addition were considered as the control. Following the 24 h incubation, the wells were washed with the PBS twice and was read at 550 nm. The percentage of biofilm eradication was calculated as per the formula presented by Chaieb *et al.* (2011)

$$\frac{\text{OD growth control} - \text{OD sample}}{\text{OD growth control}} \times 100$$

Biofilm metabolism assay by XTT: The evaluation of the metabolic activity of the biofilms, which had been formed by *A. baumannii*, was carried out through a modified tetrazolium salt XTT [2, 3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] reduction study that was accommodated from Chaieb *et al.* (2011). Reagents were mixed to obtain 50/1 XTT agent (Labeling reagent)/activation agent (electron coupling reagent) and the XTT solution was prepared. The MIC and 2 MIC of *Z. tenuior* EO were applied to the 48 h biofilm structures formed in 96 well plates. The XTT kit which was prepared after the 24 h incubation was added to each test and control well. At the end of the 5 h incubation period, 450 nm wave length was read at the microplate reader.

Data analysis: The values were obtained as Mean \pm Standard Deviation (SD) as a result of three repetitions and then their statistical analysis was conducted through the one-way analysis of variance (ANOVA), the confidence level was 95% for multiple comparisons and student's t-test for comparisons between two groups. Values were considered significant at $p < 0.05$.

RESULTS

Gas chromatography-mass spectrometry (GC-MS) analysis:

The results of GC-MS analysis of *Z. tenuior* oil were given Table 1.

Table 1: Main components of essential oil of *Ziziphora tenuior* aerial parts

| Rt ^a | (%) ^b | Compounds |
|-----------------|------------------|---------------------------------|
| 3.45 | 1.62 | 2-pentanone, 4-hydroxy-4-methyl |
| 5.32 | 0.04 | β -pinene |
| 6.11 | 0.13 | cyclohexanon-3methyl |
| 6.86 | 0.12 | alpha-limonene |
| 8.40 | 0.16 | trans-isolimonene |
| 11.64 | 11.40 | unidentified |
| 11.92 | 1.12 | unidentified |
| 12.40 | 0.24 | menthofuran |
| 13.33 | 0.21 | (+)-isomenthone |
| 13.88 | 1.72 | menthone |
| 14.18 | 0.46 | cis-isopulegone |
| 14.45 | 0.54 | trans-isopulegone |
| 14.59 | 0.31 | o-isopropyl-anisole |
| 17.11 | 74.37 | pulegone |
| 18.03 | 0.57 | menthyl acetate |
| 18.65 | 0.24 | piperitone |
| 19.52 | 0.08 | (+)-isopiperitenone |
| 21.21 | 0.04 | β -bourbonene |
| 22.33 | 4.02 | piperitenone |
| 26.68 | 0.03 | cadinene |

Rt^a: Retention time, (%)^b: Relative percentage obtained from peak area

Cytotoxic potential of *Ziziphora tenuior* EO: Cytotoxicity of EO were tested for 24 h by quantitative analysis using the WST-1 test (Fig. 1). The cytotoxic value of control were compared with that of EO 100 and 50% (v/v) and found to be statistically different (Fig. 1, $p < 0.05$). There was no significant difference among the effect of control, EO 25, 12.5 and 6.25% ($p > 0.05$).

Antioxidant capacity of *Ziziphora tenuior* EO: Their antioxidant capacities were defined after a comparison was made including the activities of known antioxidants like ascorbic acid. Whereas the DPPH scavenging activities of the *Z. tenuior* EO was $IC_{50} = 9,60 \pm 0.57 \mu\text{L mL}^{-1}$, the IC_{50} value of the ascorbic acid was found as $IC_{50} = 6,97 \pm 1.05 \mu\text{g mL}^{-1}$ (Fig. 2).

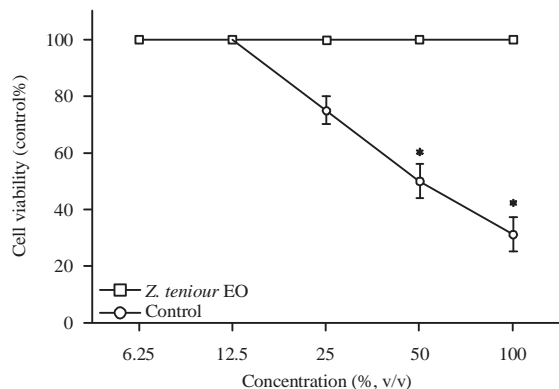


Fig. 1: *In vitro* cytotoxic effect of *Ziziphora tenuior* EO on L929 fibroblast cells. $p < 0.05$ vs control

Antimicrobial and antibiofilm activity: Disc diffusion test results of 19 *A. baumannii* strains, which have multiple resistance, were found above >30 mm in all the strains. The MIC values of the *Z. tenuior* EO were found as $0.6-1.25 \mu\text{L mL}^{-1}$ on MDR *A. baumannii* strains. The MBC values were found as $2.5-5 \mu\text{L mL}^{-1}$. While the MBIC values of the *A. baumannii* strains were determined as $0.3-1.25 \mu\text{L mL}^{-1}$, the MBEC values were found as $5-10 \mu\text{L mL}^{-1}$ (Table 2).

The percentage reduction in biofilm formation: The EO at the MIC value was applied onto the bacteria biofilm formations that emerged after the 48 h incubation. It was seen that the MIC value of the EO damaged the biofilm formations constituted by the *A. baumannii* strains by 51-84% (Table 2).

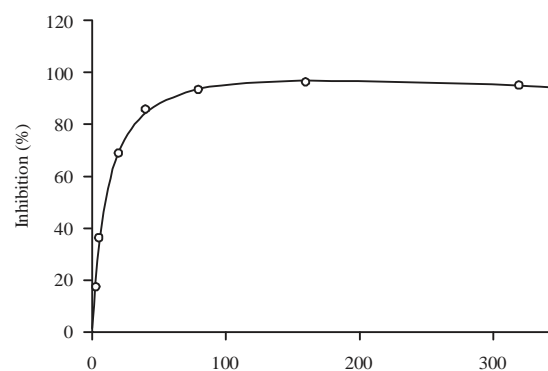


Fig. 2: Inhibition of essential oil from *Ziziphora tenuior* for scavenging DPPH (IC_{50} value was defined as the concentration of 50% radical inhibition and calculated). All data were presented as the Mean \pm SD for three replicates

Table 2: Antimicrobial and antibiofilm activity of *Ziziphora tenuior* EO on MDR *Acinetobacter baumannii* strains

| Strains | Zone Inhibition ^a | MIC ($\mu\text{L mL}^{-1}$) | MBC ($\mu\text{L mL}^{-1}$) | MBIC ($\mu\text{L mL}^{-1}$) | MBEC ($\mu\text{L mL}^{-1}$) | Reduction in biofilm formation on MIC value (%) |
|---------|------------------------------|-------------------------------|-------------------------------|--------------------------------|--------------------------------|---|
| 1 | 30.6 \pm 3.7 | 0.6 | 2.5 | 0.3 | 5 | 81.0 \pm 2.0 |
| 2 | 31.6 \pm 3.5 | 0.6 | 2.5 | 0.6 | 10 | 80.3 \pm 3.0 |
| 3 | 32.6 \pm 0.5 | 1.25 | 5 | 1.25 | 5 | 83.3 \pm 4.9 |
| 4 | 32.6 \pm 1.5 | 1.25 | 2.5 | 0.6 | 10 | 62.6 \pm 3.7 |
| 5 | 33.6 \pm 0.5 | 0.6 | 5 | 0.6 | 5 | 70.0 \pm 4.3 |
| 6 | 34.0 \pm 1.0 | 0.6 | 5 | 0.3 | 5 | 81.6 \pm 2.0 |
| 7 | 32.3 \pm 2.0 | 0.6 | 5 | 0.6 | 5 | 67.3 \pm 3.5 |
| 8 | 31.6 \pm 2.0 | 0.6 | 5 | 0.3 | 5 | 67.6 \pm 2.5 |
| 9 | 31.1 \pm 3.0 | 0.6 | 5 | 0.6 | 5 | 78.3 \pm 3.7 |
| 10 | 32.6 \pm 0.5 | 0.6 | 2.5 | 0.6 | 10 | 64.0 \pm 4.0 |
| 11 | 32.6 \pm 2.3 | 0.6 | 5 | 0.6 | 10 | 51.6 \pm 4.7 |
| 12 | 33.3 \pm 1.1 | 1.25 | 5 | 1.25 | 5 | 84.0 \pm 4.5 |
| 13 | 33.6 \pm 1.5 | 1.25 | 2.5 | 0.3 | 5 | 77.3 \pm 3.0 |
| 14 | 30.6 \pm 3.7 | 0.6 | 5 | 0.3 | 5 | 81.3 \pm 1.5 |
| 15 | 35.0 \pm 1.0 | 0.6 | 2.5 | 0.6 | 5 | 69.3 \pm 2.0 |
| 16 | 33.0 \pm 2.6 | 1.25 | 2.5 | 1.25 | 5 | 74.6 \pm 5.0 |
| 17 | 31.6 \pm 2.0 | 0.6 | 2.5 | 0.6 | 5 | 81.3 \pm 3.5 |
| 18 | 32.0 \pm 2.6 | 0.6 | 5 | 0.6 | 5 | 81.6 \pm 3.0 |
| 19 | 30.0 \pm 1.0 | 0.6 | 5 | 0.6 | 5 | 71.3 \pm 2.3 |

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, MBIC: Minimum biofilm inhibitory concentration, MBEC: Minimum biofilm eradication concentration, ^aValues are reported as Mean \pm standard deviation of three experiments, values given as mm

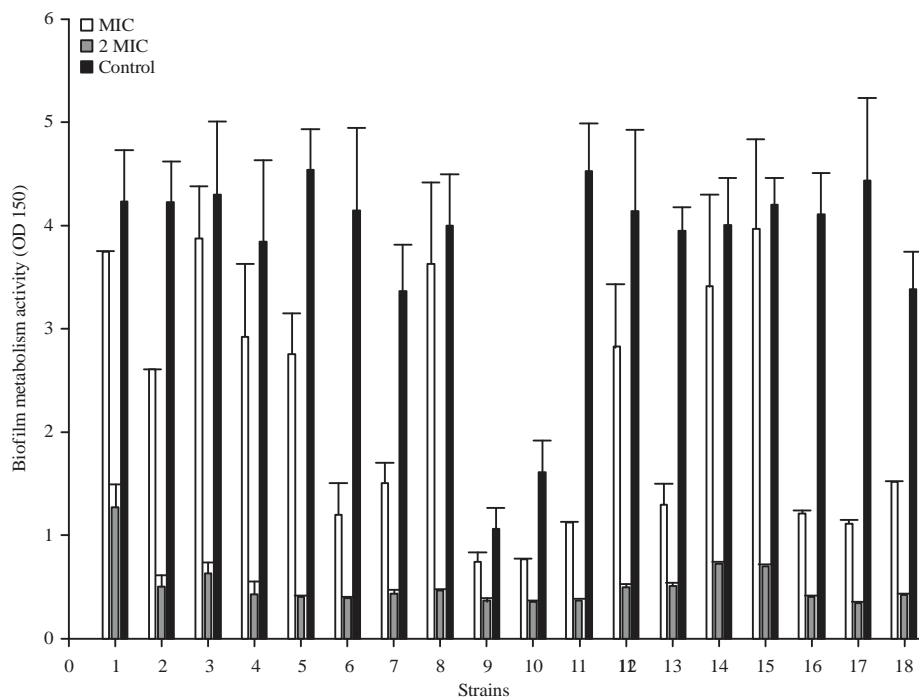


Fig. 3: Changes in metabolic activity following 48 h exposure of biofilms of MDR *Acinetobacter baumannii* strains to *Ziziphora tenuior* Essential Oil (EO) as determined by the XTT assay (control = biofilms not exposed to EO, n = 3 for each treatment and for each strain)

Inhibition of the metabolic activity assessment: The effect of the *Z. tenuior* EO on the metabolic activity within the 48 h biofilm, which was formed by the MDR *A. baumannii* strains, was assessed. Accordingly, it was seen that the metabolic activity decreased over time compared to the control at MIC and 2 MIC (Fig. 3).

DISCUSSION

The resistance developed by the microorganisms against the microbial agents occurring together with the intensive usage of the antibiotics and various factors that come together is an important problem. This problem is increasing substantially today (Kim *et al.*, 2015). The World Health Organization (WHO) describes the antimicrobial resistance as one of the most significant three problems which the human health faces with (Howard *et al.*, 2012).

In recent years, formation and rapid spread of the *A. baumannii* clinical isolates which are resistant to the antibiotics used in the treatment has been a worrying change. Especially over the past decade, terms of 'pandrug resistance', 'extensively drug resistance' and 'multidrug resistance' have started to be used to define the resistance profiles of the Acinetobacteria and these bacteria were identified as the

red-alarm human pathogen in the medical world due to the emergent high antibiotic resistance and resulting treatment difficulties (Howard *et al.*, 2012; Zarrilli *et al.*, 2013).

Acinetobacter baumannii clinical isolates are resistant to almost all the antimicrobial agents used for treatment today. The effectiveness of the limited number of recently developed antimicrobial agents on this bacterium seems limited, too (Towner, 2009). Therefore, studies related to new agents, which can be used for the treatment of these pathogens and whose efficiency can be supported *in vitro* and *in vivo*, will be significant.

The increase in the bacteria resistant to antibiotics and the insufficient number of the recently developed antibiotics require creating new strategies. It is indicated in *in vitro* studies that the EOs of many phytochemicals and other components have a lot of properties including the antimicrobial effect (Langeveld *et al.*, 2014). *Ziziphora tenuior* extracts were declared to have antimicrobial activities on many gram-positive and gram-negative bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Bacillus subtilis* and fungi like *Candida albicans* (Sarac and Ugur, 2009; Mahboubi *et al.*, 2014a). In our study, we researched the antimicrobial activity of the *Z. tenuior* EO on the MDR

A. baumannii isolates and we obtained quite interesting results. It was seen that this oil had a quite effective antimicrobial activity on the strains found resistant against especially the antibiotics which were used in treatments. The EO formed inhibition zones with the diameter of 30-35 mm on the isolates. The inhibition values at low concentrations between 0.6-1.25 $\mu\text{L mL}^{-1}$ obtained at the MIC study showed that this EO substantially prevented the reproduction of the MDR *A. baumannii* isolates. Antimicrobial effect of the *Z. tenuior* on *A. baumannii* was stated in a study (Mahboubi *et al.*, 2014b). However, the antimicrobial effect values that we obtained in our study seem better. We assume that this situation may have resulted from the difference in the chemical composition of the *Z. tenuior* EO we used in our study.

In our study, major component of the *Z. tenuior* EO was found as pulegone with the density of 74.37% according to the GC-MS analyses. Pulegone is a natural monoterpene that can be obtained from some plants. It is reported that the plants, the major component of which is pulegone, are frequently used medically besides the commercial use of the pulegone in many fields and the obtained EO can change according to the origin of the plant, weather conditions, climate, harvest time, blooming status etc. and has antimicrobial activity (Marotti *et al.*, 1994; Dhingra *et al.*, 2011). The antibacterial effect on *A. baumannii* may have resulted from this intense pulegone concentration. However, other components may also contribute to this effect together with pulegone. Therefore, more detailed studies are needed to examine the effects of pulegone and the other components on *A. baumannii*.

It was found out in our study that the *Z. tenuior* EO also had strong antioxidant characteristic besides its antimicrobial and antibiofilm effects on *A. baumannii* isolates. In a study, the antioxidant characteristic of *Z. tenuior* was researched through different methods and its strong antioxidant characteristic was revealed. Plant chemical composition reported in this study is similar to the plant composition we evaluated in our study (Gholivand *et al.*, 2014). As a result, *Z. tenuior* is seen as a good antioxidant.

Today, one of the resistance characteristics, which leads to problematic treatment of *A. baumannii* infections, is the ability of these bacteria to form a significant amount of biofilm (Longo *et al.*, 2014). Clinically applied antimicrobial susceptibility tests are conducted against the planctonic forms of the microorganisms. However, it is reported that MIC values cannot be highly effective against the sessile forms of the microorganisms in biofilm (Ceri *et al.*, 1999). Therefore, not only planctonic forms but also sessile forms of the

microorganisms in biofilm were researched in our study on antimicrobial and antibiofilm activity effects. It was discovered in our study that *Z. tenuior* EO had an effective antibiofilm activity on MDR *A. baumannii* isolates. Accordingly, it was observed that the ability of the microorganisms to form biofilm was inhibited at the MIC level in 13 isolates and below the MIC in six isolates. In addition 48 h biofilm structures that emerged in the MBEC determination study were eradicated at the concentration of 5-10 $\mu\text{L mL}^{-1}$. All the antimicrobial and antibiofilm activity data of the *Z. tenuior* EO obtained in our study are seen quite below the cytotoxic value in our study. According to these data that we obtained in our study, *Z. tenuior* EO can be considered as a significant phytotherapeutic agent in the development of new treatment agents that can be used in the treatment of *A. baumannii* infections.

CONCLUSION

One of the most important health problems is the antimicrobial resistance problem today. Options of antibiotics that can be used for the MDR *A. baumannii* treatment, a significant nosocomial pathogen, have substantially decreased today and don't even exist for some strains. For this reason, development of new antimicrobial agents for the MDR *A. baumannii* treatment is significant. It was witnessed in our study that *Z. tenuior* EO had quite strong antimicrobial and antibiofilm effects on MDR *A. baumannii*. Consequently, *Z. tenuior* can be a potential agent that can be used for the treatment of MDR *A. baumannii* infections. Therefore, we suppose that supporting the findings obtained our study with further studies will be important.

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