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# Essential oils of selected citrus fruits and spice plants as potential antibacterial and antibiofilm agents

*Original Article*

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*Abstract:*

This study evaluates the antibacterial and antibiofilm properties of essential oils (EOs) from *Citrus lemon* (L.) Osbeck, lemon; *Citrus reticulata* Blanco, mandarine; *Nigella sativa* L., black cumin, and *Foeniculum vulgare* Mill., fennel, using the disk-diffusion, broth microdilution, and tissue culture plate methods on 11 bacterial strains, including the multidrug-resistant (MDR). Results showed that tested EOs exhibit antibacterial effects, that are stronger in Gram-positive bacteria. The widest inhibition zones were achieved with the black cumin EO against MDR *Staphylococcus aureus*. Values of the minimum inhibitory concentrations ranged from 250 to 750 µg/ml, while minimum bactericidal concentrations were 500-1000 µg/ml. Black cumin EO performed strong antibiofilm features, with the total elimination of the biofilm in the case of different *S. aureus* strains (including methicillin-resistant, MRSA), and *Pseudomonas aeruginosa*. All investigated EOs exhibited strain-specific and dose-dependent antibacterial and antibiofilm activity.

*Key words:*
bacterial biofilms, antibiofilm agents, essential oils, *Citrus lemon* (L.) Osbeck, *Citrus reticulata* Blanco, *Nigella sativa* L., *Foeniculum vulgare* Mill.

*Apstrakt:*

**Eterična ulja ploda odabranih citrusa i začinskih biljaka kao potencijalni antibakterijski i antibiofilm agensi**

Ova studija evaluira antibakterijska i antibiofilm svojstva eteričnih ulja iz *Citrus lemon* (L.) Osbeck, limuna; *Citrus reticulata* Blanco, mandarine; *Nigella sativa* L., crnog kima i *Foeniculum vulgare* Mill., komorača, korišćenjem disk-difuzione, mikrodilucijske i "tissue culture plate" metode na 11 sojeva bakterija, uključujući multirezistentne (MDR) sojeve. Rezultati su pokazali da testirana eterična ulja ispoljavaju antibakterijski efekat, koji je jači kod Gram-pozitivnih bakterija. Najšire zone inhibicije su postignute delovanjem eteričnog ulja crnog kima na MDR *Staphylococcus aureus*. Vrednosti minimalne inhibitorne koncentracije su se kretale od 250 do 750 µg/ml, dok su minimalne baktericidne koncentracije bile 500-1000 µg/ml. Eterično ulje crnog kima je ispoljilo jako antibiofilm dejstvo, sa totalnom eliminacijom biofilma u slučaju različitih sojeva *S. aureus* (uključujući meticilin-rezistentni, MRSA) i *Pseudomonas aeruginosa*. Sva ispitivana eterična ulja su ispoljila antibakterijsku i antibiofilm aktivnost koja je bila specifična za soj, te dozno-zavisna.

*Ključne reči:*
bakterijski biofilmovi, antibiofilm agensi, eterična ulja, *Citrus lemon* (L.) Osbeck, *Citrus reticulata* Blanco, *Nigella sativa* L., *Foeniculum vulgare* Mill.

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## Introduction

Antimicrobial resistance is considered one of the major global health challenges of the 21st century (Hernando-Amado et al., 2019). According to Urban-Chmiel et al. (2022), antibiotic resistance is acquired via several mechanisms such as active removal of the antibiotic from the cell, enzymatic modifications of the drug, modifications of cell components which are the target of the antibiotic, overexpression of an enzyme inactivated by the antibiotic, a change in the permeability of bacteria cell membranes, production of an alternative metabolic pathway, an increase in the concentration of a metabolite which is an antagonist of the antibiotic, a reduction in the amount or activity of an enzyme activating the precursor of the antibiotic, modifications in regulatory systems not associated with the direct mechanism

of action of the antibiotic, or a reduction in the demand for the product of the inhibited metabolic pathway. Bacterial biofilm represents a structured consortium of microorganisms embedded in a self-produced matrix made from polysaccharides, proteins, and DNA, characterized by increased resistance to antimicrobial agents (Høiby et al., 2010). Studies suggest that microbial cells within the biofilm have 10–1000 times more antibiotic resistance in comparison to planktonic cells (Mah, 2012). Furthermore, bacterial biofilms are involved in approximately 80% of chronic and recurrent microbial infections in humans (Sharma et al., 2019). Numerous investigations have shown that different plant products as well as plant active ingredients can inhibit the formation and development of bacterial biofilms, and eradicate mature biofilms (Cheng et al., 2022).

Essential oils (EOs) are the volatile secondary metabolites of plants, produced by more than 17.500 plant species from many angiosperm families, but only about 300 of them are commercialized (Wińska et al., 2019). Numerous biological activities such as antiseptic, antibacterial, antiviral, antioxidant, antiparasitic, antifungal, and insecticidal effects are previously reported for EOs. Also, EOs are considered a powerful tool in the reduction of bacterial resistance (Chouhan et al., 2017). Essential oils as well as other bioactive products of medicinal plants have received excessive attention for their low toxicity, pharmacological activities, and economic viability (Auddy et al., 2003). Therefore, EOs and their components are naturally occurring antimicrobial compounds with the potential to prevent the limitations of conventional antimicrobial agents (Orhan-Yanıkan et al., 2019).

Lemon, *Citrus limon* (L.) Osbeck (Rutaceae), is a well-known and frequently used plant for different purposes, especially in cooking, but studies suggest its various biological activities such as anticancer, antioxidant, antimicrobial, etc. Lemon is also proven for many beneficial effects on the nervous, cardiovascular, respiratory, and skeletal systems (Klimek-Szczykutowicz et al., 2020). Bioactive properties such as antimicrobial, anticancer, antioxidant, antigenotoxic, hepatoprotective, etc. are also related to *Citrus reticulata* Blanco (Rutaceae), the mandarin orange (Musara et al., 2020). *Nigella sativa* L. (Ranunculaceae), known as black cumin, is a worldwide distributed plant with many curative effects on human health. The pharmacological significance of this species is mainly illustrated by its capacity to act as an antimicrobial and antioxidative agent (Tabassum et al., 2018). Fennel or *Foeniculum vulgare* Mill. (Apiaceae), is a seasonal medicinal plant, grown by humans in nearly every region. Due to its great aromaticity and spiciness, this is a popular cooking ingredient but nevertheless, pharmacological studies revealed that fennel possesses bioactive potential and could be used in the treatment of different diseases (Tripathi et al., 2012). The main goal of this study was to evaluate the antibacterial and antibiofilm potential of essential oils made from well-known edible and spice plants: lemon, *C. lemon* (lemon), *C. reticulata* (mandarin orange), *N. sativa* (black cumin), and *F. vulgare* (fennel).

## Materials and Methods

### *Essential oils*

In this investigation pure EOs derived from *Citrus lemon* (L.) Osbeck, *Citrus reticulata* Blanco, *Nigella sativa* L., and *Foeniculum vulgare* Mill. (Dea Flores d.o.o., Rijeka, Croatia) were used. Stock solutions of the test substances were prepared in 0.1% dimethyl sulfoxide ≥99% (DMSO) (Sigma-Aldrich) and kept at room temperature in the dark.

### *Bacterial species*

Investigation of antibacterial and antibiofilm properties of selected EOs comprised a total of 11 bacteria, including the multidrug-resistant (MDR), strains: *Staphylococcus aureus* ATCC 6538 (SA1); *S. aureus* ATCC 25923 (SA2); methicillin-resistant *S. aureus* (MRSA): *S. aureus* ATCC 33591 (SA3), and *S. aureus* NCTC 12493 (SA4); *Enterococcus faecalis* ATCC 29212 (EF); *Bacillus subtilis* ATCC 6633 (BS); *Escherichia coli* 14169 (EC1); *E. coli* ATCC 25922 (EC2); extended-spectrum beta-lactamase-producing (ESBL) *E. coli* ATCC 35218 (EC3); *Pseudomonas aeruginosa* ATCC 27853 (PA); *Salmonella enterica* NCTC 6017 (SE). Investigated bacterial strains were purchased from the American Type Culture Collection (ATCC) and National Collection of Type Cultures (NCTC) (MicroBioLogics, St. Cloud, Minnesota, USA). Inoculums were set in accordance with EUCAST (2017). Overnight cultures were diluted in sterile saline solution to the final turbidity of 0.5 McFarland standard, which corresponds to the bacterial concentration of $1.5 \times 10^8$ CFU/ml.

### *Determination of the inhibition zones*

The antibacterial activity of investigated essential oils was evaluated by using pure EO (100%), as well as different concentrations of EO (250, 500, and 750 µg/ml) aseptically dissolved in 0.1% DMSO. For this part of the study, the disk diffusion method was applied (Bauer et al., 1966).

Bacterial strains were cultured in Mueller Hinton (MH) medium (Fluka Biochemica; Buchs,

Switzerland), and after adjusting the final density of inoculum ($1.5 \times 10^8$ CFU/ml), spread over the growth medium plates. An amount of 10 µl of each concentration of EOs was impregnated into the paper disk which was enough to achieve saturation. A total of six disks (four concentrations of EO, positive and negative control included) were placed on one 150 mm plate. After the application of EO, plates were incubated at 35±2 °C for 16-18 hours. As the positive control, antibiotics Ampicillin (10µg), Streptomycin (10µg), and Colistin (10µg), all made by Oxoid™ (Great Britain) were used, while 0.1% DMSO served as the solvent control. The antibacterial effect was evaluated based on the diameter (mm) of inhibition zones. All tests were performed in triplicate, and the mean value ± standard deviation (STDEV) was taken for further analysis.

### *Minimum inhibitory and minimum bactericidal concentration*

The minimum inhibitory concentration (MIC) of EOs was determined through the broth microdilution method (CLSI, 2018). Pure EOs (100%) were dissolved in 0.1% DMSO to achieve the stock concentration of 1000 µg/ml. The final volume of 100 µl of two-fold dilutions of EOs ranging from 500 to 1.95 µg/ml, was applied in a 96-well microtiter plate containing the Mueller Hinton broth (Sigma-Aldrich). An amount of 10 µl of bacterial inoculum (concentration of $1.5 \times 10^8$ CFU/ml) was then added to each well. Pure bacterial culture was taken as the growth control, and uninoculated media with the DMSO was used as the negative control. After the overnight incubation, results were read on a microplate reader (Biochrom EZ Read 400) at 595 nm. Experiments were performed in quadruplets, and MIC concentrations were determined based on the generated absorbance values. Minimum bactericidal concentration (MBC) was evaluated by replating the inoculums from the wells without signs of growth on a sterile Mueller Hinton medium and observing the presence of growth after overnight incubation.

### *Evaluation of the antibiofilm activity*

In order to determine the biofilm formation in the presence of tested EOs, the tissue culture plate method (TCP) in 96 well plates (Merritt et al., 2005) was used, with the tryptic Soy Broth, TSB (Sigma-Aldrich) as the growing medium. The stock solution of EO in DMSO was two-fold diluted in TSB up to the end concentration of 1.95 µg/ml. An amount of 100 µl of such dilution was added to each well, followed by the inoculation with 10 µl of the bacterial suspension. Uninoculated media with DMSO was used as the solvent control, while wells described as the untreated biofilms controls contained media with bacterial inoculum. Inoculums were prepared as described above, i. e turbidity of 0.5 McFarland standard and bacterial concentration of $1.5 \times 10^8$ CFU/ml. The adherence of bacteria in the presence of TSB was used for the determination of the biofilm formation. After the overnight incubation, the plates were emptied, washed in Phosphate Buffered Saline, PBS (Sigma-Aldrich, USA), and stained with 0.1% crystal violet solution for 10 minutes. After washing, 96% ethanol was added to each well. Results were analysed on the microplate reader (Biochrom EZ Read 400) at 595 nm. This experiment was done in four replications, and results were given as the means ± STDEV. The biofilm-forming category was determined according to Stepanović et al. (2007) and by the Biofilm Classifier Software ver 1.1. The optical density cut-off value (ODc) was calculated as three standard deviations above the mean OD of the negative control, while the biofilm categories were determined as follows: OD ≤ ODc: non-adherent (NA), ODc < OD ≤ 2 x ODc: weakly adherent (W), 2 x ODc < OD ≤ 4 x ODc: moderately adherent (M), and 4 x ODc < OD: strongly adherent (S).

### *Statistical analysis*

For the calculation of the descriptive statistical parameters (mean values and standard deviation) and the percentage of biofilm inhibition Microsoft Office 2019 Excel (Microsoft Corporation, USA) was used. Data were further analyzed by one-way ANOVA and *post-hoc* Fisher's LSD test (STATISTICA 10; StatSoft. Inc.) at the significance level of $p<0.05$.

## Results and discussion

The antibacterial activity of essential oils is mainly associated with their hydrophobic properties, which ensure their distribution in the lipids of the cell membranes and mitochondria, which further affects the structural integrity and function of those structures and after all results in leakage of cell contents (Tang et al., 2020). Such events interfere with the ATP balance and among others, have an impact on pH, protein synthesis, coagulation of the cytoplasmic material, DNA disruption, and *quorum sensing* inhibition (El-Tarabily et al., 2021). Obtained results regarding the antibacterial activity of investigated EOs through the measuring of inhibition zones are presented in Table 1. Overall observation showed that all investigated EOs exhibited antibacterial potential against all tested bacteria except *E. coli* ATCC 14169, *E. coli* ATCC 35218 (ESBL strain), and *S. enterica* NCTC 6017. The most sensitive bacterial species was *B. subtilis*, with achieved inhibition zones with all tested EOs, in all concentrations except the lowest dilution

**Table 1.** Diameter of inhibition zones of investigated essential oils and standard antibiotics

| Sample | | Bacterial strains | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SA1 | SA2 | SA3* | SA4* | EF | BS | EC1 | EC2 | EC3* | PA | SE |
| | | Growth inhibition (mm) | | | | | | | | | | |
| Pure EO | Lemon | NI$^{a}$ | NI$^{b}$ | NI$^{c,d}$ | 19.00±0.0$^{e}$ | 11.00±0.0$^{f}$ | 20.00±0.0$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| | Mandarine | 13.30±0.6$^{a}$ | NI$^{b}$ | NI$^{c,d}$ | NI$^{e}$ | NI$^{f}$ | 12.30±1.2$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| | Black cumin | 19.70±1.2$^{a}$ | 21.00±0.0$^{b}$ | 38.30±2.9$^{c,d}$ | 32.00±1.7$^{e}$ | NI$^{f}$ | 30.50±0.7$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | 30.00±0.0$^{h,k,l}$ | NI$^{e,k}$ | 12.00±1.0$^{i}$ | NI$^{d,j,l}$ |
| | Fennel | NI$^{a}$ | NI$^{b}$ | 8.50±1.4$^{c,d}$ | NI$^{e}$ | NI$^{f}$ | 11.30±1.5$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| 250 µg/ml | Lemon | NI$^{a}$ | NI$^{b}$ | NI$^{c,d}$ | NI$^{e}$ | NI$^{f}$ | NI$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| | Mandarine | NI$^{a}$ | NI$^{b}$ | NI$^{c,d}$ | NI$^{e}$ | NI$^{f}$ | NI$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| | Black cumin | NI$^{a}$ | NI$^{b}$ | 17.30±2.5$^{c,d}$ | NI$^{e}$ | NI$^{f}$ | NI$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| | Fennel | NI$^{a}$ | NI$^{b}$ | NI$^{c,d}$ | NI$^{e}$ | NI$^{f}$ | NI$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| 500 µg/ml | Lemon | NI$^{a}$ | NI$^{b}$ | NI$^{c,d}$ | NI$^{e}$ | 10.00±0.0$^{f}$ | 13.00±0.0$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| | Mandarine | 10.70±0.6$^{a}$ | NI$^{b}$ | NI$^{c,d}$ | NI$^{e}$ | NI$^{f}$ | 8.30±0.6$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| | Black cumin | 7.30±0.6$^{a}$ | 9.00±2.6$^{b}$ | 30.30±2.8$^{c,d}$ | 20.30±2.3$^{e}$ | NI$^{f}$ | 27.00±1.4$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | 21.70±2.9$^{h,k,l}$ | NI$^{e,k}$ | 9.30±0.6$^{i}$ | NI$^{d,j,l}$ |
| | Fennel | NI$^{a}$ | NI$^{b}$ | NI$^{c,d}$ | NI$^{e}$ | NI$^{f}$ | 7.00±1.7$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| 750 µg/ml | Lemon | NI$^{a}$ | NI$^{b}$ | NI$^{c,d}$ | 16.50±0.7$^{e}$ | 9.00±0.0$^{f}$ | 14.00±0.0$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| | Mandarine | 8.00±1.7$^{a}$ | NI$^{b}$ | NI$^{c,d}$ | NI$^{e}$ | NI$^{f}$ | 8.70±0.6$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| | Black cumin | 16.70±1.2$^{a}$ | 17.30±2.$^{b}$ | 35.20±1.7$^{c,d}$ | 26.30±2.5$^{e}$ | NI$^{f}$ | 29.00±2.6$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | 28.30±1.5$^{h,k,l}$ | NI$^{e,k}$ | 10.00±0.0$^{i}$ | NI$^{d,j,l}$ |
| | Fennel | NI$^{a}$ | NI$^{b}$ | 6.60±1.1$^{c,d}$ | NI$^{e}$ | NI$^{f}$ | 8.30±1.1$^{a,b,c,e,f,g,h,i,j}$ | NI$^{g}$ | NI$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| **Ampicillin** | | 11.00±0.0$^{a}$ | 12.00±0.0$^{b}$ | NI$^{c,d}$ | NI$^{e}$ | 9.00±0.0$^{f}$ | 22.00±0.0$^{a,b,c,e,f,g,h,i,j}$ | 14.00±0.0$^{g}$ | 16.00±0.0$^{h,k,l}$ | NI$^{e,k}$ | NI$^{i}$ | NI$^{d,j,l}$ |
| **Streptomycin** | | 9.00±0.0$^{a}$ | 10.00±0.0$^{b}$ | NI$^{c,d}$ | NI$^{e}$ | 15.00±0.0$^{f}$ | 20.00±0.0$^{a,b,c,e,f,g,h,i,j}$ | 15.00±0.0$^{g}$ | 17.00±0.0$^{h,k,l}$ | 12.00±0.0$^{e,k}$ | 14.00±0.0$^{i}$ | 15.00±0.0$^{d,j,l}$ |
| **Colistin** | | NI$^{a}$ | NI$^{b}$ | NI$^{c,d}$ | NI$^{e}$ | NI$^{f}$ | 10.00±0.0$^{a,b,c,e,f,g,h,i,j}$ | 16.00±0.0$^{g}$ | 15.00±0.0$^{h,k,l}$ | 10.00±0.0$^{e,k}$ | 17.00±0.0$^{i}$ | 11.00±0.0$^{d,j,l}$ |

SA1: *Staphylococcus aureus* ATCC 6538; SA2: *S. aureus* ATCC 25923; SA3: *S. aureus* ATCC 33591 (MRSA); SA4: *S. aureus* NCTC 12493 (MRSA); EF: *Enterococcus faecalis* ATCC 29212; BS: *Bacillus subtilis* ATCC 6633; EC1: *Escherichia coli* ATCC 14169; EC2: *E. coli* ATCC 25922; EC3: *E. coli* ATCC 35218 (ESBL); PA: *Pseudomonas aeruginosa* ATCC 27853; SE: *Salmonella enterica* NCTC 6017.
*MDR strains.
Letters in superscript indicate statistically significant differences after performing *post-hoc* Fisher's LSD test. Values with the same letter differ significantly at $p<0.05$.
Results are mean±SDEV.
NI=No inhibition.
DMSO=NI.

(250 µg/ml). In comparison to the antibacterial activity of standard antibiotics, the black cumin EO performed the greater growth inhibition of *B. subtilis* (**Tab.1**). Black cumin is recognized for its antimicrobial, immunomodulatory, anti-inflammatory, and antioxidant features (Ugur et al., 2016). Dalli et al. (2021) investigated the chemical profile of the black cumin EO from different geographical areas. As the major components *α*-phellandrene, *β*-pinene, *β*-cymene, and 4-caranol were identified, and the antibacterial activity of this plant could be associated with them. According to previous findings (Oussalah et al., 2006; Saad et al., 2013), volatile compounds possess the capacity to inhibit the synthesis of structural macromolecules and growth regulators.

In general, Gram-positive bacteria included in this study were more susceptible to the activity of tested EOs. Growth inhibition zones observed in Gram-positive strains ranged from 6.60±1.10 (*S. aureus* ATCC 33591, MRSA strain; achieved by the activity of fennel EO at 750 µg/ml) to 38.30±2.90 (same strain; inhibited by the pure EO of black cumin). A possible explanation of such results could be related to the more complex and rigid outer membrane of Gram-negative bacteria, where the lipopolysaccharide layer limits the diffusion of hydrophobic constituents, unlike the Gram-positive bacteria, where peptidoglycan cell wall provides less resistance (Patterson et al., 2019). Suggested mechanisms of the antibacterial activity of EOs are increased cell permeability and toxic effects on membrane structure (Swamy et al., 2016; Chouhan et al., 2017). Furthermore, there should be mentioned that EOs are composed of 20 to 60 different compounds (Nazzaro et al., 2013), and specific ones could be responsible for the particular antibacterial activity. Adequate diffusion of the active molecules through the agar medium requires optimization of medium concentration to achieve optimum activity of some chemical compounds. The results of Uzair et al. (2017) suggest that essential oils exhibit a strong synergism with certain antibiotics, even in MDR bacterial strains. Therefore, some essential oils that weren't so effective alone, could be discussed as synergistic candidates. Both MRSA strains included in this study were susceptible to EOs, while the same strains were resistant to all standard antibiotics (**Tab. 1**). The number of bacterial strains inhibited by the activity of investigated EOs and antibiotics is presented in **Fig. 1**.

Broth microdilution protocol was performed after all the results from the disk-diffusion method were gained. Only those bacterial species where the inhibition with the investigated EO was achieved, were further analyzed in the sense of determining the minimum inhibitory concentration. Minimum inhibitory concentrations of investigated EOs against tested bacterial species ranged from 250 µg/ml, recorded in the case of the black cumin EO against *S. aureus* ATCC 33591 (MRSA strain), while the highest MIC values (750 µg/ml) are related to the fennel and lemon EOs against *S. aureus* ATCC 33591 and *S. aureus* NCTC 12493, both MRSA strains. MIC values of the fennel and lemon EOs against MRSA strains (*S. aureus* ATCC 33591 and *S. aureus* NCTC 12493, respectively) were extrapolated from the disk-diffusion method since there was bacterial growth at 500 µg/ml, and no growth at 1000 µg/ml (pure oil in DMSO), but a particular concentration of 750 µg/ml was not included in the decimal dilutions used in the broth microdilution method. Obtained values are presented in **Tab. 2**.

In order to establish the MBC value, the results of the previously performed methods were taken into account. The MBC was determined for the EOs with known MIC. With the exception of the black cumin EO against the *S. aureus* ATCC 33591 (MRSA), where MBC was determined at 500 µg/ml, other samples

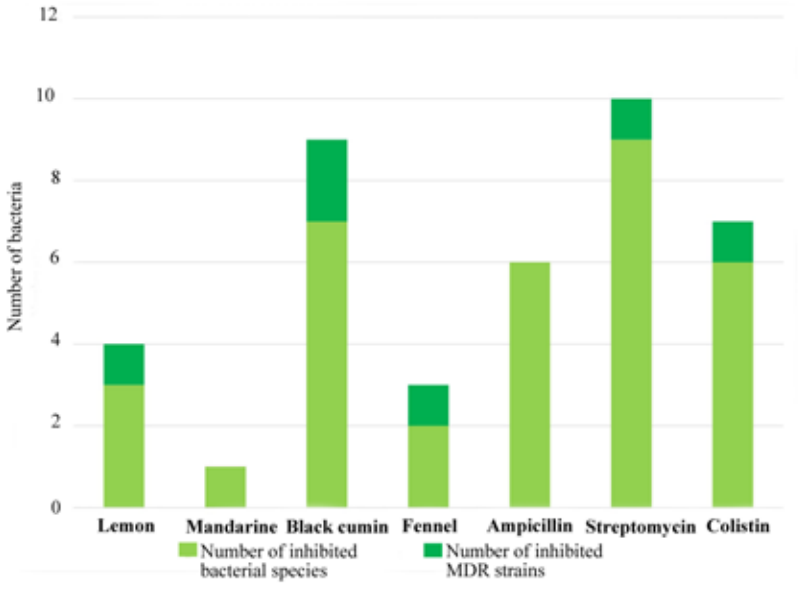


**Fig. 1.** Inhibited bacterial strains after the application of essential oils and antibiotics

performed bactericidal activity as the pure EO, at 1000 µg/ml of DMSO.

For the evaluation of the antibiofilm potential of tested EOs, the first step included the determination of the biofilm-forming category of investigated bacterial strains based on the results generated in positive controls. Obtained results showed that strong biofilm-formers were: *S. aureus* ATCC 6538 (SA1), *S. aureus* NCTC 12493 (SA4; MRSA), *E. coli* ATCC 35218 (EC3; ESBL), and *P. aeruginosa* ATCC 27853 (PA). Moderately adherent biofilm was detected in *S. aureus* ATCC 25923 (SA2), *S. aureus* ATCC 33591 (SA3; MRSA), *B. subtilis* ATCC 6633 (BS), and *E. coli* 14169 (EC1). Weakly adherent biofilm was formed by *E. faecalis* ATCC 29212 (EF), *E. coli* ATCC 25922 (EC2), and *S. enterica* NCTC 6017 (SE). Mean absorbance values with standard deviations are presented in **Fig. 2**.

**Table 2.** Minimum inhibitory concentration (µg/ml) of tested essential oils and standard antibiotics

| Essential oils | Bacterial strains | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | SA1 | SA2 | SA3 | SA4 | EF | BS | EC1 | EC2 | EC3 | PA | SE |
| **Lemon** | - | - | - | 750* | 500 | 500 | - | - | - | - | - |
| **Mandarine** | - | - | - | - | - | 500 | - | - | - | - | - |
| **Black cumin** | 500 | 500 | 250 | 500 | - | 500 | - | 500 | - | 500 | - |
| **Fennel** | - | - | 750* | - | - | 500 | - | - | - | - | - |

SA1: *Staphylococcus aureus* ATCC 6538; SA2: *S. aureus* ATCC 25923; SA3: *S. aureus* ATCC 33591 (MRSA); SA4: *S. aureus* NCTC 12493 (MRSA); EF: *Enterococcus faecalis* ATCC 29212; BS: *Bacillus subtilis* ATCC 6633; EC1: *Escherichia coli* ATCC 14169; EC2: *E. coli* ATCC 25922; EC3: *E. coli* ATCC 35218 (ESBL); PA: *Pseudomonas aeruginosa* ATCC 27853; SE: *Salmonella enterica* NCTC 6017.

*MIC is extrapolated according to the results of the disk-diffusion method.

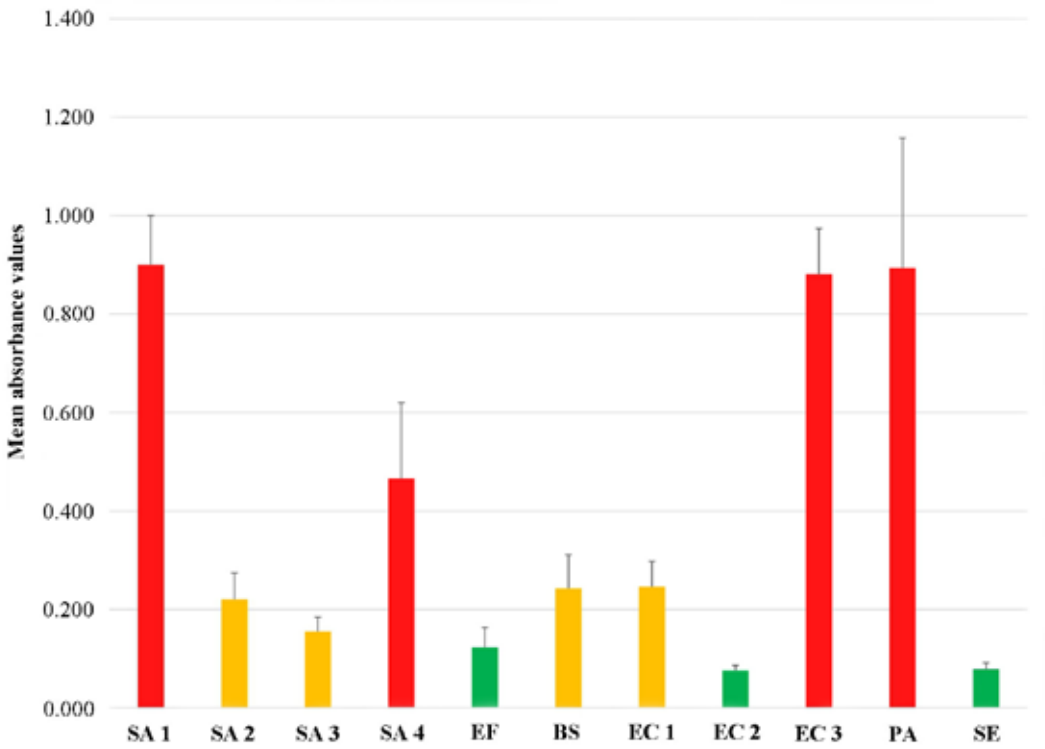


**Fig. 2.** Biofilm-forming categories of investigated bacteria (Red columns, strong biofilm-formers: SA1, *Staphylococcus aureus* ATCC; SA4, methicillin-resistant (MRSA) *S. aureus* NCTC 12493; EC3, extended-spectrum beta-lactamase-producing (ESBL) *E. coli* ATCC 35218; PA, *Pseudomonas aeruginosa* ATCC 27853. Yellow columns, moderate biofilm-formers: SA2, *S. aureus* ATCC 25923; SA3, methicillin-resistant *S. aureus* (MRSA) ATCC 33591; BS, *Bacillus subtilis* ATCC 6633; EC1, *Escherichia coli* 14169. Green columns, weak biofilm-formers: EF, *Enterococcus faecalis* ATCC 29212; EC2, *E. coli* ATCC 25922; SE, *Salmonella enterica* NCTC 6017)

Subinhibitory concentrations of tested EOs were examined for antibiofilm properties. Changes in the biofilm-forming capacity of investigated bacteria due to the activity of EOs are presented in **Tab. 3**. Lemon EO decreased the biofilm-forming capacity of *S. aureus* NCTC 12493 (MRSA), with full inhibition of the biofilm formation at a concentration of 62.5 µg/ml. The same sample removed the weakly adherent biofilm of *E. faecalis* ATCC 29212 and the moderately adherent biofilm of *B. subtilis* ATCC 6633 at 125 µg/ml. Lemon EO is known for its antibacterial potential, but recently there are also studies regarding its antibiofilm effects (Sun et al., 2018; Luciardi et al., 2021; Jamil et al., 2022). Our investigation confirmed that lemon EO has the ability to inhibit bacterial growth, including the MDR strains. Furthermore, antibiofilm activity is recorded against Gram-positive bacteria. As with other EOs, biological activity is typically associated with the most abundant compound. Lawrence & Palombo (2009) noted that particular constituents of EO don't show the same level of antibacterial activity as the complete oils, which suggests the involvement of the minor components, that can act synergistically with major compounds. In the lemon EO the most abundant monoterpene hydrocarbons are D-limonene, *γ*-terpinene, and *β*-pinene, while among the oxygenated monoterpenes, the most abundant are *α*-terpineol, nerol, and geraniol (Yazgan et al., 2019). Mandarin orange EO leads to a reduction of biofilm formation of *S. aureus* ATCC 6538 which is otherwise a good biofilm producer, and in *B. subtilis* ATCC 6633 which has a moderate biofilm-forming capacity, to weakly adherent at 125 µg/ml. Mandarin orange EO consists mainly of limonene and γ-terpinene, while other compounds include α-and β-pinene, β-myrcene, *o*-cymene, and b-thujene, with the limonene being considered the main constituent involved in bioactive and antimicrobial properties (Song et al., 2020). Song et al. (2021) noted that mandarin orange EO can inhibit biofilm formation and destroy mature biofilms, but these authors also emphasize the lower activity of EO against Gram-negative bacteria. In accordance with other results of this study, black cumin EO showed great antibiofilm potential, and eradication of biofilm was recorded in the case of *S. aureus* ATCC 6538, *S. aureus* ATCC 33591 (MRSA), *S. aureus* NCTC 12493 (MRSA), and *P. aeruginosa* ATCC 27853. At the concentration of 125 µg/ml of black cumin EO, there was no bacterial cell adherence in the abovementioned strains. Bourgou et al. (2010) also noted differences in the chemical composition of the black cumin EO from various geographical areas, while Chaieb et al. (2011) defined thymoquinone as the active principle of the black cumin with anti-biofilm potential. A review by Forouzanfar et al. (2014) stands out the antimicrobial properties of black cumin, with an accent of wide medicinal use of this plant without reported side effects. Fennel EO leads to the eradication of pre-formed biofilms of *S. aureus* ATCC 33591 (MRSA) and *B. subtilis* ATCC 6633 at the concentration of 125 µg/ml. Antibacterial properties of fennel investigated earlier (Diao et al., 2014; Mutlu-Ingok, 2021; Alam et al., 2022) are recently supported by antibiofilm studies of this plant and its derivates (Kwiatkowski et al., 2020). The major ingredient of the fennel EO is *p*-metoxypropenylobenzene, which is a by-product of terpene synthesis (Kwiatkowski

**Table 3.** Impact of the tested EOs on the biofilm-forming capacity of investigated bacteria

| Essential oil | Bacterial strains | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **SA1** | **SA2** | **SA3** | **SA4** | **EF** | **BS** | **EC1** | **EC2** | **EC3** | **PA** | **SE** |
| **Lemon** | - | - | - | +++* | +* | ++* | - | - | - | - | - |
| **Mandarine** | ++ | - | - | - | - | + | - | - | - | - | - |
| **Black cumin** | +++* | + | ++* | +++* | - | + | - | + | - | +++* | - |
| **Fennel** | - | - | ++* | - | - | ++* | - | - | - | - | - |

SA1: *Staphylococcus aureus* ATCC 6538; SA2: *S. aureus* ATCC 25923; SA3: *S. aureus* ATCC 33591 (MRSA); SA4: *S. aureus* NCTC 12493 (MRSA); EF: *Enterococcus faecalis* ATCC 29212; BS: *Bacillus subtilis* ATCC 6633; EC1: *Escherichia coli* ATCC 14169; EC2: *E. coli* ATCC 25922; EC3: *E. coli* ATCC 35218 (ESBL); PA: *Pseudomonas aeruginosa* ATCC 27853; SE: *Salmonella enterica* NCTC 6017.

Changes in the biofilm-forming category:

+++ decreasing up to three biofilm-forming categories

++ decreasing up to two biofilm-forming categories

+ decreasing in one biofilm-forming category

* Elimination of the biofilm in presence of a particular concentration of tested EO

- No antibiofilm activity

et al., 2019).

## Conclusion

Essential oils represent bioactive compounds of natural origin, where the proportion of active constituents vary due to geographical distribution and many environmental conditions. These slight differences in the chemical profile of the essential oil could be crucial in terms of the occurrence of bacterial resistance. The antibacterial potential of EOs derived from four edible and spice plants: lemon, mandarin orange, black cumin, and fennel is emphasized through the results of this research. Performed investigation showed that essential oils derived from *C. lemon*, *C. reticulata*, *N. sativa*, and *F. vulgare* exhibited antibacterial properties which are proved through different microbiological assays. Gram-positive bacteria were more susceptible to the activity of EOs. For instance, MDR *S. aureus* treated with black cumin EO had the greatest growth inhibition, which was not achieved by the activity of tested antibiotics. Additionally, *B. subtilis* was successfully inhibited by all four EOs. Besides inhibition, investigated EO performed bactericidal properties. The antibiofilm activity was noted for all examined EOs, with the most prominent activity of the black cumin EO, where complete inhibition of the biofilm formation was noted for strong biofilm-formers, including MDR strains. It could be concluded that all investigated essential oils displayed strain-specific and dose-dependent antibacterial activity.

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