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# Synergistic potential of *Juniperus communis* and *Helichrysum italicum* essential oils against nontuberculous mycobacteria

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**Abstract**

**Objective**. The present study evaluated the possible synergistic antimycobacterial interactions of *Juniperus communis* and *Helichrysum italicum* essential oils (EO).

**Methods**. Antimycobacterial potential was tested against *Mycobacterium avium* and *Mycobacterium intracellulare* using broth and water dilution method and checkerboard synergy method. Antiadhesion and antibiofilm effect of EOs was evaluated on biotic (HeLa cells) and abiotic surface (polystyrene). To evaluate the possible mechanisms of action, cellular leakage of proteins and DNA was tested and structural changes were visualized with a transmission electron microscope.

**Results.** MIC, minimum bactericidal concentration (MBC) and minimal effective concentration (MEC) were 1.6 mg $ml^{-1}$ for *J. communis* EO and 3.2 mg $ml^{-1}$ for *H. italicum* EO against both mycobacteria. All combinations of EOs in checkerboard synergy method produced fractional inhibitory concentration index values ranging from 0.501 to 1.5, corresponding to synergistic, additive or indifferent effects. *Mycobacterium avium* showed a greater tendency to create biofilm but these EOs at subinhibitory concentrations (sMIC) effectively blocked the adhesion and the establishment of biofilm. The exposure of both mycobacteria to MICs and sMICs lead to significant morphological changes: acquired a swollen form, ghost-like cell, disorganized cytoplasm detached from the cell wall. OD value of supernatant for both mycobacteria exposed to EOs have confirmed that there is a leakage of cellular material.

**Conclusion**. The leakage of the cellular material is noticeably higher in sMIC, which is probably due to cell wall damage. sMIC of both EOs have an additive or synergistic effect, reducing MICs, limiting adhesion and preventing the formation of biofilms.

## INTRODUCTION

Nontuberculous mycobacteria (NTM) are ubiquitous opportunistic micro-organisms, present in soil, surface water and water systems. Recently, NTM were included in the group of opportunistic premise plumbing pathogens (OPPPs) [1] together with *Legionella pneumophila*, *Pseudomonas aeruginosa, Acinetobacter baumannii,* etc. These bacteria are considered normal residents of premise plumbing, but also the agents of infections, esspecially in individuals with predisposing conditions. Some of the common features shared by OPPPs are dissinfectant resistance, tendency to adhere to surfaces and proliferate in biofilms. Biofilms generated by NTM can be defined in the same way as any other biofilms. It begins by attachement of mycobacteria to abiotic or biotic surfaces, production of extracellular matrix and maturation of biofilm [2]. Biofilm-forming mycobacteria are more resistant to environmental stresses, disinfectants and

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**Keywords:** antiadhesion; antibiofilm; checkerboard synergy method; essential oils; nontuberculous mycobacteria; opportunistic premise plumbing pathogens.

**Abbreviations:** Ad, additive; ADC, albumin-dextrose-catalase; An, antagonistic; ATCC, American Type Culture Collection; DMEM, Dulbecco's Eagle Medium; EO, essential oil; FBS, fetal bovine serum; FIC, fractional inhibitory concentration; FICi, fractional inhibitory concentration indeks; *H. italicum*, *Helichrysum italicum*; 7H9S, supplemented Middlebrook 7H9 broth; 7H10S, supplemented Middlebrook 7H10 agar; 7H10S, supplemented Middlebrook 7H10 agar; In, indifferent; *J. communis*, *Juniperus communis*; *M. avium*, *Mycobacterium avium*; MBC, minimal bactericidal concentration; MEC, minimal effective concentration; MIC, minimal inhibitori concentration; *M. intracellulare*, *Mycobacterium intracellulare*; NTM, nontuberculous mycobacteria; OADC, oleic acid-albumin-dextrose-catalase; OPPP, opportunistic premise plumbing pathogens; PTA, phosphotungstic acid; sMIC, subinhibitory concentrations; Sy, synergistic; Tw 80, Tween 80.

antimicrobial drugs. NTM share habitats and can grow within free-living amoeba ranking them into a group of amoebae-resisting micro-organisms [3]. From natural sources, NTM are usually transmitted by aerosol inhalation, ingestion of contaminated food and water or inoculation through the skin. NTM infections has been increasing globally, most commonly presented as pulmonary mycobacteriosis, lymphadenitis in children, skin infections or disseminated form of disease in immunocompromised persons [4]. Because NTM infections represent an emerging clinical and public health threat it is necessary to explore and develop new strategies to block the biofilm formation as an important cause of human infection. Particularly, new light is cast on natural sources such as plants that have an antimicrobial effect. Essential oils (EOs) are volatile and fragrant substances of oily consistency [5]. *Juniperus communis* L. is a shrub or small evergreen tree from the family *Cupressaceae*. According to the literature, the main components of *J. communis* EO are α-pinene, sabinene, β-pinene, β-myrcene and limonene [6–8]. The EO of juniper berries are attributed to different effects such as diuretic, antidiabetic and antihyperlipidemic activity, antimicrobial activity, antioxidant activity, etc. [9]. *Helichrysum italicum* is a small perennial aromatic shrub from the family *Asteraceae* with yellow flowers. Grows on dry cliffs and sandy soils in the Mediterranean region and plays an important role in the traditional medicine. Its various biological effects are described: antimicrobial, antifungal and anti-inflammatory properties, antierythematous and photoprotective activities, intestinal antispasmodic effects [10, 11]. The antimycobacterial effect of different EOs or their main components has already been confirmed in several studies that were mostly focused on *Mycobacterium tuberculosis* [12, 13]. Our study was focused on the antibacterial and antibiofilm potential of *J. communis* and *H. italicum* EOs on NTM.

## METHODS

### Essential oils

The EOs of the aromatic plants *J. communis* and *H. italicum* were purchased from 'Ireks aroma d.o.o.', Zagreb, Croatia. Both EOs were dissolved in dimethyl sulfoxyde (DMSO, Kemika, Croatia). We have already used them in our previous experiments and tested their composition. The main components of *J. communis* EO were α-pinene (31.13%), sabinene (9.95%), *β*-pinene (8.50%), *β*-myrcene (4.80%), limonene (4.53%), germacrene D (4.21%), δ-cadinene (3.3%), trans-*β*-caryophyllene (2.95%), germacrene B (2.83%), α-humulene (2.44%) and β-elemene (2.34%) [7]. The chemical composition of *H. italicum* EO characterized by gas chromatography/mass spectrometry analyses was α-pinene (21.6%), ϒ-curcumene (21.6%), neryl-acetate (7.9%), *β*-selinene (6.5%), isoitalicene (5.4%), *β*-caryophyllene (4.9%) and α-curcumene (4.5%) [14].

### Strains and growth media

Bacterial strains were purchased from the American Type Culture Collection (ATCC, VA, USA). *Mycobacterium avium* ssp. *avium* (serotype 2) ATCC 25291 (*M. avium*) and *Mycobacterium intracellulare* ATCC 13950 (*M. intracellulare*) strains were subcultivated twice in supplemented Middlebrook 7H9 broth (7H9-S; Difco, Detroit, Michigan, USA). Furthermore, 7H9-S was supplemented with 10% albumin–dextrose–catalase (ADC, Biolife Italiana, Milano, Italy) and 0.05% Tween 80 (Tw 80, Biolife Italiana, Milano, Italy) at 37 °C for 2 successive weeks to obtain $10^8$ c.f.u. $ml^{-1}$. The bacteria were then frozen at −80 °C dispensed with addition of 10% glycerol. For each experiment, an aliquot was thawed and subcultured in 7H9-S for 2 weeks and then on Middlebrook 7H10 agar (7H10-S; Difco, Detroit, Michigan, USA) supplemented with 10% oleic acid–albumin–dextrose–catalase (OADC, Biolife Italiana, Milano, Italy) and 0.05% Tw 80 at 37 °C for 2 more weeks. Initial inocula were later verified by diluting and plating the culture onto 7H10-S agar and incubated at 37 °C for 4 to 6 weeks before colonies were counted [15].

### Determination of MIC and minimum bactericidal concentration (MBC)

A broth microdilution method was used to determine MIC and MBC of the *J. communis* and the *H. italicum* EOs as has been previously described [7]. Briefly, twofold dilutions of EO in the 7H9-S broth starting from 0.1 to 51.2 mg $ml^{-1}$ in the sterile 96-well microtitre plate (Vacutest Kima s.r.l., Italy) were performed. In each well mycobacterial suspension ($1\times10^6$ c.f.u. $ml^{-1}$ per well) and resazurin (0.015% solution) (Sigma, Germany) [16] were added. Amikacin (Sigma, Germany) in concentrations from 0.001 to 0.128 mg $ml^{-1}$ served as a positive control. The highest concentration of DMSO used in the samples of EOs was also tested to eliminate the bactericidal effect of the solvent. After 24 , 72 and 96 h of incubation at 37 °C plates were read visually. The lowest concentration that did not show change in colour was defined as MIC. All dilutions were inoculated on 7H10-S agar and incubated for 4 weeks at 37 °C. The MBC was defined as the lowest concentration of EO that killed ≥99% of the bacteria. The results are expressed in mg $ml^{-1}$ [15].

### Determination of minimal effective concentration (MEC)

MEC was determined in the same manner as MIC but the dilutions were made in sterile tap water instead in 7H9-S [7]. Positive (sterile tap water with bacterial inoculum) and negative (sterile tap water allone) growth controls were performed. After 24 h of incubation at 37 °C the test dilutions from each well were inoculated on 7H10-S and incubated further for 4 weeks at 37 °C. The MEC was defined as the lowest concentration of EO that killed ≥99% of bacteria. The results are expressed in mg $ml^{-1}$ .

### Checkerboard synergy method

The stock solutions and serial twofold dilutions of each EO to at least double the MIC were prepared in 7H9-S and was distributed into the wells of the sterile microdilution plates. The first EO of the combination was serially diluted along the ordinate, while the second EO was diluted along

the abscissa. An inoculum equal to $1\times10^6$ c.f.u. $ml^{-1}$ was prepared from each *Mycobacterium* isolate in 7H9-S and added, with resazurin (0.015% solution), to wells with diluted EO to reach the final volume of 200 µl. The plates were incubated at 37 °C for 4 days under aerobic conditions. The resulting checkerboard contains each combination of two EOs, with wells that contain the highest concentration of each EO at opposite corners. The test dilutions from each well were inoculated on 7H10-S and incubated further for 4 weeks at 37 °C [15].

Calculation of fractional inhibitory concentration (FIC):

FIC of EO A=MIC of EO A in combination with EO B / MIC of EO A alone

FIC of EO B=MIC of EO B in combination with EO A / MIC of EO B alone.

Calculation of fractional inhibitory concentration index (FICi):

FICi=FIC EO A+FIC EO B=$MIC_{AB}/MIC_A+MIC_{BA}/MIC_B$ [5, 17].

The effect of FICi was interpreted as follows: combination were considered synergistic (**Sy**) if the FICi was ≤0.5, antagonistic (**An**) if the FICi was >4, additive (**Ad**) when FICi was >0.5 and≤1.0, indifferent (**In**) when FICi was >1.0 and ≤4 [18].

## Leakage of cellular metabolites

After 2 weeks' incubation in 7H9-S culture *M. avium* or *M. intracellulare* were transferred into sterile tubes and were centrifuged at 3500 ***g*** for 10 min. and washed twice in sterile tap water. $OD_{600}$ of each bacterial suspension was adjusted to 1.0 corresponding to $1\times10^8$ c.f.u. $ml^{-1}$. Bacterial numbers were verified by plating tenfold serial dilutions. Aliquots of each bacterial suspension were dispensed in a sterile flask and EOs of *J. communis* and *H. italicum* were added in a concentration that showed the synergistic effect. The flask containing only bacterial cultures served as growth controls. After incubation of 24 h at 37 °C, the suspensions were centrifuged at 3500 ***g*** for 10 min. Supernatants were used for quantitation of nucleic acid, at absorbance of 260 nm (A260), and proteins at 280 nm (A280) [7, 19].

## Antiadhesion assay on biotic surface

HeLa cervical cancer cell lines (ATCC, VA, USA) were kept in Dulbecco's Modified Eagle Medium (DMEM, Lonza, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Invitrogen, NY, USA), 2 mM L-glutamine (Lonza, Belgium), 100 U $ml^{-1}$ penicillin and 100 mg $ml^{-1}$ streptomycin (Sigma, Germany) in humidified atmosphere with 5% $CO_2$ at 37 °C. For the experiments $10^5$ HeLa cells were seeded into 24-well microtitre plates (Falcon, Becton Dickinson, USA) for 2 h until a confluent monolayer was formed. Then, cells were washed with DMEM and their intact state was checked under the microscope. For blanks HeLa cells were treated only with DMEM ($10^5$/well). Mycobacterial suspension in DMEM ($10^5$/well) were added to the following wells together with *J. communis* EO or *H. italicum* EO or combination of both EOs. EOs were added in 1/4xMIC. The control wells were the mycobacterial suspension in DMEM. The plate was incubated for 1 h in a humidified atmosphere with 5% $CO_2$ at 37 °C. After incubation all wells were three times washed with PBS to eliminate unbound mycobacteria, filled with PBS and sonificated in a water bath at 40 kHz (Bactosonic, Bandelin, Berlin, Germany) for 1 min. Mycobacteria were enumerated by plating on 7H10-S at 37 °C for 14 days. Cell viability was checked during experiment using 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide colorimetric assay (MTT, Sigma-Aldrich, MO, USA). The percentage of adhesion inhibition on HeLa cells was determined according to the formula [20]:

$$\text{Percentage of inhibition (\%)} = 1 - \frac{CFU\ of\ sample\ treated\ with\ the\ EO}{CFU\ of\ negative\ control\ sample} \times 100$$

## Antiadhesion and antibiofilm effect of *J. communis* EO and *H. italicum* EO

Suspensions of each mycobacterial isolate, were made in sterile tap water and approximately $10^5$ c.f.u. $ml^{-1}$ were inoculated per well of sterile polystyrene microtitre plate (Vacutest Kima s.r.l., Italy). Both mycobacteria were treated with subinhibitory concentrations of *J. communis* EO and *H. italicum* EO, separately and in combination (0.8 and 0.012 mg $ml^{-1}$, respectively). Plates were then incubated in antiadhesion or in antibiofilm assay for 24 h at 37 °C and 72 h at 37 °C, respectively. Antreated mycobacterial cells served as the control. At the end of the incubation, plates were washed three times with sterile tap water and sonificated in a water bath at 40 kHz for 1 min. Mycobacteria were enumerated by culturing on 7H10-S at 37 °C for 14 days until colonies were observed. The percentage of polystyrene adhesion inhibition or biofilm inhibition was determined as previously described for antiadhesion assay on biotic surface according to Teanpaisan *et al.* [20].

## Transmission electron microscopy

To evaluate structural changes the morphologies of the bacteria exposed to the selected EO and their synergistic combinations were analysed. Briefly, *M. avium* and *M. intracellulare* were grown on 7H10-S for 4 to 6 weeks and then exposed to the *J. communis* and *H. italicum* EOs, as well as their synergistic combinations for 24 h at 37 °C. Then, 10 µl of bacterial suspension ($1\times10^8$ c.f.u. $ml^{-1}$) was placed on Formvar-coated copper grids (Agar Scientific Ltd, Essex, UK) for 2 min. After that, the excess of liquid was wicked off the grids with Watman no. 3 filter paper (Macherey-Nagel, Duren, Germany). The bacteria remaining on the grids were stained with 1% phosphotungstic acid (PTA; Sigma-Aldrich, St. Louise, USA) for 1 min, and the excess of PTA carefully removed with filter paper. The grids were then left dry on air for a few minutes. The bacteria were inspected on a transmission electron microscope (JEM-2100F, Jeol, Japan).

### Statistical analysis

The data were analysed using STATISTICA commercial software, 12.0 (StatSoft, Tulsa, OK, USA). Results are expressed as mean value±SD. Normality of the data distribution was assessed by the Kolmogorov–Smirnov normality test. The distribution qualified the normality test, so nonparametric tests were applied. Differences between groups of samples were analysed by the Kruskal–Wallis ANOVA on ranks test, while the influence of EO on mycobacterium were tested by Mann–Whitney U test. Differences with $P<0.05$ were considered to be statistically significant.

## RESULTS

The chemical composition of *J. communis* EO and *H. italicum* EO was previously determined by GC/MS analysis [7, 14]. Both oils were rich in α-pinene. The *H. italicum* EO, along with it, contained the same concentration of ϒ-curcumene.

### MIC, MBC and MEC

The MIC values obtained for *J. communis* EO and for *H. italicum* EO against both NTM was 1.6 and 3.2 mg ml$^{-1}$, respectively. MIC, MBC and MEC in this assay were of equal value so the result is further shown only as MIC. MIC and MBC for amikacin against *M. avium* and *M. intracellulare* were 0.016 and 0.008 mg ml$^{-1}$, respectively.

### FICi for combinations of *J. communis* EO and *H. italicum* EO

All combinations of EOs produced $FIC_i$ values ranging from 0.501 to 1.5, corresponding to synergistic, additive or indifferent effects (Table 1). The MIC is reduced for at least one of the paired substances of all the combinations. However, the synergistic effect only occurred against *M. avium* when *J. communis* EO (in concentration of 0.8 mg ml$^{-1}$ or 1/2xMIC) was combined with *H. italicum* EO in concentration of 0.006 mg ml$^{-1}$ (533-fold reduction in MIC) or 0.012 mg ml$^{-1}$ (267-fold reduction in MIC). For *M. intracellulare* additive effect of *H. italicum* and *J. communis* EO, both in concentration of 1/2xMIC, was observed.

### Leakage of cellular metabolites

When cell membrane leakage is monitored by *M. avium* supernatant absorbance measurement, combination of the subinhibitory concentration of *H. italicum* EO and *J. communis* EO in the concentration of 1/2xMIC leads to a substantial increase in OD at 260 and 280 nm (Fig. 1a). For *M. intracellulare*, the same effect is visible at MIC and 1/2xMIC for *J. communis* EO, at 1/2xMIC for *H. italicum* EO and in combination of these EOs at concentrations of 1/2xMIC at $OD_{260}$ and $OD_{280}$ (Fig. 1b). *J. communis* EO at 1/2xMIC significantly influences the increase of OD at both wavelengths of *M. intracellulare* compared to *M. avium*.

### Antiadhesion on biotic surface

As shown in Fig. 2a, the adhesion of *M. avium* and *M. intracellulare* to HeLa cells under the influence of EOs at concentrations of 1/4xMIC, added individual or in combination, was significantly inhibited, compared to the control, except for *M. intracellulare* when both EOs are combined. *H. italicum* EO and *J. communis* EO in combination, significantly reduced the adhesion of *M. avium* to HeLa cells compared to *M. intracellulare*. But the combination of both EOs in relation to the treatment with only *J. communis* EO in *M. intracellulare* leads to significant inhibition of adhesion to HeLa cells. There was significant antiadhesion activity for each of these EOs using adhesion inhibition in the range of 66.5 to 71.5% for *M. avium* and in the range of 64.7 to 92.8% for *M. intracellulare* (Table 2).

**Table 1.** Checkerboard synergy assay with *J. communis* EO and *H. italicum* EO against *M. avium* and *M. intracellulare*

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***M. avium*** | *HI* EO* | **0.006** | **0.012** | 0.025 | 0.05 | 0.1 | 0.2 | 0.4 | 0.8 | 1.6 | 1.6 | 1.6 | 3.2 | 3.2 | |
| | *JU* EO* | **0.8** | **0.8** | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.4 | 0.2 | 0.2 | 0.1 | |
| | $FIC_{HI}$ | 0.001 | 0.004 | 0.008 | 0.02 | 0.03 | 0.06 | 0.13 | 0.25 | 0.5 | 0.5 | 0.5 | 1.0 | 1.0 | |
| | $FIC_{JU}$ | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.25 | 0.13 | 0.13 | 0.06 | |
| | $FIC_i$ | **0.501** | **0.504** | 0.508 | 0.52 | 0.53 | 0.56 | 0.63 | 0.53 | 1.0 | 0.75 | 0.63 | 1.13 | 1.06 | |
| | Interaction | **Sy** | **Sy** | Ad | Ad | Ad | Ad | Ad | Ad | Ad | Ad | Ad | In | In | |
| ***M. intracellulare*** | *HI* EO* | **0.006** | 0.012 | 0.025 | 0.05 | 0.1 | 0.2 | 0.4 | 0.8 | 1.6 | **1.6** | 3.2 | 3.2 | 3.2 | 3.2 |
| | *JU* EO* | **1.6** | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | **0.8** | 0.8 | 0.4 | 0.2 | 0.1 |
| | $FIC_{HI}$ | 0.001 | 0.004 | 0.008 | 0.02 | 0.03 | 0.06 | 0.13 | 0.25 | 0.5 | 0.5 | 1.0 | 1.0 | 1.0 | 1.0 |
| | $FIC_{JU}$ | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 0.5 | 0.5 | 0.25 | 0.13 | 0.06 |
| | $FIC_i$ | **1.001** | 1.004 | 1.008 | 1.02 | 1.03 | 1.06 | 1.13 | 1.25 | 1.5 | **1.0** | 1.5 | 1.25 | 1.13 | 1.06 |
| | Interaction | **Ad** | Ad | In | In | In | In | In | In | In | **Ad** | In | In | In | In |

*Concentration of EO in mg ml$^{-1}$.
Ad, additive; $FIC_{HI}$, fractional inhibitory concentration for *H. italicum* EO; FICi, fractional inhibitory concentration index; $FIC_{JU}$, fractional inhibitory concentration for *J. communis* EO; HI EO, *H. italicum* EO; In, indifferent; JU EO, *J. communis* EO; Sy, synergistic.

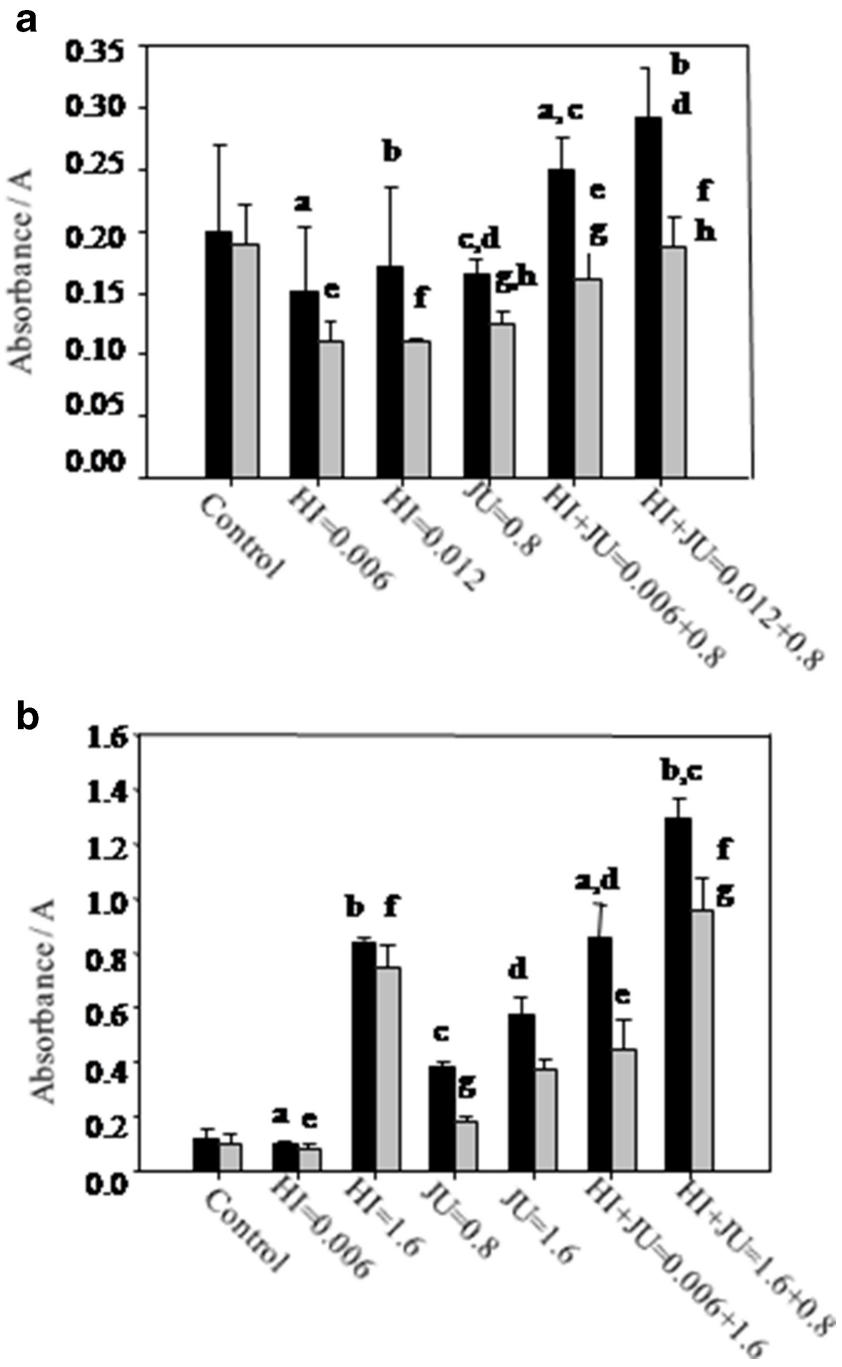


**Fig. 1.** Absorbance of supernatant of *M. avium* (a) and *M. intracellulare* (b) for quantitation of nucleic acid, at OD of 260 nm (■), and proteins at 280 nm (▥) after treatment with *J. communis* EO and *H. italicum* EO, separately and in combination (concentrations of EOs in mg ml$^{-1}$; HI=*H. italicum* EO; JU=*J. communis* EO). Data are means±SD. Statistically significant differences between groups bearing the same letter in each subfigure ($P$<0.05; versus control, and between the equally labelled groups).

### Antiadhesion and antibiofilm on abiotic surface

*M. avium* exhibits a higher tendency to adherence to polystyrene microtitre plates than *M. intracellulare* (Fig. 2b). *J. communis* EO at 1/2xMIC remarkably prevents adhesion of both NTM after 24 h. Subinhibitory concentration of *H. italicum* EO, compared to control, significantly decreased the number of adhered NTMs ($P$<0.05). The influence of both EOs, individually or in combination, on biofilm formation after 72 h in water is significantly less on *M. avium* than that on *M. intracellulare* (Fig. 2c). Percentage of inhibition of adhesion on polystyrene and percentage of inhibition of biofilm formation of *M. avium* and *M. intracellulare* under the

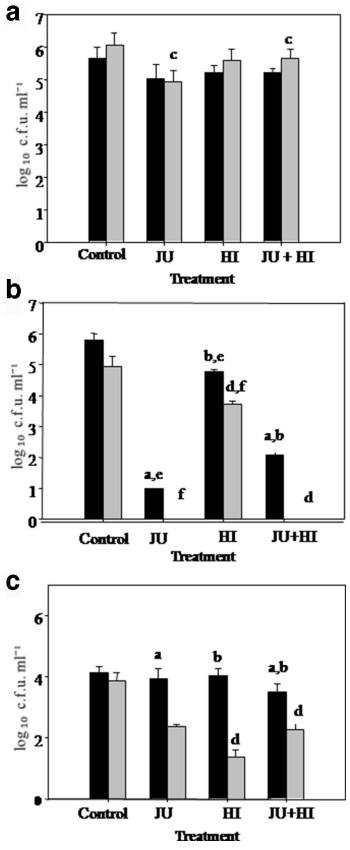


**Fig. 2.** Effect of *J. communis* EO and *H. italicum* EO on adhesion and biofilm formation of *M. avium* (■) and *M. intracellulare* (▥): adhesion to HeLa cells (a), adhesion on polystyrene (b), biofilm formation on polystyrene (c). *J. communis* EO and *H. italicum* EO at concentrations of 1/4xMIC, separately and in combination (a); *J. communis* EO in concentration of 0.8 mg ml$^{-1}$ (1/2xMIC) and *H. italicum* EO in concentration of 0.012 mg ml$^{-1}$, separately and in combination (b and c). Data are means±SD. Statistically significant differences between groups bearing the same letter in each subfigure ($P$<0.05; versus control, and between the equally labelled groups).

influence of *J. communis* EO and *H. italicum* EO at concentrations of 1/4xMIC is shown in Table 2. *J. communis* EO significantly inhibits adhesion and biofilm formation of both mycobacteria ($P$<0.05), with *M. intracellulare* being more sensitive to the effect of both EOs ($P$<0.05).

## TEM examinations

The morphological change was apparent in the cells of *M. avium* (Fig. 3b) and *M. intracellulare* (Fig. 3e) that were exposed to *J. communis* EO. There was disorganized cytoplasm, cells were more clustered, forming small buds on the cell wall and they change morphology from bacilli form to oval, which were not seen in untreated cells (Fig. 3a and d). Mycobacteria exposed to synergistic concentrations of *J. communis* EO and *H. italicum* EO acquired a swollen form, ghost-like cell and the cytoplasm was detached from the cell wall (Fig. 3c and f).

**Table 2.** Inhibition of adhesion and biofilm formation of *M. avium* and *M. intracellulare* on abiotic and biotic surface under the influence of *J. communis* EO and *H. italicum* EO

| **Percentage of inhibition of adhesion on** | **Abiotic surface (polystyrene)*** | | **Biotic surface (HeLa cells)†** | |
|---|---|---|---|---|
| | ***M. avium*** | ***M. intracellulare*** | ***M. avium*** | ***M. intracellulare*** |
| *J. communis* EO | 100% | 100% | 71.5% | 92.8% |
| *H. italicum* EO | 91.5% | 95.6% | 66.5% | 66.4% |
| *J. communis* EO+*H. italicum* EO | 100% | 100% | 68.9% | 64.7% |
| **Percentage of inhibition of biofilm formation on abiotic surface (polystyrene)*** | | | | |
| | ***M. avium*** | ***M. intracellulare*** | | |
| *J. communis* EO | 23.6% | 98% | | |
| *H. italicum* EO | 15% | 99.7% | | |
| *J. communis* EO+*H. italicum* EO | 74.3% | 99.1% | | |

**J. communis* EO in concentration of 0.8 mg ml$^{-1}$ and *H. italicum* EO in concentration of 0.012 mg ml$^{-1}$, separately and in combination.
†*J. communis* EO and *H. italicum* EO in concentrations of 1/4xMIC separately and in combination.

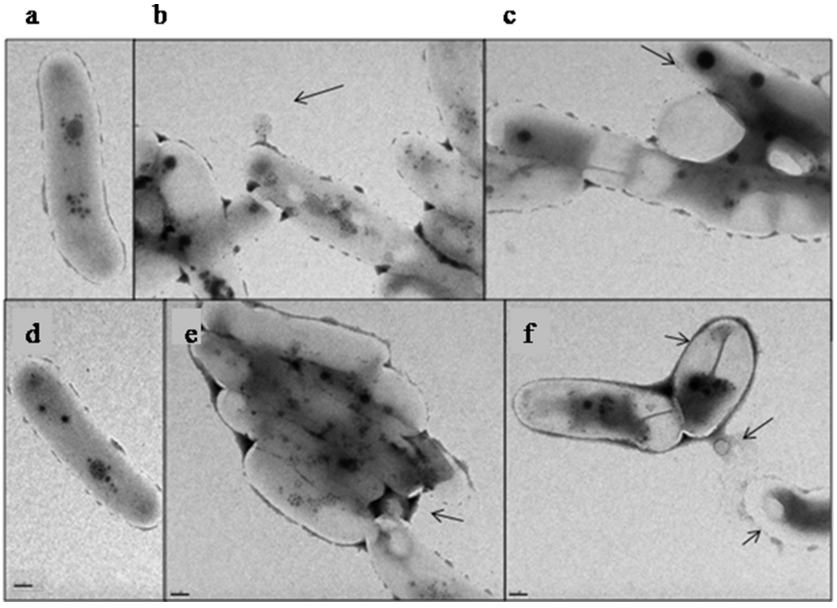


**Fig. 3.** Morphological analysis by transmission electron microscopy. (a) Standard cell morphology of *M. avium*. (b) Cells of *M. avium* after 24 h of exposure to the *J. communis* EO (1xMIC). (c) Cells of *M. avium* after 24 h of exposure to the *J. communis* EO (1/2xMIC) and *H. italicum* EO (1/256xMIC). (d) Standard cell morphology of *M. intracellulare*. (e) Cells of *M. intracellulare* after 24 h of exposure to the *J. communis* EO (1xMIC). (f) Cells of *M. intracellulare* after 24 h of exposure to the *J. communis* EO (1/2xMIC) and *H. italicum* EO (1×256 MIC). Arrows indicate cellular leakage (b, c, e, f), 'swollen form' of mycobacteria (c, f), ghost-like cell and the detached cytoplasm from the cell wall (f).

## DISCUSSION

Presence of NTM in freshwater has been recorded in many environmental studies [21]. Various methods for reducing exposure to NTM have been explored: disinfection with clorine preparations, filtration, ultraviolet radiation, etc. *M. avium* is perfectly adapted for maintenance in the water distribution system because it is resistant to disinfection, has thermal resistance, the ability to create biofilm and growth under stagnation conditions [3]. The six most common NTM clinical isolates are *M. avium* complex (47%), *M. gordonae* (11%), *M. xenopi* (8%), *M. fortuitum* complex (7%), *M. kansasii* (4%) and *M. abscessus* (3%) [22]. In our study, we tested the influence of *J. communis* EO and *H. italicum* EO on *M. avium* and *M. intracellulare*. MIC, MBC and MEC in this assay were of equal value, which indicates their bactericidal activity (MBC/MIC ratio must be less than or equal to 4) [23]. The calculated MBC/MIC ratio for *J. communis* EO and *H. italicum* EO was 1 and for amikacin 2, which confirms their strong bactericidal activity. Antimicrobial activity of the EOs is determined by their chemical composition, functional groups and the possible synergistic effect between the various components [5]. The main component of both EOs is α-pinen. More than two-thirds of the chemical composition of *J. communis* EO consisted of monoterpenes, while those in *H. italicum* EO make up slightly more than one-quarter, and the rest are sesquiterpenes, primarily ϒ-curcumene. Previous studies have shown that monoterpenes may cause morphological changes in the cell wall of mycobacteria, which leads to their increased permeability and cellular outflow [7, 24]. By measuring the OD value of the supernatant for both NTM we have confirmed that there is a leakage of cellular material. Particularly, this is noticeable in *M. intracelulare* while for *M. avium* a higher concentration of the EO is required [7]. It has been shown in several reports that the hydrophobic biologically active components present in the EO can be attached to the surface of the bacterial cell and accumulate in the cell membrane, thereby impairing its structural integrity, which can adversely affect cell metabolism, causing cell death [25]. Morphological changes are even more noticeable by observation under an electron microscope. Mycobacteria exposed to synergistic concentrations of both EOs have shown significant changes in the overall form of NTM cells and cytoplasm homogeneity, which is consistent with our previous findings and findings of other authors [7, 24]. Similar changes were observed with mycobacteria exposed to antibiotics isoniazid, ciprofloxacin, rifabutin, ethambutol and clarithromycin [26]. Synergistic effects were measured with the checkerboard synergy assay, which was conducted to evaluate the antimycobacterial effect of EO combinations. A synergistic effect of the combination of *J. communis* EO and *H. italicum* EO in subinhibitory concentration on *M. avium* was observed, while in *M. intracellulare* the same concentration resulted in an additive effect. Significantly, the antagonistic effect is not observed in any combination of EOs. It has been confirmed that some clinical isolates of *M. avium* formed more biofilms when incubated in water than in 7H9 due to increased upregulation of proteins that regulate the synthesis of other proteins involved in biofilm formation [27]. In our study, *M. avium* showed greater ability to create biofilms compared to *M. intracellulare* corresponding

to available researches [28]. Moreover, with *M. intracellulare* both oils, individually or in combination, almost completely inhibited adhesion to polystyrene, while on *M. avium* this effect is somewhat weaker but still significant. It is important that we use subinhibitory concentrations that are not bactericidal and led to a significant reduction in adhesion to polystyrene and reduction of the biofilm formation. To study potential inhibition of adhesion to biotic surface we used HeLa cells. Both EOs significantly reduced the adhesion of *M. avium* and *M. intracellulare* on HeLa cells. The additive and synergistic effects of these EOs are important as they reduced the MIC of combined oils, maintained the antimycobacterial activity and decreased potential toxicity. The antimycobacterial activity was even more promising against biofilm formation, which makes *J. communis* EO and *H. italicum* EO a promising candidate in combat strategies to limit NTM biofilm formation.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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