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## Article

# Combined Application of *Juniperus communis* Essential Oil and Amikacin, Clarithromycin and Rifampicin against *Mycobacterium avium* and *Mycobacterium intracellulare*

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**Abstract:** The group of nontuberculous mycobacteria (NTM) includes about 200 mycobacteria that are widespread in the natural environment as free-living saprophytic bacteria, commensals or symbionts. NTM, also referred to as atypical mycobacteria, are mostly apathogenic; nowadays, they are increasingly important environmental opportunistic pathogens. This study continues the work of previous studies which investigated the individual and synergistic effect of different essential oils (EOs) on NTM. The aim was to investigate the effect of the interaction of the common juniper (*Juniperus communis*) EO and the antimicrobials, amikacin, clarithromycin and rifampicin, against *Mycobacterium avium* and *M. intracellulare* using the checkerboard synergy method in an enriched Middlebrook 7H9 broth. Morphological changes of treated NTM cells were observed with a transmission electron microscope. The most synergistic combinations were found at subinhibitory concentrations of the common juniper EO and rifampicin against both tested NTM and this EO and clarithromycin against *M. avium*. A slightly smaller number of synergistic effects on both NTM were found using a combination of this EO and amikacin. Combinations of clarithromycin and the common juniper EO showed no synergism against *M. intracellulare*. The exposure of both NTM to synergistic combinations of this EO and antimicrobials caused significant morphological changes in mycobacterial cells. Synergism with the combined use of EOs and antimicrobials allows the use of low effective concentrations via the sustained antimicrobial effect of the tested substances, but with potentially reduced toxicity.

**Keywords:** amikacin; checkerboard synergy method; clarithromycin; *Juniperus communis*; nontuberculous mycobacteria; rifampicin



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## 1. Introduction

NTM are environmental opportunistic bacteria most often isolated from different water sources, soil and animals [1]. They are characterized by a distinct ability to form biofilms in water and on various surfaces. In the middle of the last century, many authors noticed the pathogenic potential of NTM, which especially came to the fore with the appearance of acquired immunodeficiency syndrome (AIDS) and other immunodeficiency conditions in patients with oncological, hematological, autoimmune and rheumatological diseases and after organ transplantation [2–9]. A recent study of the use of inhaled corticosteroids in chronic respiratory diseases, especially chronic obstructive pulmonary disease and asthma, indicates that such patients have a 16.5 times higher risk of NTM infection [10].

Members of the *Mycobacterium avium* complex (MAC) are the most frequently isolated pathogenic species of NTM from respiratory samples. These are non-pigmented, slow-growing mycobacteria with smooth, flat and transparent colonies [11]. Historically, the

MAC included two species, *Mycobacterium avium* (*M. avium*) and *Mycobacterium intracellulare* (*M. intracellulare*). *M. avium* has four subspecies, *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *silvaticum* [12,13]. As a rule, NTM are transmitted from the environment via ingestion, inhalation and inoculation into an immunocompromised host [14]. The outcome of respiratory exposure to NTM depends on the type of mycobacteria and its significance for general clinical conditions including the exposure dose and length of exposure, disruption of the local and general immunity host, as well as associated lung diseases and the extent of previous damage [15]. The treatment of infections caused by NTM is still not sufficiently standardized and is a challenge for several reasons. After the isolation of some of the NTM species from a clinical sample, it is necessary to determine their clinical significance and decide whether treatment is necessary or that it is a case of contamination/colonization. When an indication for treatment is established, it is usually long-term and complicated by intolerance, toxicity and drug interactions, and often requires the modification of therapeutic regimens. There are significant differences between different species and strains' virulence and response to treatment. Antimicrobial drugs used in therapeutic regimens are combinations of macrolides (clarithromycin or azithromycin), rifamycin (rifampicin or rifabutin), ethambutol, quinolone (ciprofloxacin or moxifloxacin) and aminoglycosides (amikacin or streptomycin) [16]. Essential oils (EOs) are volatile, natural, complex compounds characterized by a strong smell, formed via the secondary metabolism within plants [17]. They are mixtures of different terpenes and terpenoids, especially hemiterpenes, monoterpenes, sesquiterpenes and diterpenes. In addition, there may be present different aromatic and aliphatic compounds: acids, alcohols, aldehydes, acyclic esters or lactones [17–20]. Other components of plant origin for which antimicrobial activity has been described are phenolic acids, quinones, flavones, flavonoids, tannins, coumarins, alkaloids, lectins, polypeptides and polyacetylenes [21]. Although they may contain a large number of ingredients in their chemical composition, the characteristic properties of EOs usually arise from only one or a few dominant constituents.

The share of individual ingredients is determined by geographical origin, vegetative period, the part of the plant used, the maturity of the plant and fruits, and the method of EO extraction. Chemical composition, functional groups and potential synergistic effects between individual components determine the antimicrobial effect of a certain EO [17,18,21]. The needles and dried fruit of the common juniper (*Juniperus communis*; *J. communis*) are used in traditional medicine as a diuretic, uroantiseptic, carminative, digestive and antioxidant [22]. The analysis of the composition of *J. communis* EO most often reveals that the main active components are  $\alpha$ - and  $\beta$ -pinene,  $\beta$ -myrcene, sabinene, limonene, terpinen-4-ol and  $\beta$ -caryophyllene [23–28]. The aim of this research was to examine the effect of the interactions between *J. communis* EO and the antimicrobial drugs, amikacin, clarithromycin and rifampicin, on NTM.

## 2. Materials and Methods

### 2.1. Essential Oil

The natural *J. communis* EO that we used in the research was purchased from “IREX AROMA d.o.o.”, Zagreb, Croatia. The EO was dissolved in dimethylsulfoxide (DMSO; Kemika, Zagreb, Croatia) to obtain a stock suspension, which was stored in a sterile glass vial in the dark at 4 °C prior to use. The chemical composition of *J. communis* EO used in this study was determined in an earlier study [28].

### 2.2. Strains and Growth Media

As previously described for the experiments, ATCC strains were used: *Mycobacterium avium* ssp. *avium* (serotype 2) ATCC 25291 (*M. avium*) and *Mycobacterium intracellulare* ATCC 13950 (*M. intracellulare*) [28–31]. Middlebrook 7H9 broth (7H9S, Difco, Detroit, MI, USA) with 10% albumin-dextrose-catalase enrichment (ADC, Biolife Italiana, Milano, Italy) and 0.05% Tween 80 (Tw 80, Biolife Italiana, Milano, Italy) was used for subcultivation of bacterial strains at 37 °C for at least 14 days to obtain  $10^8$  CFU mL<sup>-1</sup>. The bacteria were

kept frozen at  $-80\text{ }^{\circ}\text{C}$  in 7H9S with 10% glycerol (Kemika, Zagreb, Croatia). An aliquot was thawed for each experiment and subcultured in 7H9S for at least 14 days and then on Middlebrook 7H10 agar (7H10S, Difco, Detroit, MI, USA) with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC, Biolife Italiana, Milano, Italy) and 0.05% Tw 80 at  $37\text{ }^{\circ}\text{C}$  for another 14 days. The number of bacteria in the initial inoculum was verified by diluting and plating the culture onto 7H10S and incubated at  $37\text{ }^{\circ}\text{C}$  for four to six weeks before colonies were counted.

### 2.3. Antimicrobial Drugs

The antimicrobial drugs, amikacin, clarithromycin, rifampicin and ethambutol (Sigma, Taufkirchen, Germany), were used in the study. The basic suspension (stock solution with a concentration of  $1000\text{ }\mu\text{g mL}^{-1}$ ) was prepared in the medium recommended by the manufacturer and stored in aliquots at  $-20\text{ }^{\circ}\text{C}$  until use. Clarithromycin and rifampicin were dissolved in DMSO, while amikacin was dissolved in sterile distilled water.

### 2.4. Sterile Tap Water

Water from the public water supply of the city of Rijeka was used in the research. The physicochemical properties of this water were determined in an authorized laboratory, and were as follows: colorless, odorless, low turbidity, neutral to slightly alkaline pH (from 7.5 to 8.0), low conductivity ( $0.211\text{--}0.250\text{ mS cm}^{-1}$  at  $20\text{ }^{\circ}\text{C}$ ) and moderate total hardness ( $135\text{ mg L}^{-1}$ ), so it was medium-hard water. A sample of tap water was collected in a sterile glass bottle and left at room temperature for two days to allow dechlorination to take place. It was then autoclaved for 15 min at  $121\text{ }^{\circ}\text{C}$  and allowed to cool to room temperature and stored at  $4\text{ }^{\circ}\text{C}$  until use.

### 2.5. Determination of the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

To determine the minimum inhibitory concentration (MIC) of *J. communis* EO and that of the antimicrobial drugs, amikacin, clarithromycin and rifampicin, against *M. avium* and *M. intracellulare*, the previously described microdilution method in broth was used [29]. Briefly, in sterile microtiter plates (Vacutest Kima s.r.l., Arzergrande, Padua, Italy) twofold serial dilutions of the tested EO were made in 7H9S starting from  $100$  to  $51,200\text{ }\mu\text{g mL}^{-1}$ . Rifampicin and amikacin were diluted to concentrations from  $0.125$  to  $64\text{ }\mu\text{g mL}^{-1}$ , and clarithromycin from  $0.015$  to  $8\text{ }\mu\text{g mL}^{-1}$ . A suspension of *M. avium* or *M. intracellulare* and  $0.015\%$  resazurin solution (Sigma-Aldrich, Saint Louis, MO, USA) was added to each dilution of the EO or antimicrobial drug to a final volume of  $200\text{ }\mu\text{L}$  and  $1 \times 10^5\text{ CFU mL}^{-1}$  of each mycobacterial species. Resazurin is a blue, non-fluorescent redox indicator used to control bacterial growth. It changes color to fluorescent pink after reduction to resorufin [32]. Plates were visually read after incubation for 96 h at  $37\text{ }^{\circ}\text{C}$  with mixing at 120 rpm (UNI-MAX 1010 shaker, Heidolph, Schwabach, Germany). The lowest concentration of the EO or antimicrobial drug at which there was no visual change in the color of resazurin was the MIC. The minimum bactericidal concentration (MBC) was determined by inoculating the test dilutions from each well without a color change on 7H10S and incubated for at least four weeks at  $37\text{ }^{\circ}\text{C}$ . The MBC was defined as the lowest concentration of EO that killed  $\geq 99\%$  of the CFU. The results are expressed in  $\mu\text{g mL}^{-1}$  [33].

### 2.6. Checkerboard Synergy Method

To determine the effect of the interaction between *J. communis* EO and amikacin, clarithromycin or rifampicin on NTM, the checkerboard synergy method was used, as described previously [30,32,33]. Briefly, stock solutions and serial twofold dilutions of the EO and each antimicrobial drug were prepared in 7H9S. *J. communis* EO was serially diluted along the ordinate, while the amikacin, clarithromycin or rifampicin was diluted along the abscissa. An inoculum of *M. avium* or *M. intracellulare* ( $10^6\text{ CFU mL}^{-1}$ ) was prepared in 7H9S and added along with  $0.015\%$  resazurin solution to wells with a combination of diluted *J. communis* EO and individual antimicrobial drugs. The final concentration of

DMSO as a solvent was approximately 10% and its effect was tested against the selected mycobacteria. The plates were incubated for four days under aerobic conditions at 37 °C and then, dilutions from each well were inoculated into 7H10S in duplicate and incubated for a further four weeks. As previously described by Bassole et al. and White et al., the fractional inhibitory concentration (FIC) and fractional inhibitory concentration index were determined (FIC<sub>i</sub>) [18,34]. According to the FIC<sub>i</sub> value, a combination of *J. communis* EO and individual antimicrobial drug was considered synergistic if the FIC<sub>i</sub> was  $\leq 0.5$ , additive when the FIC<sub>i</sub> was  $>0.5$  and  $\leq 1.0$ , indifferent when the FIC<sub>i</sub> was  $>1.0$  and  $\leq 4$  and antagonistic if the FIC<sub>i</sub> was  $>4$  [35].

### 2.7. Determination of Cellular Content Leakage

The leakage of the cellular contents of mycobacterial cells after the action of *J. communis* EO has been described previously [28]. In this study, the leakage of the cellular contents of *M. avium* and *M. intracellulare* was monitored after the action of selected antimicrobial drugs and after the action of the synergistic combinations of *J. communis* EO and each antimicrobial drug. In short, after two weeks of cultivation in enriched 7H9 broth, NTM were centrifuged at  $3500 \times g$  for 10 min, washed twice and resuspended in sterile tap water (STW) with 0.05% Tw80. The density (OD<sub>600</sub>) of the suspension was adjusted to 1.0 corresponding to  $1 \times 10^8$  CFU mL<sup>-1</sup>. The MIC and  $2 \times$  MIC of amikacin, clarithromycin or rifampicin were then added to the aliquots. Then, *J. communis* EO was tested in combination with the antimicrobial drugs at concentrations that showed a synergistic effect in the checkerboard synergy method. After 24 h of incubation at 37 °C, with mixing at 120 rpm, the suspensions were centrifuged at  $3500 \times g$  for 10 min. The leakage of nucleic acids at a wavelength of 260 nm and proteins at a wavelength of 280 nm was measured in the supernatant using a spectrophotometer (Eppendorf BioPhotometer 6131, Eppendorf, Hamburg, Germany) [36].

### 2.8. Transmission Electron Microscopy

In order to visualize possible structural and morphological cell changes, ultrastructural analysis was performed. We observed the morphological changes upon treatment with *J. communis* EO and the antimicrobials, amikacin, clarithromycin and rifampicin. The cells were conditioned for ultrastructural analysis, and electron microscopy analysis was applied as previously described [29]. A transmission electron microscope (TEM) (JEM-2100F, Jeol, Japan) was used.

### 2.9. Statistical Analysis

All assays were repeated three times. The experimental data are expressed as means with standard deviations and analyzed using the STATISTICA commercial software, 12.0 (StatSoft, Tulsa, OK, USA). Differences between groups of samples were analyzed using the Kruskal–Wallis ANOVA on ranks test, while the effects of the EO and antimicrobials on NTM were tested using the Mann–Whitney U test. Differences with  $p < 0.05$  were considered to be statistically significant.

## 3. Results

### 3.1. Susceptibility of Nontuberculous Mycobacteria to Juniperus communis Essential Oil and Selected Antimicrobial Drugs

The tested *J. communis* EO showed equal antimycobacterial effects on *M. avium* and *M. intracellulare* with the MIC and MBC value of 1600 µg mL<sup>-1</sup> (Table 1). The lowest MIC and MBC values for both NTM were observed for clarithromycin.

**Table 1.** Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of *J. communis* EO and selected antimicrobial drugs against nontuberculous mycobacteria.

	MIC/MBC ( $\mu\text{g mL}^{-1}$ )	
	<i>M. avium</i>	<i>M. intracellulare</i>
<i>J. communis</i> EO	1600/1600	1600/1600
Amikacin	2/4	1/4
Clarithromycin	0.5/2	0.062/0.5
Rifampicin	2/8	1/2

### 3.2. Synergistic Effect of *J. communis* EO and Selected Antimicrobial Drugs on Nontuberculous Mycobacteria

The results of the interaction between *J. communis* EO and amikacin, rifampicin and clarithromycin against *M. avium* carried out by using the checkerboard synergy method in enriched 7H9 are shown in Table 2.

**Table 2.** Checkerboard synergy method for *J. communis* EO and selected antimicrobials against *M. avium*.

<i>J. communis</i> EO *	MIC = 1600	400	400	400	800	800	800
Amikacin *	MIC = 2	0.25	0.5	1	0.25	0.5	1
	FIC <sub>JU</sub>	0.25	0.25	0.25	0.5	0.5	0.5
	FIC <sub>Amika</sub>	0.125	0.25	0.50	0.125	0.125	0.5
	FIC <sub>i</sub>	0.375	0.5	0.75	0.625	0.75	1
Interaction		Si	Si	Ad	Ad	Ad	Ad
<i>J. communis</i> EU *	MIC = 1600	200	200	200	400	400	400
Clarithromycin *	MIC = 0.5	0.062	0.125	0.25	0.031	0.062	0.125
	FIC <sub>JU</sub>	0.125	0.125	0.125	0.25	0.25	0.25
	FIC <sub>CLR</sub>	0.124	0.25	0.5	0.062	0.124	0.25
	FIC <sub>i</sub>	0.249	0.375	0.625	0.312	0.374	0.5
Interaction		Si	Si	Ad	Si	Si	Si
<i>J. communis</i> EU *	MIC = 1600	400	800	800	800	800	
Clarithromycin *	MIC = 0.5	0.25	0.031	0.062	0.125	0.25	
	FIC <sub>JU</sub>	0.25	0.5	0.5	0.5	0.5	
	FIC <sub>CLR</sub>	0.5	0.062	0.124	0.25	0.5	
	FIC <sub>i</sub>	0.75	0.562	0.624	0.75	1	
Interaction		Ad	Ad	Ad	Ad	Ad	
<i>J. communis</i> EO *	MIC = 1600	25	50	100	200	200	200
Rifampicin *	MIC = 2	1	1	1	0.25	0.5	1
	FIC <sub>JU</sub>	0.0156	0.03125	0.0625	0.125	0.125	0.125
	FIC <sub>RIF</sub>	0.5	0.5	0.5	0.125	0.25	0.5
	FIC <sub>i</sub>	0.516	0.531	0.562	0.250	0.375	0.625
Interaction		Ad	Ad	Ad	Si	Si	Ad
<i>J. communis</i> EO *	MIC = 1600	400	400	400	800	800	800
Rifampicin *	MIC = 2	0.25	0.5	1	0.25	0.5	1
	FIC <sub>JU</sub>	0.25	0.25	0.25	0.5	0.5	0.5
	FIC <sub>RIF</sub>	0.125	0.25	0.50	0.125	0.125	0.5
	FIC <sub>i</sub>	0.375	0.5	0.75	0.625	0.75	1
Interaction		Si	Si	Ad	Ad	Ad	Ad

\* Concentration of essential oils and antimicrobial drugs in  $\mu\text{g mL}^{-1}$ ; MIC—minimum inhibitory concentration; EO—essential oil; JU—*Juniperus communis*; Amika—amikacin; RIF—rifampicin; CLR—clarithromycin; FIC—fractional inhibitory concentration; FIC<sub>i</sub>—fractional inhibitory index; Ad—additive impact; In—indifferent action; Si—synergistic action.

Amikacin at a concentration of  $0.5 \mu\text{g mL}^{-1}$  (1/4 of the MIC) and  $0.25 \mu\text{g mL}^{-1}$  (1/8 of the MIC) in combination with *J. communis* EO at a concentration of  $400 \mu\text{g mL}^{-1}$  (1/4 of the MIC) showed a synergistic effect against *M. avium* with FIC<sub>i</sub> values of 0.375 and 0.5. *J. communis* EO at a concentration of  $400 \mu\text{g mL}^{-1}$  or  $800 \mu\text{g mL}^{-1}$  (1/4 or 1/2 of the MIC

value) with amikacin at a concentration of  $1 \mu\text{g mL}^{-1}$  (1/2 of the MIC),  $0.5 \mu\text{g mL}^{-1}$  (1/4 of the MIC) or  $0.25 \mu\text{g mL}^{-1}$  (1/8 of the MIC value) showed an additive effect.

Clarithromycin at concentrations from  $0.031 \mu\text{g mL}^{-1}$  to  $0.125 \mu\text{g mL}^{-1}$  (1/16 to  $1/4 \times \text{MIC}$  values) with  $1/4 \times \text{MIC}$  of *J. communis* EO and  $1/4$  and  $1/8 \times \text{MIC}$  of clarithromycin with  $1/8$  of the MIC value of *J. communis* EO achieved a synergistic effect against *M. avium*. *J. communis* EO and clarithromycin in the remaining combinations tested showed an additive effect.

Rifampicin and *J. communis* EO achieved a synergistic effect against *M. avium* in four combinations in which both were at a concentration of  $1/4 \times \text{MIC}$  or  $1/8 \times \text{MIC}$  value. Rifampicin at a concentration of  $1 \mu\text{g mL}^{-1}$  (1/2 of the MIC) in combination with *J. communis* EO at concentrations from  $400$  to  $25 \mu\text{g mL}^{-1}$  ( $1/4 \times \text{MIC}$  to  $1/64 \times \text{MIC}$ ) showed an additive effect against *M. avium*. The same effect was also observed in combinations of  $0.5 \mu\text{g mL}^{-1}$  or  $0.25 \mu\text{g mL}^{-1}$  ( $1/4$  and  $1/8$  of the MIC) of rifampicin with  $800 \mu\text{g mL}^{-1}$  ( $1/2 \times \text{MIC}$ ) of *J. communis* EO.

Table 3 shows the results of the interaction between *J. communis* EO and amikacin, clarithromycin and rifampicin against *M. intracellulare* carried out using the checkerboard synergy method.

**Table 3.** Checkerboard synergy method for *J. communis* EO and selected antimicrobials against *M. intracellulare*.

<i>J. communis</i> EO *	MIC = 1600	400	400	400	800	800	800		
Amikacin *	MIC = 1	0.25	0.5	1	25	0.5	1		
	FIC <sub>JU</sub>	0.25	0.25	0.25	0.5	0.5	0.5		
	FIC <sub>Amika</sub>	0.25	0.5	1	0.25	0.5	1		
	FIC <sub>i</sub>	0.5	0.75	1.25	0.75	1	1.5		
	Interaction	Si	Ad	In	Ad	Ad	In		
<i>J. communis</i> EO *	MIC = 1600	800	800	800	800	1600	1600	1600	1600
Clarithromycin *	MIC = 0.062	0.003875	0.00775	0.0155	0.031	0.003875	0.00775	0.0155	0.031
	FIC <sub>JU</sub>	0.5	0.5	0.5	0.5	1	1	1	1
	FIC <sub>CLR</sub>	0.063	0.125	0.25	0.5	0.063	0.125	0.25	0.5
	FIC <sub>i</sub>	0.563	0.625	0.75	1	1.063	1.125	1.25	1.5
	Interaction	Ad	Ad	Ad	Ad	In	In	In	In
<i>J. communis</i> EO *	MIC = 1600	400	400	400	400	400	400		
Rifampicin *	MIC = 1	0.015	0.031	0.062	0.125	0.25	0.5		
	FIC <sub>JU</sub>	0.25	0.25	0.25	0.25	0.25	0.25		
	FIC <sub>RIF</sub>	0.015	0.031	0.062	0.125	0.25	0.5		
	FIC <sub>i</sub>	0.265	0.281	0.312	0.375	0.5	0.75		
	Interaction	Si	Si	Si	Si	Si	Ad		
<i>J. communis</i> EU *	MIC = 1600	800	800	800	800	800	800		
Rifampicin *	MIC = 1	0.015	0.031	0.062	0.125	0.25	0.5		
	FIC <sub>JU</sub>	0.015	0.031	0.062	0.125	0.25	0.5		
	FIC <sub>RIF</sub>	0.5	0.5	0.5	0.5	0.5	0.5		
	FIC <sub>i</sub>	0.515	0.531	0.562	0.625	0.75	1		
	Interaction	Ad	Ad	Ad	Ad	Ad	Ad		

\* Concentration of essential oils and antimicrobial drugs in  $\mu\text{g mL}^{-1}$ ; MIC—minimum inhibitory concentration; EO—essential oil; JU—*Juniperus communis*; AMIKA—amikacin; RIF—rifampicin, CLR—clarithromycin; FIC—fractional inhibitory concentration; FIC<sub>i</sub>—fractional inhibitory index; Ad—additive impact; In—indifferent action; Si—synergistic action.

In testing the effects of *J. communis* EO and amikacin against *M. intracellulare*, one synergistic combination was recorded with a FIC<sub>i</sub> of 0.5. It contained *J. communis* EO at a concentration of  $400 \mu\text{g mL}^{-1}$  ( $1/4 \times \text{MIC}$ ) and amikacin at a concentration of  $25 \mu\text{g mL}^{-1}$  ( $1/4 \times \text{MIC}$ ). By increasing the concentrations of amikacin or *J. communis* EO to  $1/2 \times \text{MIC}$  ( $0.5 \mu\text{g mL}^{-1}$  and  $800 \mu\text{g mL}^{-1}$ , respectively), the effect against *M. intracellulare* was additive. The MIC of amikacin in combination with *J. communis* EO at a concentration of  $800 \mu\text{g mL}^{-1}$  and  $400 \mu\text{g mL}^{-1}$  ( $1/2$  and  $1/4$  of the MIC) acted indifferently towards *M. intracellulare*.

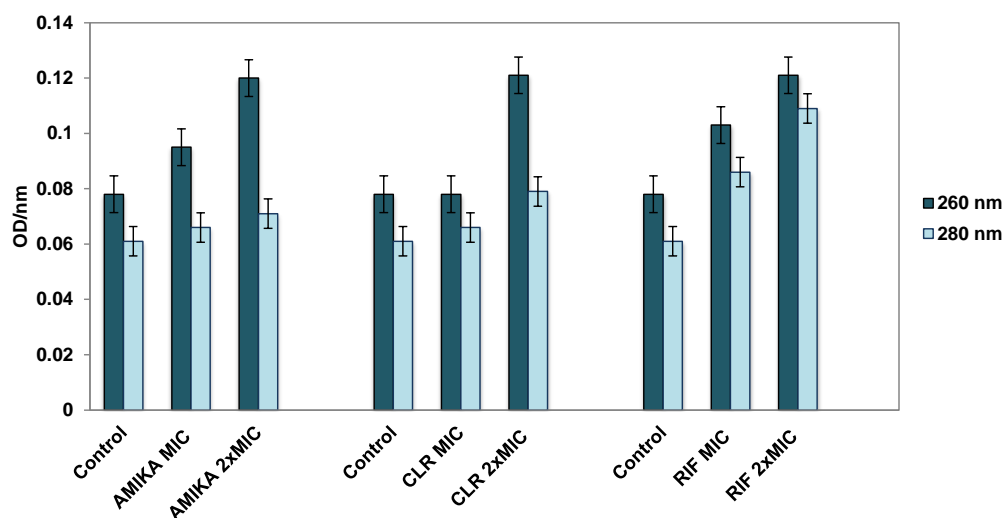
*J. communis* EO at a concentration of  $1/2 \times \text{MIC}$  in combination with clarithromycin at concentrations from  $0.031 \mu\text{g mL}^{-1}$  to  $0.003875 \mu\text{g mL}^{-1}$  (from  $1/2$  to  $1/16$  of the MIC) had an additive effect on *M. intracellulare*, while the same concentrations of clarithromycin in combination with *J. communis* EO at the MIC worked indifferently to *M. intracellulare*.

Rifampicin at concentrations from  $0.25 \text{ mg mL}^{-1}$  to  $0.015 \mu\text{g mL}^{-1}$  (from  $1/4 \times \text{MIC}$  to  $1/64 \times \text{MIC}$ ) in combination with *J. communis* EO at a concentration of  $400 \mu\text{g mL}^{-1}$  ( $1/4 \times \text{MIC}$ ) achieved a synergistic effect against *M. intracellulare* with a  $\text{FIC}_i$  from 0.265 to 0.5. By increasing the concentration of *J. communis* EO to  $800 \mu\text{g mL}^{-1}$  ( $1/2$  of the MIC) in combination with rifampicin at concentrations from  $1/4 \times \text{MIC}$  to  $1/64 \times \text{MIC}$ , their mutual effect became additive towards *M. intracellulare*. An additive effect was also observed with the combination of  $1/4 \times \text{MIC}$  of *J. communis* EO with  $1/2 \times \text{MIC}$  ( $0.5 \mu\text{g mL}^{-1}$ ) of rifampicin.

### 3.3. Leakage of Cellular Contents after Exposure to Selected Antimicrobial Drugs

After exposure to the MIC and  $2 \times \text{MIC}$  of the antimicrobial drugs, amikacin, clarithromycin and rifampicin, the leakage of the cellular contents of *M. avium* and *M. intracellulare* was determined. The measurement was made using a spectrophotometer at two wavelengths, 260 nm and 280 nm. The leakage of nucleic acids from the cell was determined by measurement at a wavelength of 260 nm, and the leakage of cellular proteins at a wavelength of 280 nm.

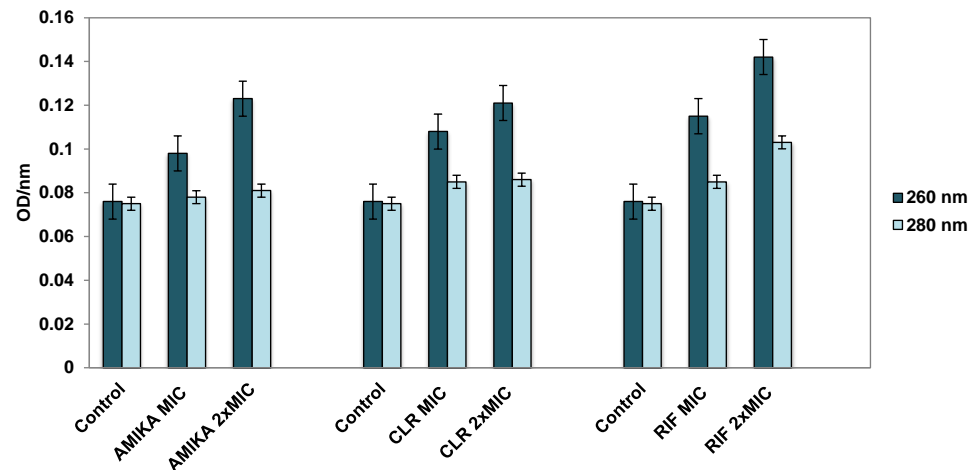
The leakage of nucleic acids was the same as for the application of  $2 \times \text{MIC}$  of the selected antimicrobial drugs against *M. avium* (Figure 1). When using the MIC, the highest leakage of nucleic acids was observed for rifampicin, followed by amikacin and clarithromycin at their MIC values. Protein leakage to the greatest extent was caused by rifampicin at a concentration of  $2 \times \text{MIC}$  and the MIC, while the remaining two antimicrobial drugs at both concentrations caused the same protein leakage.



**Figure 1.** Leakage of nucleic acids (260 nm) and proteins (280 nm) from *M. avium* after application of selected antimicrobial drugs at MIC and  $2 \times \text{MIC}$  (MIC—minimum inhibitory concentration; AMIKA—amikacin; CLR—clarithromycin; RIF—rifampicin). The experiment was repeated three times in duplicate and the mean value  $\pm$  SD is shown.

The leakage of nucleic acids from bacterial cells was the highest in *M. intracellulare* after application of  $2 \times \text{MIC}$  of rifampicin (Figure 2). The MIC of all three applied antimicrobial drugs caused the same leakage of nucleic acids from *M. intracellulare*. The protein leakage was the highest when using rifampicin at a concentration of  $2 \times \text{MIC}$ , while other antimicrobials at the MIC and  $2 \times \text{MIC}$ , as well as in *M. avium*, caused the same amount of protein leakage.

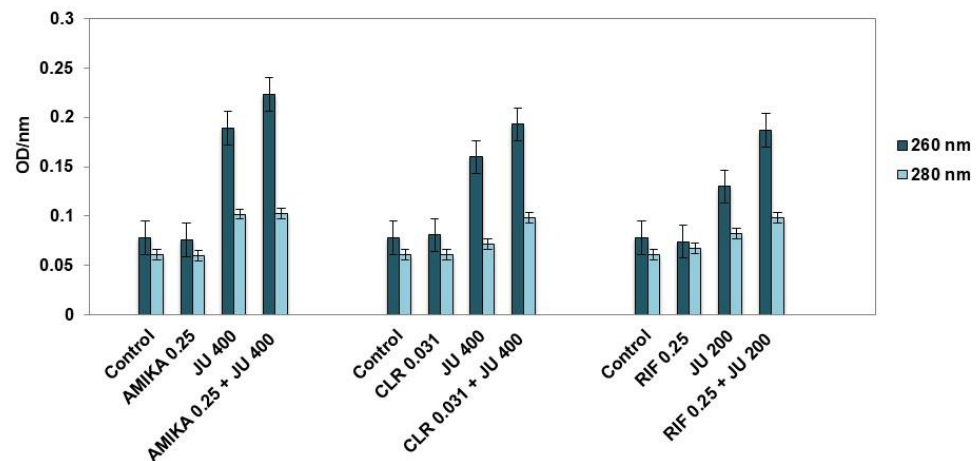




**Figure 2.** Leakage of nucleic acids (260 nm) and proteins (280 nm) from *M. intracellulare* after application of selected antimicrobial drugs at MIC and  $2 \times$  MIC (MIC—minimum inhibitory concentration; AMIKA—amikacin; CLR—clarithromycin; RIF—rifampicin). The experiment was repeated three times in duplicate and the mean value  $\pm$  SD is shown.

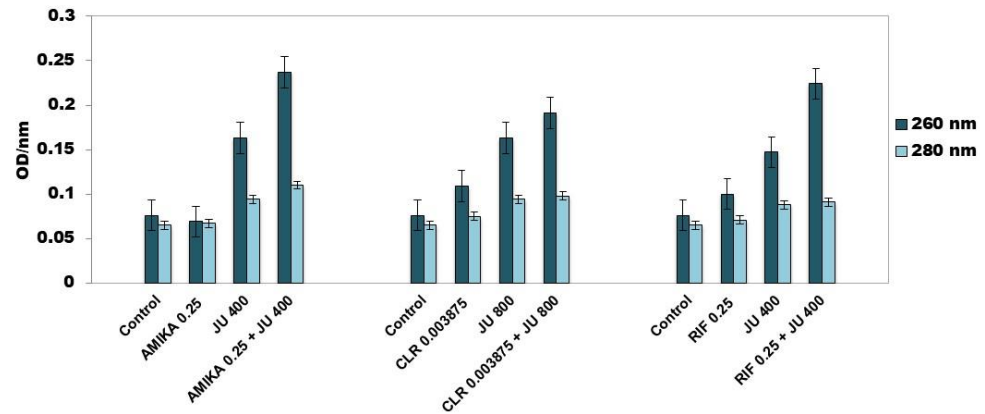
### 3.4. Leakage of Cellular Contents after Exposure to Synergistic Combinations of *J. communis* EO and Selected Antimicrobial Drugs

When comparing the individual influence by the synergistic combinations of *J. communis* EO and selected antimicrobial drugs on the leakage of the cellular contents of *M. avium*, it was observed that the leakage of nucleic acids measured at 260 nm was the highest for all synergistic combinations of antimicrobial drugs and EO. Slightly lower values than for synergistic combinations were shown by *J. communis* EO for each combination tested individually (Figure 3). The protein efflux from the cells of *M. avium* measured at 280 nm showed the same pattern as for the nucleic acid efflux, i.e., the highest efflux was recorded for synergistic combinations, followed by *J. communis* EO for each combination tested individually.



**Figure 3.** Leakage of nucleic acids (260 nm) and proteins (280 nm) from *M. avium* after application of synergistic subinhibitory concentrations of antimicrobial drugs and *J. communis* EO (in  $\mu\text{g mL}^{-1}$ ), individually and in combination (AMIKA—amikacin; CLR—clarithromycin; RIF—rifampicin; JU—*Juniperus communis* essential oil). The experiment was repeated three times in duplicate and the mean value  $\pm$  SD is shown.

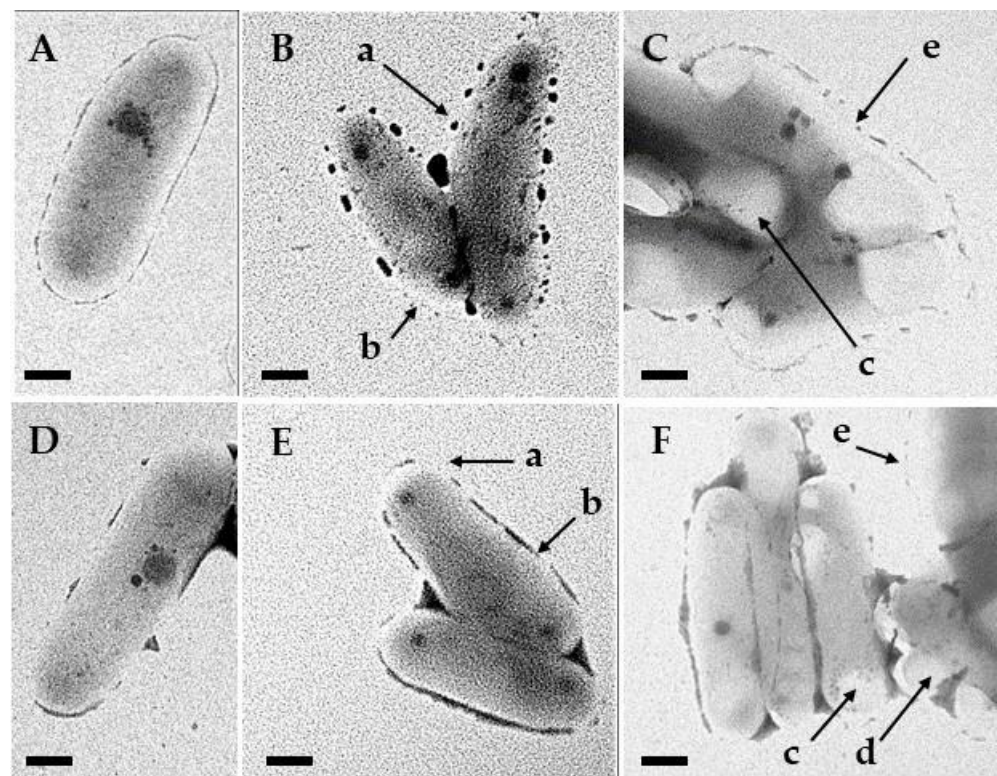
In *M. intracellulare*, synergistic combinations of antimicrobial drugs and *J. communis* EO at subinhibitory concentrations showed the greatest effect on the leakage of cellular contents, especially nucleic acids (Figure 4). All tested antimicrobial drugs in combination with *J. communis* EO caused a significantly higher leakage of nucleic acids than proteins.



**Figure 4.** Leakage of nucleic acids (260 nm) and proteins (280 nm) from *M. intracellulare* after application of synergistic subinhibitory concentrations of antimicrobial drugs and *J. communis* EO (in  $\mu\text{g mL}^{-1}$ ), individually and in combination (AMIKA—amikacin; CLR—clarithromycin; RIF—rifampicin; JU—*Juniperus communis* essential oil). The experiment was repeated three times in duplicate and the mean value  $\pm$  SD is shown.

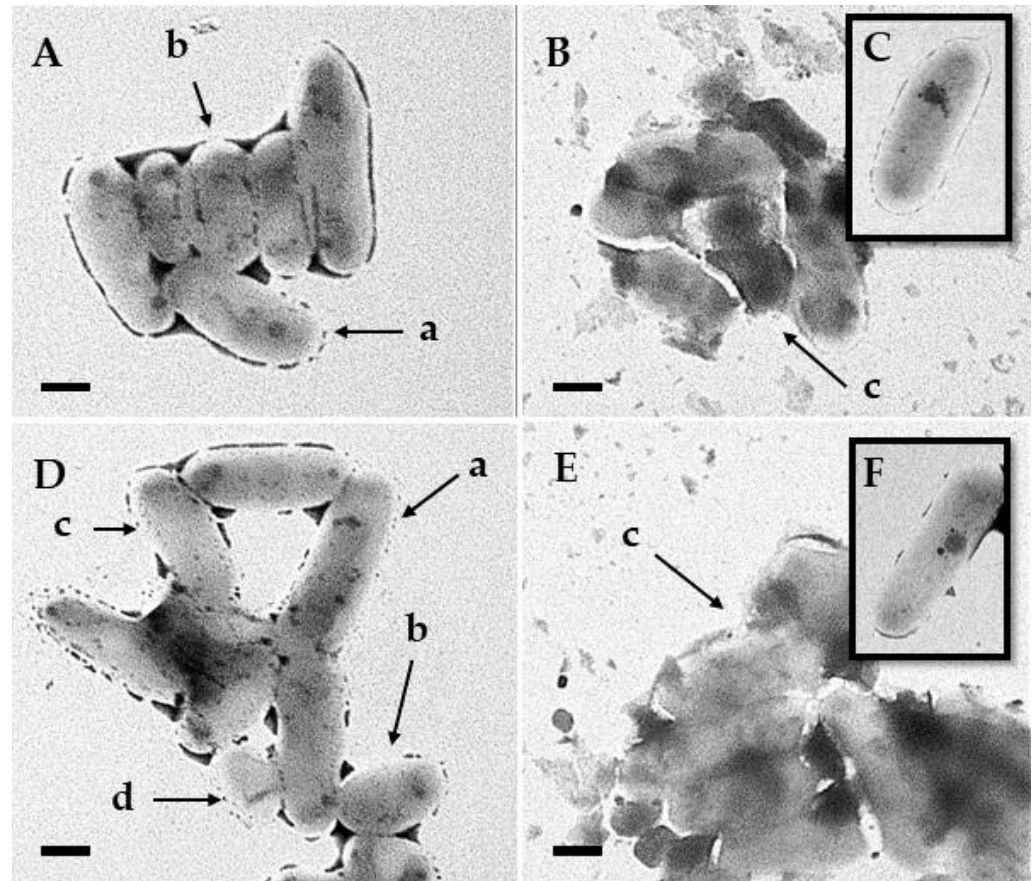
### 3.5. Transmission Electron Microscopy

Using TEM, the morphological changes in *M. avium* and *M. intracellulare* exposed to the action of antimicrobials were detected. The intensity of the observed ultrastructural morphological changes increased significantly with the use of synergistic combinations of *J. communis* EO and selected antimicrobial drugs (Figures 5–7).



**Figure 5.** Morphological analysis by transmission electron microscopy,  $\times 20\text{K}$ . Standard cell morphology of *M. avium* (A) cell and *M. intracellulare* (D). Cells of *M. avium* (B) and *M. intracellulare* (E) after 24 h of exposure to amikacin at  $1 \times \text{MIC}$ . Cells of *M. avium* (C) after 24 h of exposure to amikacin at a

concentration of  $0.25 \mu\text{g mL}^{-1}$  ( $1/8 \times \text{MIC}$ ) in combination with *J. communis* EO at a concentration of  $400 \mu\text{g mL}^{-1}$  ( $1/4 \times \text{MIC}$ ). Cells of *M. intracellulare* (F) after 24 h of exposure to amikacin at a concentration of  $0.25 \mu\text{g mL}^{-1}$  ( $1/4 \times \text{MIC}$ ) in combination with *J. communis* EO at a concentration of  $400 \mu\text{g mL}^{-1}$  ( $1/4 \times \text{MIC}$ ). MIC—minimum inhibitory concentration; EO—essential oil. Arrows indicate thinned cell wall (a), “swollen form” of mycobacteria (b), lipidic inclusions (c), ghost-like cell (d) and the detached cytoplasm from the cell wall (e).

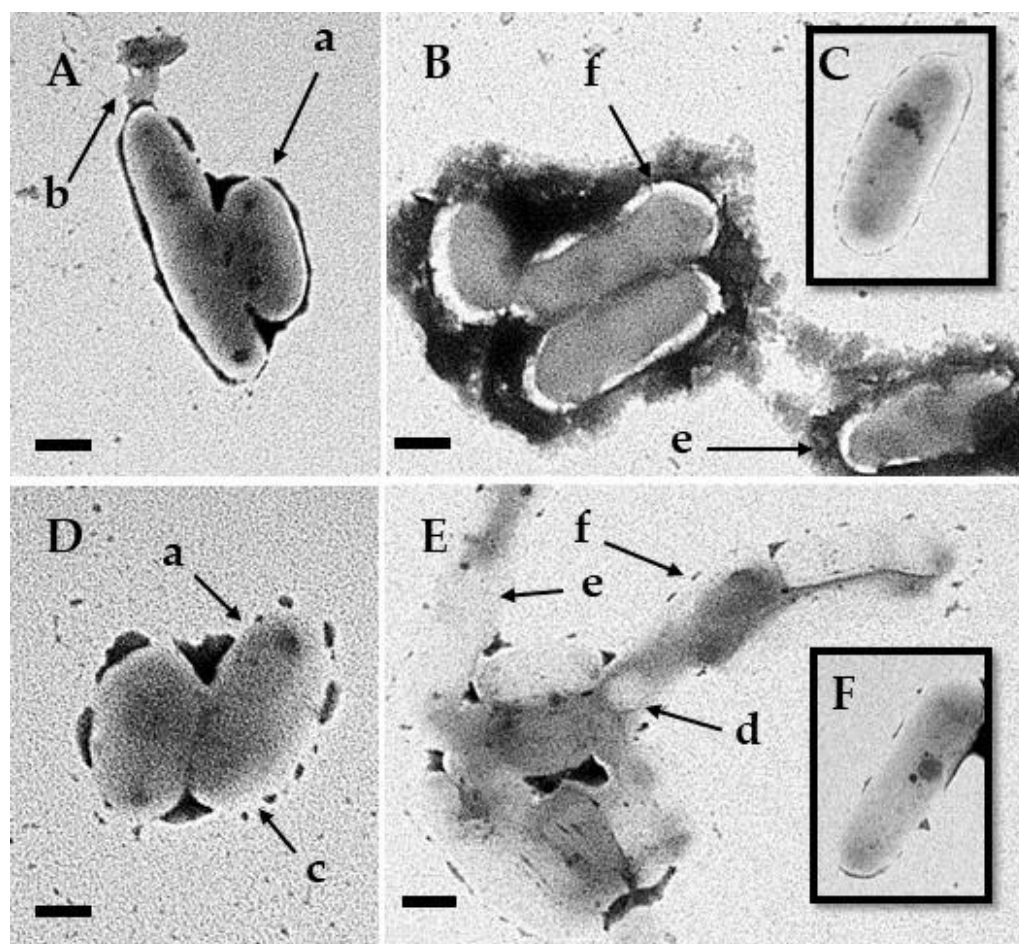


**Figure 6.** Morphological analysis using transmission electron microscopy,  $\times 20\text{K}$ . Standard cell morphology of *M. avium* (C) cell and *M. intracellulare* (F). Cells of *M. avium* (B) and *M. intracellulare* (D) after 24 h of exposure to clarithromycin at  $1 \times \text{MIC}$ . Cells of *M. avium* (A) after 24 h of exposure to clarithromycin at a concentration of  $0.125 \mu\text{g mL}^{-1}$  ( $1/4 \times \text{MIC}$ ) in combination with *J. communis* EO at a concentration of  $400 \mu\text{g mL}^{-1}$  ( $1/4 \times \text{MIC}$ ). Cells of *M. intracellulare* (E) after 24 h of exposure to clarithromycin at a concentration of  $0.003875 \mu\text{g mL}^{-1}$  ( $1/16 \times \text{MIC}$ ) in combination with *J. communis* EO at a concentration of  $800 \mu\text{g mL}^{-1}$  ( $1/2 \times \text{MIC}$ ). MIC—minimum inhibitory concentration; EO—essential oil. Arrows indicate thinned and/or disrupted cell wall (a), “swollen form” of mycobacteria (b), ghost-like cell (c) and the detached cytoplasm from the cell wall (d).

In regard to the NTM exposed to the MIC of selected antimicrobial drugs, several distinct morphological changes were noted including a thinned cell wall, changed shape from bacillary to oval (“swollen form”), more frequent grouping together, and inhomogeneous cytoplasm, suggestive of metabolic changes.

The cell walls of mycobacteria treated with synergistic combinations of *J. communis* EO and the selected antimicrobial drugs became extremely thin, and the cells were enlarged and rounded with lipid inclusions and intracytoplasmic vacuoles. Separation of the cytoplasm from the cell wall was observed together with the disruption of the integrity and permeability of the cell membrane, which consequently led to the leakage of cell contents and the lysis of mycobacterial cells. Debris from disintegrated cells could be occasionally

seen. The described changes were most pronounced when using synergistic combinations of *J. communis* EO and clarithromycin or rifampicin.



**Figure 7.** Morphological analysis using transmission electron microscopy,  $\times 20K$ . Standard cell morphology of *M. avium* (C) cell and *M. intracellulare* (F). Cells of *M. avium* (A) and *M. intracellulare* (D) after 24 h of exposure to rifampicin at  $1 \times \text{MIC}$ . Cells of *M. avium* (B) after 24 h of exposure to rifampicin at a concentration of  $0.25 \mu\text{g mL}^{-1}$  ( $1/8 \times \text{MIC}$ ) in combination with *J. communis* EO at a concentration of  $400 \mu\text{g mL}^{-1}$  ( $1/4 \times \text{MIC}$ ). Cells of *M. intracellulare* (E) after 24 h of exposure to rifampicin at a concentration of  $0.015 \mu\text{g mL}^{-1}$  ( $1/64 \times \text{MIC}$ ) in combination with *J. communis* EO at a concentration of  $400 \mu\text{g mL}^{-1}$  ( $1/4 \times \text{MIC}$ ). MIC—minimum inhibitory concentration; EO—essential oil. Arrows indicate focal thinned cell wall (a), leakage of cellular contents (b), “swollen form” of mycobacteria (c), lipidic inclusions (d), ghost-like cell (e) and the detached cytoplasm from the cell wall (f).

#### 4. Discussion

Due to the prevalence of NTM in various natural habitats shared with humans and animals, including natural waters and soil and drinking water distribution systems, the environment has been accepted as the source of human infections with NTM [37]. Improved healthcare and an extended life expectancy of patients at risk as well as an aging population largely burdened with various chronic diseases, a reduction in cross-immunity due to the reduced frequency of tuberculosis, abolition of mandatory BCG vaccination, which is believed to have a protective effect against NTM, and more sensitive microbiological and radiological diagnostic methods, are among the factors which significantly contribute to the more frequent isolation of NTM [38–41]. The increasing frequency of the isolation of NTM from different clinical samples and their clinical significance (the most common MAC or *M. avium* and *M. intracellulare*) as well as their prevalence urge the need to research new ways

of treating mycobacteriosis and preventing NTM. It is a known fact that plants contain many bioactive substances. Research on the antimicrobial effect of certain plant species and natural substances, and their effective concentrations which do not harm the human body, represents an important contribution to the improvement of therapeutic and preventive protocols. Constituent components of the EOs of wild plants of the Mediterranean climate, such as those of *J. communis*, have strong medicinal and antimicrobial properties [18,42–44]. Earlier studies have shown that monoterpenes can lead to morphological changes in the cell wall of mycobacteria that cause its increased permeability and leakage of cell contents [28,29,45].

A method often used to test the antimicrobial activity of plant extracts is the agar or disk diffusion method. The limitation of this method is the hydrophobic nature of most EOs, which prevents the uniform diffusion of these substances through the agar [46]. The disk diffusion method is only suitable as a preliminary screening test before the quantitative determination of the MIC using dilution methods [47]. The dilution method in agar or broth is commonly used. The results obtained by each of these methods may differ, as they may be affected by many factors, such as differences in microbial growth, exposure of microorganisms to the EO, solubility of the EO or its components, and the use and amount of emulsifiers. The above can partly explain the differences in MIC values in published research [46]. The microdilution method in broth, which we also used in our research, stands out as the most accurate way of assessing the antimicrobial effect of plant extracts [47]. After testing individual EOs and their components, we started testing their combinations and combinations with antimicrobial drugs with the aim of finding the lowest concentrations that show a mutual effect on NTM inhibition [28,29,48]. Different EOs, their components or antimicrobial drugs can interact with each other and reduce or increase their antimicrobial activity. The interaction between the tested compounds can produce an indifferent, additive, antagonistic or synergistic effect [18]. The FIC<sub>i</sub> value is used to define the nature of the interaction. However, the values used differ between publications and make it difficult to compare studies. We decided on the values defined by the majority of available research in which synergistic interaction is described by a FIC<sub>i</sub> ≤ 0.5, while antagonism is indicated by a FIC<sub>i</sub> ≥ 4 [18,35]. It should be emphasized that we did not find a single antagonistic effect in all the tested combinations of the EO and selected antimicrobial drugs in relation to NTM. We investigated the mutual effect of *J. communis* EO and the antimicrobial drugs, amikacin, clarithromycin and rifampicin, against *M. avium* and *M. intracellulare* and determined a number of synergistic combinations in subinhibitory concentrations. We did not find a synergistic effect only in the combination of *J. communis* EO and clarithromycin against *M. intracellulare*. Only standard strains were used in the study, but it can be assumed that different clinical isolates of the MAC could have different MICs for the EO and antibiotics used, which represents a certain limitation of this study. Sieniawska et al. investigated the synergistic effect of α-pinene, α-bisabolol, (S)-limonene, (R)-limonene, sabinene, β-elemene and myrcene and the antimicrobial drugs, rifampicin, ethambutol and isoniazid, against virulent and avirulent strains of *M. tuberculosis*. They recorded a synergistic effect of all components in combination with rifampicin and ethambutol against avirulent strains. However, the low MIC values for α-pinene and bisabolol were not proportionally reflected in the morphological changes in the mycobacterial cell observed using TEM [45,49]. In general, compounds with similar chemical structures show additive rather than synergistic effects. The antagonistic effect is attributed to the interaction between non-oxygenated and oxygenated monoterpenes, combinations of bactericidal and bacteriostatic components, and components that have the same target site of action [18].

The MBC/MIC ratio for the antimicrobial drugs which we tested against *M. avium* and *M. intracellulare* was 2 or 4, except for clarithromycin against *M. intracellulare*. Such a MBC/MIC ratio ≤ 4 is an indicator of the strong bactericidal effect of these antimicrobial drugs against *M. avium* and *M. intracellulare* [50]. In our research, in all synergistic combinations with antimicrobial drugs against *M. avium* and *M. intracellulare*, *J. communis* EO was at a concentration of  $1/4 \times \text{MIC}$  or  $1/8 \times \text{MIC}$ . Of the antimicrobial drugs,

rifampicin proved to be the most effective, which in all concentrations from  $1/4 \times \text{MIC}$  to  $1/64 \times \text{MIC}$  in combination with *J. communis* EO at a concentration of  $1/4 \times \text{MIC}$  acted synergistically against *M. intracellulare*. There is considerable variability in the sensitivity of mycobacteria to rifamycins and amikacin, which probably results in large part from the impermeability of the mycobacterial cell wall and depends on the content of cell wall wax, especially phthiocerol dimycocerosate, which is crucial for host cell membrane invasion, increased phagocytosis, intracellular survival by reducing acidification and its increased production in strains of mycobacteria resistant to rifampicin, which was confirmed [51]. We hypothesize that the synergistic effect of rifampicin or amikacin and *J. communis* EO is based on damage to the structure of the cell wall of mycobacteria by the action of *J. communis* EO, increased permeability of the cell wall and greater influx of rifampicin or amikacin after *J. communis* EO exposure. We also visualized the mentioned damage with the help of TEM. The same synergistic effect was attributed to ethambutol.

In relation to *M. avium*, clarithromycin showed as many as five synergistic combinations with *J. communis* EO. The first studies that analyzed the improvement of the effectiveness of antimicrobial drugs against *M. tuberculosis* and *M. avium* by adding EO components were conducted by Rastogi et al. [52,53]. They reported that trans-cinnamic acid with amikacin had a synergistic effect against both species of mycobacteria as measured by the radiometric growth index. They asserted that in *M. avium*, due to the chemical similarity between trans-cinnamic acid and phenylalanine, which is a component of its outer wall, their replacement may occur during the formation of the cell wall. Such a mechanism of action has been ruled out for *M. tuberculosis* [53,54]. Sherry et al. recently presented the case of a patient with pulmonary tuberculosis in whom inhalation therapy using *Eucalyptus globulus* EO, three times a day for three weeks, was administered in addition to antituberculosis drugs, leading to the clinical improvement of symptoms and negative sputum culture [55]. The presumed mechanisms of this synergistic action are the different target sites of the two combined components, each acting on a different target site, and then improved diffusion and distribution of the EO and its components in the bacterial cell, inhibition of a common biochemical pathway, inhibition of protective enzymes, and action on a specific resistance mechanism [18,54].

Antimicrobial drugs at the MIC and  $2 \times \text{MIC}$  caused a significant leakage of nucleic acids and proteins from the mycobacterial cells, which was determined via measurement at wavelengths of 260 nm and 280 nm. In *M. avium*, clarithromycin, rifampicin and amikacin at  $2 \times \text{MIC}$  induced identical levels of nucleic acid efflux. Rifampicin and amikacin caused an increase in the leakage of macromolecules proportional to the increase in their concentration, while in the case of clarithromycin, an increase in the leakage of nucleic acids was noticeable when applying  $2 \times \text{MIC}$ . For macrolides that are lipophilic and enter mycobacteria via passive diffusion through the cell wall, an important factor for their effectiveness is the selective permeability of the cell wall. It was found that clarithromycin enters mycobacteria faster than the less lipophilic erythromycin, which is associated with the higher level of antimycobacterial activity of clarithromycin. This is where the effect of the mycobacterial cell wall of limiting the activity of macrolides comes to the fore, and it can be reduced by the synergistic effect of ethambutol, which inhibits the synthesis of the mycobacterial cell wall [56]. Since, according to our observations, *J. communis* EO leads to damage to the cell wall of mycobacteria, it can facilitate the entry of clarithromycin into the mycobacterial cell. Clarithromycin did not show a synergistic effect with *J. communis* EO against *M. intracellulare* since its MBC/MIC ratio was 8, which according to Sawicki et al. is a sign of low bactericidal activity [50]. It is accepted that macrolides are more active in vitro in slightly alkaline conditions (pH 7.3 to 7.4) than in an acidic medium of pH 6.8, so the pH of the medium is also a factor that can affect the MIC and MBC [56]. With *M. avium*, we did not record such an effect.

The cell envelope is crucial for the physiological processes of mycobacteria. The main feature of mycobacteria is the abundance of lipids, which make up to 40% of the dry weight of tuberculosis bacilli. The cell wall of mycobacteria contains up to 60% lipid, compared to

about 20% in the cell wall of Gram-negative microorganisms. Many biological properties of mycobacteria have been attributed to these lipids. This includes the very high resistance of most mycobacterial species to most broad-spectrum antibiotics, except, for example, streptomycin and rifampicin [57]. Like our earlier research, this also confirmed that *J. communis* EO leads to a significant disruption of the structure of the cell wall of mycobacteria, which makes it more sensitive to environmental influences and more permeable to various substances, including antimicrobial drugs.

The observed patterns of ultrastructural changes with the consequent leakage of cellular macromolecules induced by cell wall damage is in agreement with the observations of Bakker-Woudenberg et al. [58]. According to these authors, the bactericidal activity of clarithromycin and ethambutol is positively correlated with concentration and time, while the bactericidal effect of rifampicin in the MAC depends on time and concentration, which is also evident from our results. In our study, compared to rifampicin at the same concentrations, amikacin caused noticeably less leakage of nucleic acids. Under the action of amikacin, protein leakage in *M. avium* did not change significantly compared to the control sample group. This is probably the result of amikacin's mechanism of action, i.e., the inhibition of protein synthesis, due to which the cellular content of proteins is reduced, and thus their amount in the expired content.

In *M. intracellulare*, as in *M. avium*, we observed the same principle of leakage of proteins and nucleic acids caused by the action of antimicrobial drugs. The exception was clarithromycin, which did not show a dependence on the increase in concentration. In our study examining the mutual effect of antimicrobial drugs in combination with *J. communis* EO, only clarithromycin did not show a synergistic effect against *M. intracellulare*.

When testing the synergistic effect on the leakage of cellular contents using combinations of *J. communis* EO and antimicrobial drugs, we noted an increased leakage of nucleic acids in all synergistic combinations in relation to *M. avium* and *M. intracellulare*. However, by comparing the individual application of only antimicrobial drugs with the application of their combinations with *J. communis* EO, we saw that under the influence of subinhibitory concentrations of *J. communis* EO, applied individually or in combination, there was a significantly higher leakage of nucleic acids, while there was no change in the leakage of proteins.

Changes in the ultrastructural morphology of *M. avium* and *M. intracellulare* under the influence of *J. communis* EO and the selected antimicrobial drugs were investigated using TEM. NTM that were exposed to the action of antimicrobials and the action of synergistic concentrations of *J. communis* EO and antimicrobial drugs, showed significant changes in cell shape and structure. Changes in morphology were more pronounced in synergistic combinations of *J. communis* EO with antimicrobial drugs. Initial changes include cell enlargement and gradual, multifocal thinning of the cell wall. Progressing changes reflect predominately macro structural shape remodeling, and cells become more rounded and oval with inhomogeneous cytoplasm, followed by an increase in lipid inclusions. From the thinned cell wall begins the cell content leakage, and the cytoplasm separates from the cell wall with the final stage characterized by lysis of the mycobacterial cell which occurs terminally. Reisner et al. exposed the MAC to the antimicrobial drugs, clarithromycin, ciprofloxacin, ethambutol or rifabutin, and then observed the resulting changes in the mycobacterial cell using TEM. They observed nucleoid condensation, a disorganized cytoplasm, separation of the cell wall from the cytoplasm, intact cytoplasmic lipid inclusions and cell lysis. Changes were observed after 24 h of MAC exposure to antimicrobial drugs, and the intensity of the changes increased with drug concentration above the MIC value [59]. The changes that we observed via TEM in NTM cells after exposure to antimicrobial drugs, individually and in combination with *J. communis* EO, are almost identical to the changes described by Reisner et al.

## 5. Conclusions

In our research, we found a number of synergistic combinations of *J. communis* EO and antimicrobial drugs that can have potential application in new ways of preventing adherence and biofilm formation of NTM in infections associated with biofilm formation, such as skin infections and secondary wound infections. The synergistic effect of the combined application of EOs or their combination with antimicrobial drugs could improve the therapeutic outcome of mycobacteriosis, reduce the minimum effective dose of the antimicrobial drug, i.e., reduce the possible side effects and toxic effects of the antimicrobial drug, and prevent the emergence of resistance to antimicrobial drugs.

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