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ANTI-MICROBIAL, ANTI-BIOFILM-FORMING PROPERTIES OF *ORIGANUM VULGARE* L. ESSENTIAL OILS ON *STAPHYLOCOCCUS AUREUS* AND ITS ANTIOXIDANT ACTION

***M. V. Kryvtsova*[1]*, *O. K. Fedkiv*[1], *M. R. Hrytsyna*[2], *I. Salamon*[3]**

[1] *Uzhhorod National University, Biological Faculty, Department of Genetics, Plant Physiology and Microbiology, 32, Voloshyn St., Uzhhorod 88000, Ukraine*

[2] *Stepan Gzhytskyj Lviv National University of Veterinary Medicine and Biotechnologies 50, Pekarska St., Lviv 79010, Ukraine*

[3] *University of Presov, Faculty of Humanities and Natural Sciences, Department of Ecology 1, 17th November St., Presov 08001, Slovakia*

**Corresponding author: e-mail: maryna.krivcova@uzhnu.edu.ua*

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Essential oils (EOs) have been widely used in folk and traditional medicine, cooking, cosmetology and other sectors of the economy. The antimicrobial properties of essential oils determine the relevance of the study of their effectiveness against antibiotic-resistant biofuel clinical isolates. Our research was aimed to determine the antimicrobial and anti-biofilm properties of essential oil of *Origanum vulgare* L. on clinical isolates of bacteria of the genus *Staphylococcus* and its antioxidant effect. The raw materials of *Origanum vulgare* L. for large-scale essential oil isolation were donated by established growers in Slovakia (Agrokarpaty, Ltd.). EOs were obtained by the large-scale distillation apparatus specifically designed for the aromatic and medicinal plants. Calendula Co., in Nova Lubovna, Slovakia. The analysis of EOs was carried out using a gas chromatograph Varian 3090. Antimicrobial activity of EOs was determined using well agar diffusion method. Quantification of biofilm formation was carried out using a microtiter plate assay and the spectrophotometric method. Biofilm formation was quantified by measuring optical density at 550 nm. Biochemical analysis of *O. vulgare* essential oil showed a high content of carvacrol and thymol (72%). All strains of *Staphylococcus aureus* were sensitive to this essential oil. At the same time, the lowest indicators of growth retardation zones were characteristic of *S. aureus* MRSA clinic biofilm creation. We have determined

the antibiofilm effect of *O. vulgare* essential oil on *S. aureus* isolates. The study showed the degradation of the biofilm under the influence of essential oil decreased with decreasing concentration. At the same time, even with the action of essential oil, the concentration of 0.01% showed a 42% decrease in the process of biofilm formation. At the highest concentration of 0.10%, a destruction of the biofilm by 78% was found. The study of the influence of essential oils on the formed biofilm showed that 0.1% solution of oil causes degradation of the biofilm by 73.2%; 0.05% – by 58% and 0.01% – by 51.5%. *O. vulgare* essential oil also showed high antioxidant properties. The neutralization rate of free radicals was 83%. The revealed antimicrobial properties of *O. vulgare* essential oil make it promising to be used as an antistatic agent, including as a component that contributes to the disintegration of biofilm in the treatment of boils, carbuncles, panaritiums, phlegmon, wound suppuration, inhalation for respiratory diseases, as a component of rinses in inflammatory periodontal diseases.

Keywords: antimicrobial activity, essential oil, *Origanum vulgare* L., oregano, bacterial biofilm, staphylococci, antioxidant action

INTRODUCTION

Despite significant advances in science with the introduction of new antibiotics, infectious diseases caused by antibiotic-resistant microorganisms are widespread today [2]. The lack of effectiveness of antibiotics is due to the rapid pace of formation of antibiotic resistance, their irrational use in various sectors of the economy. Even though antibiotic drugs remain the main means in the treatment of infectious diseases, the question of finding new alternative agents that have high bactericidal or bacteriostatic activity is topical. Plant substances, including essential oils, are very promising in this respect [4]. The biological activity of essential oils of different species has much in common, but at the same time, even within one plant species, the composition of essential oils depending on the place of growth, climatic and geographical conditions, the chemotype of the plant can vary greatly, and accordingly, the biological activity varies.

Essential oils are used in folk and traditional medicine, cosmetology and cooking. Antimicrobial and antiviral properties of essential oils have been presented in literature [11, 13, 14]. The effect of essential oils as an antiseptic does not decrease with repeated use. Their antimicrobial activity is due to the ability to disrupt the structure of the cytoplasmic membrane, which leads to the disruption of the transport of substances and metabolic processes [30]. Medicinal plants and essential oils attract attention as sources for the production of drugs with antimicrobial, anti-inflammatory and immunomodulatory effects [15, 21].

Origanum genus is important medicinally as it has antimicrobial, antifungal, antioxidant, antibacterial, antithrombin, antimutagenic, angiogenic, antiparasetic and antihyperglycaemic properties. Phytochemical investigations of the species of this genus have resulted in the extraction of a number of important bioactive compounds. This emphasizes the need of extensive study for reporting the additional information on the medicinal importance of other unattended species of genus *Origanum* [6]. It is known that thymol and carvacrol are the main active components of essential oil oregano [11, 16]. At the same time, several chemotypes of *O. vulgare* populations are distinguished by the qualitative content of essential oils, which depends on the ecological conditions of growth.

Lukas *et al.*, 2015 [17] established a geographical distribution of the accumulation of *O. vulgare* essential oils in Europe. In general, the content of essential oils was 0.03%–4.6%. The plants growing in the Mediterranean region are characterized by cymil- and acyclic linalool / linalyl acetate chemotype. Populations growing in regions with continental climate are dominated by sabinyl chemotype.

In populations of *O. vulgare* ssp. *hirtum* from the Campaign on the Sunshine Coast, carvacrol/thymol chemotype dominated, total phenol content is 45.70% oil (23.34% carvacrol and 22.16% thymol). The other, thymol/α-terpineol chemotype is characteristic of populations located on the mainland (40.50% of the oil is phenols, with a predominance of thymol and its derivatives (29.33%). The phenolic fraction was 6.30% of the oil, half of which was represented by thymol (3.24%), and the main content was 15.90% linalacetate and 12.50% linalool [7].

Plants of *O. vulgare* ssp*. hirtum* from northern Greece is rich in thymol, while from southern Greece – is carvacrol [12]. Their composition revealed: γ-terpinene (0.6% to 3.6%), *p*-cymeme (17.3% to 51.3%), thymol (0.2% to 42.8%) and carvacrol (1.7% to 69.6%). The study of the content of *O. vulgare* essential oils grown in southeastern Spain was dominated by carvacrol (58.7–77.4%), and also revealed: (E) -β-caryophyllene (0.5–4.9%), thymol (0.2–5.8%), *p*-cemene (3.8–8.2%), γ-terpinene (2.1–10.7%) [5].

Antimicrobial properties are provided by the aromatic essential oils, in particular carvacrol, whereas phenolic compounds exhibit antioxidant properties [10]. Another important components of *O. vulgare* is flavonoids and phenolic acids. In this species, high content of rosemary acid (23 mg/g), as well as, luteolin-7-*O*-β-D-glucuronide was detected, whereas protocatechin, 3-*O*-caffeoylquinic acid and caffeic acid were present in low quantities [19].

Thus, for the preparation and use of essential oils as components of agents with antimicrobial properties, it is important to study the antimicrobial and antioxidant properties of the essential oil, including *O. vulgare*, obtained from raw materials at a particular site. Our study aimed to determine the antimicrobial, antibiofilm-forming properties of *Origanum vulgare* L. essential oil on clinical isolates of bacteria of the genus *Staphylococcus* and its antioxidant action.

MATERIALS AND METHODS

Isolation of essential oils. The raw materials of different aromatic plant species for large-scale essential oil isolation were donated by established growers in Slovakia (Agrokarpaty, Ltd.). EOs were obtained by the large-scale distillation apparatus [22] specifically designed for aromatic and medicinal plants. Calendula Co., in Nova Lubovna, Slovakia.

GC-FID analyses. The analysis of EOs was carried out using a gas chromatograph Varian 3090, connected to MS Saturn 2100T integrator. The following operating conditions were used: capillary column: RX-5MS, 30 m×0.250 mm i.d., film thickness: 0.25 μm, carrier gas: He_2, adjusted to a flux of 1.5 mL/min, injection and FID-detector temperatures: 220 °C and 250 °C respectively, a capacity of sample injection: 2 μL, MS-detector with automatic injector type 1177.

Components were identified by their GC retention times, and the resulting values were comparable to those in literature. Oil component standards for comparison were supplied by Extrasynthese, Merck, Fulka, Sigma a Roth [27].

Antimicrobial activity. Antimicrobial activity of EOs was determined using well agar diffusion method. The sensitivity of microorganisms to EO was determined by the

agar diffusion test. The bacterium inocula 100 μL in the physiological solution were adjusted to the equivalent of 0.5 McFarland standard, and evenly spread on the surface of Muller–Hinton agar (incubated at 37±2 °C for 24 hours). The extracts of 20 μL were introduced into wells 6 mm in diameter. The diameters of the inhibition zones were measured in millimetres including the diameter of the well. Antimicrobial activity of EOs was determined using well agar diffusion method. Antimicrobial activity of *O. vulgare* L. essential oil was performed using clinical isolates of bacteria of the genus *Staphylococcus: S. aureus* ATCC 25923*, S. aureus, S. aureus* MRSA*, S. aureus* clinical biofilm creation, *S. epidermidis, S. haemolyticus, S. saprophyticus.*

Destruction of bacterial biofilm. The bacterial biofilm degradation ability was tested in 96-well microplates (Greiner-BioOne, Austria) according to the O'Toole technique [23]. A bacterial suspension of *S. aureus* biofilm isolates in 0.5 McFarland standard 1.5×108 CFU/mL broth was prepared. 180 μL of the broth suspension was added to the wells and 20 μL of essential oil in DMSO were added to the wells; serial dilutions were prepared so that the concentration of essential oil solutions in the wells would be 0.01%, 0.05%, and 0.1%. 24 hours after culturing at 37 °C, the plates were washed with distilled water 3–5 times and stained with 0.1% crystal violet for 20 min. They were dried at room temperature for 2 hours, after which 200 μL of 95 alcohol was added. The optical density was determined spectrometrically at a wavelength of 550 nm. Negative control: DMSO.

Destruction of the formed bacterial biofilm. The sensitivity of the microorganisms in biofilm form to the essential oils was tested on microbial biofilms grown in 96 well microplates. After 24 h of incubation, the microplates were washed three times from planktonic (unattached) microorganisms with sterile water. 180 μL of broth and 20 μL of essential oils were added to the wells so that the concentration of the solutions would be 0.01%, 0.05%, and 0.1%. After 24 hours of exposure, the liquid was drained, the wells were washed with sterile water and stained for 20 min with 0.1% crystal violet solution. Then, the wells were washed again four times with water using 200 μL of water per well. For the extraction of the dye from the cells in the wells, 200 μL of 95% ethanol was added. The exposure lasted for 20 min at room temperature. The optical density was measured spectrophotometrically at a wavelength of 550 nm. Negative control: dimethyl sulfoxide. More than 50% of the suppression of the biofilm formation was considered a valid suppression [26].

The antioxidant activity of the essential oil extracts was determined by the spectrophotometric (DPPH) method. It is based on the ability of a stable free radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH) to react with antioxidants (proton donors) including those of phenolic nature. Because these compounds can produce intense peak light absorption in the 514–517 nm range, their concentration in the solution can be determined spectrophotometrically. The results were expressed as a percentage of antiradical activity, ie the ability to neutralize the radicals, compared with trolox.

RESULTS AND DISCUSSION

According to the European Plant Genetic Resources Cooperation Program (EPGRCP), oregano (*Origanum vulgare* L.) is included in the list of priority species of medicinal and aromatic plants [29]. It is known that oregano oil contains a whole complex of biologically active substances, in particular, its composition includes: thymol, carvacrol, sesquiterpenes, free alcohols, geranyl acetate, flavonoids [11].

Biochemical analysis of *O. vulgare* essential oil showed a high content of carvacrol and thymol in it (Table 1).

Table 1. **Indicators of the biochemical composition of the essential oil of *Origanum vulgare*,%**

Таблиця 1. **Показники біохімічного складу ефірної олії *Origanum vulgare*, %**

Index	Results
Density	0.949 ± 0.002 g/cm^3
Refractive index	1.551 ± 0.001
Carvacrol	56.0 ± 3.0
Thymol	16.01 ± 1.2

Due to the study of the antioxidant properties of *O. vulgare* by inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH), a high rate of 83% of neutralization of free radicals by the extract of this plant was established.

High antioxidant activity of *O. vulgare* was established [28]. Two indicators were used to evaluate the level of oxidative stress reduction: the content of lipid peroxides and the level of protein oxidative modification. When using the meal of *O. vulgare,* there was a 71% reduction in the content of thiobarbituric products (TBACP) compared to the control and the standard that was ascorbic acid. The latter at a concentration of 10^{-4} mg/mL reduces TBACP by 17% compared to the control. The reduction of carbonyl groups by the use of *O. vulgare* extract was 89% compared to the control and 77% compared to the standard. The antioxidant properties of *Daucus carota* and *Humulus lupulus* were several times smaller than the ore. Gutiérrez-Grijalva, 2018 [10], based on the literature analysis, showed that the antioxidant properties were established by the DPPH method, due to the content of phenolic compounds in *O. vulgare.* In particular, the presence in the aqueous extract of eriodictyol, apigenin, caffeic acid and kaempferol, as well as rosmarinic, chicory, coffee and *p*-coumaric acids.

Due to carvacrol, essential oils of oregano (*Thymbra capitata* and *O. vulgare*) grown in southeastern Spain show inhibitory activity against lipoxygenase (LOX) and acetylcholinesterase (AChE), as well as anti-inflammatory and anti-Alzheimer's supporting potential [5].

Chinese scientists have linked the antioxidant properties of plants to phenol content. Of the twenty-one compounds tested in the ethanol extract of *O. vulgare* using 2,2-diphenyl-1-picrylhydrazyl (DPPH), which binds free radicals, twelve substances exhibited high antioxidant activity. Several compounds had antiviral activity against the respiratory syncytial virus (RSV), Coxsackie virus B3 (CVB3) and herpes simplex virus type 1 (HSV-1) [32].

Other studies have found the antioxidant activity of volatile glycosides of *O. vulgare* ssp. *hirtum*, which equates the author to the action of α-tocopherol. In particular, fourteen volatile aglycones identified with thymoquinone were detected in this species. Other important aglycones were benzyl alcohol, eugenol, 2-phenyl-ethanol, thymol, 3-hexen-1-ol and carvacrol [20].

The biochemical composition of the essential oils extracted from *O. vulgare* may vary to a certain extent, primarily due to the existence of different chemotypes and the effects of major factors that specifically affect the composition: seasonality, geographical

origin, genetic variation, growth stages, drying and storage after collection [3]. It was argued that it is the high content of aromatic essential oils – thymol, paraminem, and carvacrol as dominant components that give Origano essential oil antimicrobial properties [18].

Antimicrobial activity of *O. vulgare* L. essential oil was carried out using clinical isolates of bacteria of *Staphylococcus* genus: *S. aureus* ATCC 25923*, S. aureus, S. aureus* MRSA*, S. aureus* clinical biofilm creation.

Studies have shown that all strains of staphylococci were sensitive to *O. vulgare* essential oil. At the same time, the highest activity was more characteristic of coagulase-negative species than coagulase-positive ones. At the same time, the lowest indicators of growth retardation zones were characteristic of *S. aureus* MRSA clinic biofilm creation. The results of the study of antimicrobial activity are presented in Table 2.

Table 2. **Antimicrobial activity of *Origanum vulgare* essential oil (hole diameter 6 mm, material amount 0.02 μL)**

Таблиця 2. **Антимікробна активність ефірної олії *Origanum vulgare* (діаметр лунки 6 мм, кількість матеріалу 0,02 мкл)**

Test culture	Growth retardation zone (mm)
Staphylococcus aureus ATCC 25923	26.7 ± 0.58
Staphylococcus aureus MRSA clinic	25.5 ± 0.5
Staphylococcus aureus clinic biofilm creation	25.7 ± 0.58
Staphylococcus aureus MRSA clinic biofilm creation	24.0 ± 0.58

The results of our study are consistent with those of other authors regarding the antimicrobial action of *O. vulgare* L. essential oil. According to the literature, *Oregano vulgare* L. essential oil inhibited bacterial growth in bacteria such as *E. coli, K. pneumoniae, L. monocytogenes, P. mirabilis , P. vulgaris, P. aeruginosa, C. albicans, S. pyogenes, S. mutans, S. enteritidis, B. cereus, S. aureus, S. pneumoniae, M. luteus*. Gram-positive bacteria were shown to be more sensitive to antimicrobial agents than Gram-negative ones. Oregano oil had more pronounced antimicrobial activity than antibiotic [24]. Antimicrobial activity of oregano essential oil, according to another work, does not cover a wide range of microorganisms, in particular it does not show inhibitory effect on *P. aeruginosa* [4]. We have shown that oregano oil has a bactericidal effect on the antibiotic resistant staphylococci, including MRSA and biofilm isolates of staphylococci.

Bacteria of *Staphylococcus* genus is the etiological factor of many inflammatory processes, including respiratory tract, oral cavity, skin, catheter-associated infections. Recent studies have shown that staphylococci biofilm are the causative agents of chronic inflammatory processes and have a much higher resistance to antibiotics and the immune system of the host [25]. It is the ability to *S. epidermidis* and *S. aureus* biofilm is considered as a factor determining the chronicity of the infectious process [23].

We have established the antibiofilm effect of essential oil of *O. vulgare* L. to isolate *S. aureus* (Fig. 1, 2). It was shown that the degradation of the biofilm under the influence of essential oil decreased with decreasing concentration. At the same time, even with the action of essential oil at a concentration of 0.01%, there was a 42% decrease in the

process of biofilm formation. The highest concentration of 0.1% provided the destruction of the biofilm by 78%. The study of the influence of essential oils on the formed biofilm showed that 0.1% solution of oil causes degradation of the biofilm by 73.2%; 0.05% – 58% and 0.01% 2 51.5%.

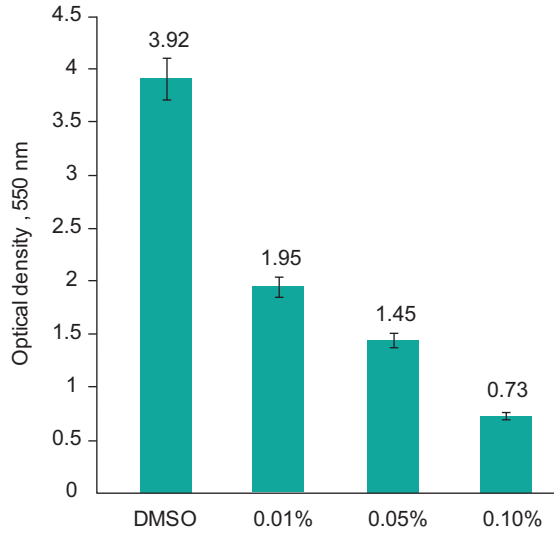

Fig. 1. Effect of different concentrations of *Origanum vulgare* essential oil on biofilm formation by *S. aureus* clinical isolates

Рис. 1. Вплив різних концентрацій ефірної олії *Origanum vulgare* на формування біоплівки клінічними ізолятами *S. aureus*

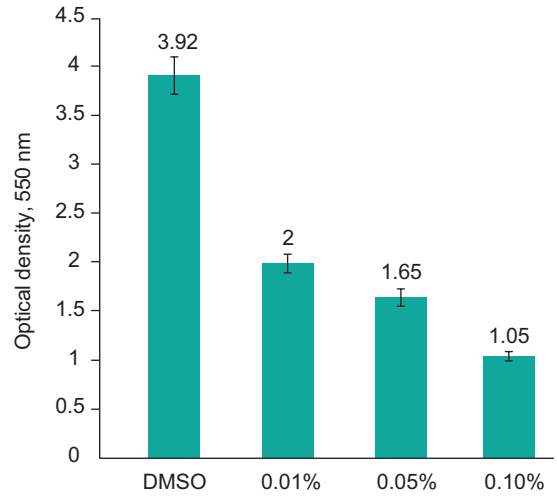

Fig. 2. Effect of different concentrations of *Origanum vulgare* essential oil on biofilm formed by clinical isolates of *S. aureus*

Рис. 2. Вплив різних концентрацій ефірної олії *Origanum vulgare* на сформовану біоплівку клінічними ізолятами *S. aureus*

The revealed antimicrobial properties of EO Origano determine the prospects of its use as an antistatic agent, including as a component that contributes to the disintegration of biofilms in the treatment of boils, carbuncles, panaritum, phlegmon, suppuration of wounds; inhalation for respiratory diseases; as a component of conditioners for inflammatory periodontal diseases [18]. Treatment of *O. vulgare* essential oils with the surface of foods and separately carvacrol showed their high efficiency in inhibiting of planktonic and sedentary *S. aureus* cells [8].

It has been proven that *O. vulgare* L. can be used as a food seasoning to reduce *S. aureus* contamination and inhibit the synthesis of staphylococcal enterotoxins since essential oil affects membrane permeability and cell wall structure [9].

De Martino *et al.*, 2009 [7] reported antimicrobial activity of *O. vulgare* plants from three different Campania (Southern Italy) populations against 10 pathogenic bacteria. The carvacrol/thymol and thymol/α-terpineol chemotypes essential oils had a predominant effect on Gram-positive pathogens, in particular *S. epidermidis* and somewhat less on *S. aureus* (ATCC 25923). Among the Gram-negative bacteria, the essential effect was the essential oils of thymol/α-terpineol chemotype on *E. coli.* The very low activity showed oil linalacetate/linalool chemotype.

The mechanism of antimicrobial action varies depending on the type of EO, its component composition and the test the microorganism. Previous studies of other researchers and ourselves show that Gram-negative bacteria are less sensitive to EO than Gram-positive ones. Therefore, the mechanism of action of essential oils is associated with the action on the cell wall of bacteria. Besides, in Gram-positive bacteria, the process of penetration of the hydrophobic compounds of EO is easier due to the lipophilic ends of lipoteichoic acid present in the cell membrane [33].

The study showed that the antimicrobial activity of EO is due to several synergistic components. The essential oil of *Origanum vulgare* contains monoterpene phenol carvacrol. Carvacrol and thymol cause an increase in the permeability of the cell membrane of bacteria, affecting both the pH gradient and the electron flow, which explains the antimicrobial activity of the essential oil [27]. It was found that *p*-cymene was ineffective as an antimicrobial agent alone, but in combination with carvacrol led to synergistic activity, which caused the destruction of bacterial cell membranes to a greater extent than carvacrol alone [1]. Essential oil components, namely: eugenol, carvone, caveol, carvacrol, and thymol, interfere with adhesion and inhibit biofilm formation of *P. aeruginosa* strains [31].

CONCLUSION

The antimicrobial properties of EO Origano for coagulase-positive (*S. aureus*, including MRSA) were detected. The antibiofilm-forming effect of essential oil has been established, which substantiates further research to study the principle possibility of creating antimicrobial effect based on the essential oil. *O. vulgare* essential oil showed high antioxidant properties. The neutralization rate of free radicals was 83%. High antimicrobial and antioxidant properties make it possible to offer oregano essential oils as natural ingredients with antimicrobial properties for the manufacture of cosmetics, medicines and food additives.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Animal Rights: This article does not contain any studies with animal subjects performed by the any of the authors.

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АНТИМІКРОБНІ, АНТИБІОПЛІВКОТВІРНІ ВЛАСТИВОСТІ ЕФІРНОЇ ОЛІЇ *ORIGANUM VULGARE* L. НА *STAPHYLOCOCCUS AUREUS* ТА ЇЇ АНТИОКСИДАНТНА АКТИВНІСТЬ

***М. В. Кривцова*[1]*, *О. К. Федьків*[1], *М. Р. Грицина*[2], *І. Саламон*[3]**

[1] *Ужгородський національний університет*
біологічний факультет, кафедра генетики, фізіології рослин і мікробіології
вул. Волошина, 32, Ужгород 88000, Україна

[2] *Львівський національний університет ветеринарної медицини та біотехнологій*
імені С.З. Гжицького, вул. Пекарська, 50, Львів 79010, Україна

[3]*Пряшівський університет, факультет гуманітарних і природничих наук, кафедра екології*
вул., 17 Листопада, Пряшів 08001, Словаччина

**Кореспондуючий автор: e-mail: maryna.krivcova@uzhnu.edu.ua*

Ефірні олії з давніх-давен широко використовують у народній, традиційній медицині, кулінарії, косметології та інших галузях народного господарства. Антимікробні властивості ефірних олій обумовлюють актуальність дослідження їхньої ефективності щодо антибіотикорезистентних біоплівкотвірних клінічних ізолятів. Метою нашої роботи було встановити антимікробні й антибіоплівкотвірні властивості ефірної олії *Origanum vulgare* L. на клінічні ізоляти *Staphylococcus aureus* та її антиоксидантну дію. Сировина *Origanum vulgare* L. для промислового виділення ефірної олії була надана відомими виробниками Словаччини (Agrokarpaty, Ltd.). Ефірна олія отримана промисловим дистиляційним апаратом, спеціально розроб-

леним для ароматичних і лікарських рослин на підприємсві Calendula Co., Nova Lubovna, Словаччина. *GC-FID аналіз.* Аналіз ефірної олії проводили з використанням газового хроматографа Varian 3090. Антимікробну активність ефірної олії визначали методом дифузії в агар. Утворення біоплівки визначали мікропланшетним методом спектрофотометрично способом вимірювання оптичної густини за 550 нм. Біохімічний аналіз ефірної олії *O. vulgare* показав високий вміст у ній карвакролу і тимолу (72%). Усі штами стафілококів були чутливими до цієї ефірної олії. Разом із тим, найнижчі показники зон затримки росту були характерні для *S. aureus* MRSA. Нами встановлено антибіоплівкотвірний ефект ефірної олії *O. vulgare* на ізоляти *S. aureus.* З'ясовано, що деструкція біоплівки під впливом ефірної олії у разі зниження концентрації зменшувалась. Водночас навіть за дії ефірної олії в концентрації 0,01% встановлено зниження процесу утворення біоплівки на 42%. Найвища концентрація 0,1% – забезпечувала деструкцію біоплівки на 78%. Дослідженням впливу ефірних олій на сформовану біоплівку з'ясовано, що 0,1% розчин олії спричиняє деградацію біоплівки на 73,2%; 0,05–58% та 0,01% – на 51,5%. Ефірна олія *O. vulgare* показала також високі антиоксидантні властивості. Показник нейтралізації вільних радикалів становив 83%. Виявлені антимікробні властивості ефірної олії *O. vulgare* обумовлюють перспективність її використання як протистафілококового засобу, зокрема, як компонента, що сприяє дезінтеграції біоплівки під час лікування фурункулів, карбункулів, панаріцію, флегмон, нагноєнь ран; інгаляторно у разі захворювання дихальних шляхів; як компонент ополіскуачів у разі запальних захворювань пародонту.

Ключові слова: антимікробна активність, ефірна олія, *Origanum vulgare* L., орегано, бактеріальна біоплівка, стафілококи, антиоксидантна дія

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