Romanian Biotechnological Letters *Vol. 21, No. 4, 2016*

ORIGINAL PAPER

Rosmarinus Officinalis Essential Oil and Eucalyptol Act as Efflux Pumps Inhibitors and Increase Ciprofloxacin Efficiency against *Pseudomonas Aeruginosa* and *Acinetobacter Baumannii* MDR Strains

Received for publication, October 13, 2015
Accepted, February 29, 2016

CRINA SAVIUC[1, 2], IRINA GHEORGHE[1], SORINA COBAN[1], VERONICA DRUMEA[2], MARIANA-CARMEN CHIFIRIUC*,[1], OTILIA BANU[3], EUGENIA BEZIRTZOGLOU[4], VERONICA LAZĂR[1]

[1]*University of Bucharest, Faculty of Biology, Department of Botany-Microbiology, Research Institute of the University of Bucharest-ICUB, Splaiul Independenței 91-95 Sector 5, cod 76201, București, Romania, Phone: 004-021-318.15.66, 004-021.310.85.03;*

[2]*S.C. Biotehnos S.A. – Gorunului Str. 3-5, 075100, Otopeni, Ilfov, Romania, Phone: 40-31-7102402, fax: 40-31-7102400;*

[3]*Emergency Institute of Cardiovascular Diseases "Prof. Dr. C.C. Iliescu", Fundeni R., No. 258, Sector 2, Bucharest, Romania;*

[4]*Department of Agricultural Development; Democritus University of Thrace, Greece;*

***Address correspondence to:** University of Bucharest, Department of Biochemistry and Molecular Biology, 91-95 Spl. Independentei, 050095, Bucharest 5th District, Romania.Tel.: +4021 318 15 75; Email: carmen_balotescu@yahoo.com*

Abstract

In this study, our aim was to assess the antimicrobial activity of Rosmarinus officinalis essential oil (EO) and eucalyptol (EU) against multiresistant strains of Acinetobacter baumannii and Pseudomonas aeruginosa. The resistance mechanisms of the used bacterial strains were characterized by phenotypic and genotypic methods. Essential oil was extracted and performed by hydrodistilation and characterized by GC-MS analysis. The intrinsic antimicrobial activity, as well as the synergic effect with ciprofloxacin was assessed by quantitative methods, while the potential mechanisms of the antimicrobial action were investigated by flow citometry.The selected P. aeruginosa strains and A. baumannii strains exhibited MDR phenotypes. The screening of beta-lactams resistance genes proved the presence of bla_{TEM} genes in 60% of the A. baumannii strains and in 37.5% of the P. aeruginosa strains, while bla_{OXA-23} was evidenced in 80% of the A. baumannii strains.The GC-MS analysis of the R. officinalis essential oil allowed the identification of 28 volatile compounds, the eucalyptol content being 5.49%. The MIC values for Rosmarinus officinalis EO ranged from 13.07 to 52.26 mg/mL, while for EU from 1.86 ÷ 41.86 mg/mL. Both EU and EO exhibited a synergic effect with ciprofloxacin. The mechanisms of action revealed by the flow cytometry analysis were the inhibition of efflux pumps activity and the cellular wall permeabilization. These results demonstrate that rosemary EO and its majoritary compound EU can be used as efflux pump inhibitors, to restore the efficiency of current antibiotics against MDR strains or even suppress the emergence of such resistance phenotypes.

Keywords: *antibiotic resistance, essential oil, antimicrobial combinations, flow cytometry*

1. Introduction

Resistance to antibiotics is used by microbial strains as a survival, competitive and adaptive mechanism to the selective pressure imposed by biotic or abiotic factors. The widespread and inappropriate use of antibiotics in medicine for the prophylaxis and treatment

of infectious diseases, agriculture and animal breeding led to the permanent increase of the number of bacterial strains resistant to more and more classes of antibiotics. In addition, the horizontal gene transfer contributed to the selection of multiple-drug (MDR), extended-drug (XDR) and pan-drug (PDR) resistance phenotypes, with major impact for the global public health, due to the severe outcome of nosocomial infections, with significant increase in morbidity, mortality and hospital costs. The ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp.) are representing a significant and increasing percent of the resistant clinical isolates involved in nosocomial infections [1, 2].

Plants and plant extracts have been used for centuries in traditional medicine for their different therapeutic effects by 80% of the world population. Also, many medications contain compounds directly isolated from plants, or modified versions of natural products [3, 4]. Nowadays, plant extracts and essential oils (EOs), or their fractions/ isolated compounds are under investigation, representing viable solutions for developing new anti-infective strategies. In this respect, our aim was to assess the intrinsic antimicrobial activity, mechanisms of action and synergic effect with antibiotics of *Rosmarinus officinalis* essential oil and one of its majoritary compounds, eucalyptol, against multiresistant strains of *Acinetobacter baumannii* and *Pseudomonas aeruginosa*.

2. Materials and Methods

2.1. Bacterial strains

We have selected for this study eight *P. aeruginosa* and six *A. baumannii* clinical isolates collected from The Institute of Cardiovascular Diseases Prof. C.C. Iliescu, Bucharest in September 2014 - March 2015, as well as one *P. aeruginosa* ATCC 27853 reference strain (table 1). The identification of bacterial strains was performed using the automated VITEK 2 Compact system.

2.2. Phenotypic and genotypic characterization of antibiotic resistance profiles of the tested strain

Evaluation of the antibiotic susceptibility of *P. aeruginosa* and *Acinetobacter baumannii* strains was performed by disk diffusion method recommended by CLSI [5].The β-lactamase producer phenotypes (metallo-β-lactamase-MBL, extended spectrum β-lactamase, inducible cephalosporinase) were highlighted by double disk diffusion. Several multiplex or simplex PCR assays were performed to detect the MBL genes - bla_{VIM}, bla_{IMP} and the extended spectrum β-lactamase ESBL genes – bla_{TEM}, $bla_{CTX\text{-}M}$in *P. aeruginosa* and the MBL genes - bla_{VIM}, bla_{IMP}, the extended spectrum β-lactamase genes – bla_{TEM}, $bla_{CTX\text{-}M}$ and the oxacillinase genes $bla_{OXA\text{-}23}$ and $bla_{OXA\text{-}24}$ in *A. baumannii* strains.

2.3. Essential oil (EO) extraction

Four samples (200g each) of dry plant material were hydrodistillated in a Neo-Clevenger-type apparatus for 4 hours according to European Pharmacopoeia 7th ed. The extraction yield was considered as the medium yield of the four experiments, expressed as the EO volume per plant material weight (v/w). The EOs portions were mixed, dried with anhydrous magnesium sulfate and stored in brown vessels at 4^{o}C prior to the physico-chemical analysis and antimicrobial properties assessment.

2.4. Physico-chemical characterization of the volatile oils extracted fromRosmarinus officinalis herba

For the GC-MS analysis a 1:50 dilution of the EO sample in hexane was made. Chemical components identification was conducted on a GC-MS system, 7890A coupled with 5975C MSD (Agilent Techonologies, Santa Clara, CA). A DB-5 MS capillary column (60 m x 0.25 mm x 0.25 μm) was used. The acquisition was conducted with different oven temperature ramps, ranging between 90º and 280ºC. Split mode injection was selected and the split ratio was 1:300. The injection volume was set up at 1 μl and the flow rate at 1.3 mL/min. MS acquisition parameters were scan mode; relative EMV mode; scanning range (50 ÷ 400) a.m.u.; MS source 230ºC; MS quad 150ºC. The compounds' identification was made by using the NIST mass spectral library.

2.5. Qualitative screening for the antimicrobial activity of EO and eucalyptol (EU)

An adapted diffusion method was used for the qualitative screening of the antimicrobial activity. The *Rosmarinus officinalis* EO and EU diluted in dimethyl sulfoxide (DMSO) 1:1 (10 μl) were used for spotting the plates previously seeded as recommended by CLSI. The plates were incubated for 24 h at 37ºC and the results were quantified according to the growth inhibition diameters.

2.6. Quantitative assay of the antimicrobial activity of EO and EU

The quantitative assay of the antimicrobial activity was performed by serial binary micro dilution (ranging from 1.86 to 52.26mg/mL) method in Müller-Hinton liquid medium distributed in 96-well plates. Dimethyl sulfoxide (DMSO) was used to facilitate mixing the essential oil and EU with the broth in a 1:1 ratio.

2.7. Flow citometry assessment of the bacterial efflux pump activity for the natural compounds and antimicrobial combinations of natural compounds and antibiotics

Flow citometry was carried out in order to evaluate the possible mechanisms of the antimicrobial activity of the natural compounds and of antimicrobial mixtures. For the cell staining two intercalant fluorochromes with affinity for DNA were used: propidium iodide (PI 10μg/mL) and ethidium bromide (EB 5μg/mL). PI was used for the cellular viability's determination, as living cells are impermeable to this dye, due to their intact membranes. EB was used for the detection and quantification of bacterial efflux activity.

The study was conducted in two different experiments: (1) for the EO/EU and (2) for the combinations EO/EU: antibiotic (i.e. ciprofloxacin – 2μg/mL). Two concentrations of natural compounds, corresponding to the MIC and MIC/8 values were tested. Staining procedures were applied to the harvested cells grown in the presence of the tested compounds. The cells were centrifuged at 13000 rpm for 3 minutes, washed 2 times, resuspended in phosphate buffered saline (PBS), stained with 10μL PI or EB and incubated for 10 minutes at 4ºC in dark. Heat-treated cells for 30 minutes at 100ºC were used as positive controls and viable cells were used as negative controls. The samples were analyzed with a FACS Calibur instrument equipped with a 488 nm Argon laser, using 670 nm long pass filter for the samples stained with PI and (585±42) nm band pass filter for the samples stained with EB. The measured parameters were forward scatter FSC, side scatter SSC and fluorescence and the back gating procedure was used, excluding all non-fluorescent particles. Typical photomultiplier tube voltages were SSC 550 V (log scale), 670 nm long pass filter (log scale) and (585±42) nm band pass filter 550V (log scale). 10000 events were collected in all runs.

3. Results and Conclusions

3.1. Phenotypic and genotypic characterization of antibiotic resistance profiles of the tested strains

The *A. baumannii* and *P. aeruginosa* strains exhibited resistance to the main classes of antibiotics used to treat infections caused by nonfermentative Gram-negative bacteria (i.e., β-lactam antibiotics, aminoglycosides and quinolones) [6, 7-11]. Thus, in *P. aeruginosa* there were identified the following β-lactam resistance phenotypes: constitutive cephalosporinases (2 strains), carbapenemases or another resistance mechanism to carbepenems (6 strains), 1 strain resistant to all the tested β-lactams, 1 strain resistant to the β-lactamase inhibitors, and two strains isolated from tracheal secretions presented a MDR phenotype. All *A. baumannii* strains exhibited a MDR phenotype, being resistant to the majority of the tested antibiotics, excepting for tobramycin and polymyxin B.

The results of PCR assays for beta-lactams resistance genes proved the presence of bla_{TEM} genes in 60% *A. baumannii* strains and in 37.5% *P. aeruginosa* strains and the presence of bla_{OXA-23} in 80% *A. baumannii* strains (Table 1).

As the carbapenems resistance in Gram-negative non-fermentative bacteria is often associated with mutations in porins, which induce decreased outer membrane permeability, the presence of *oprD* gene was tested in *P. aeruginosa* strains. The OprD porin allows the passive uptake across the outer membrane of basic amino acids and dipeptides with a basic residue and forms pores that are permeable for carbapenems. The external loops 2 and 3 were identified as entrances for basic amino acids and binding sites for imipenem. Any substitution or deletion within loop 2 or 3 induces changes in conformation and can cause imipenem resistance, as well as the loss of the OprD porin [12].The PCR assay results proved the absence of this gene in all analyzed *P. aeruginosa* strains.

Table 1 –The resistance profiles of the selected strains

No.	Bacterial strain	Clinical specimen	Resistance phenotype	Resistance genes
1	*Pseudomonas aeruginosa* 49 (Ps49)	stool	C+	$\boldsymbol{bla_{TEM}}$
2	*Pseudomonas aeruginosa* 147 (Ps147)	tracheal secretion	MDR	$\boldsymbol{bla_{TEM}}$
3	*Pseudomonas aeruginosa* 156 (Ps156)	tracheal secretion	MDR	
4	*Pseudomonas aeruginosa* 157 (Ps157)	tracheal secretion		$\boldsymbol{bla_{TEM}}$
5	*Pseudomonas aeruginosa* 160 (Ps160)	urinary tract infection	C+	
6	*Pseudomonas aeruginosa* 164(Ps164)	tracheal secretion	β-lactam resistance	
7	*Pseudomonas aeruginosa* 165 (Ps165)	tracheal secretion		
8	*Pseudomonas aeruginosa* 234 (Ps234)	urinary tract infection	C+	
9	*Acinetobacter baumannii* 64 (Ac64)	catheter	MDR	$\boldsymbol{bla_{OXA-23}}$
10	*Acinetobacter baumannii* 110 (Ac110)	tracheal secretion	MDR	$\boldsymbol{bla_{TEM}, bla_{OXA-23}}$
11	*Acinetobacter baumannii* 230 (Ac230)	urinary tract infection	MDR	
12	*Acinetobacter baumannii* 286 (Ac286)	nasal exudate	MDR	$\boldsymbol{bla_{TEM}, bla_{OXA-23}}$
13	*Acinetobacter baumannii* 288 (Ac288)	nasal exudate	MDR	$\boldsymbol{bla_{TEM}, bla_{OXA-23}}$
14	*Acinetobacter baumannii* 388 (Ac388)	pharyngeal exudate	MDR	
15	***Pseudomonas aeruginosa* ATCC 27853**	**reference strain**		

C+ - carbapenemase production

3.2. Extraction andphysico-chemical characterization of the EO

The extraction yield considered as the medium yield of the four experiments was 0.987% (V/w). Our results in extraction yield are in accordance with the literature data, taking into account the extraction time and vegetal material grounding state [13].The GC-MS analysis of the EO extracted from *R. officinalis* herba allowed the identification of around 28 volatile

compounds accounting for about 94.934% of the total compounds (table 2, fig. 1). The major compound was α-pinene, with 19.01% of the relative aria, followed by the 1,8-cineole (EU) (5.49%), L-camphor (5.71%), verbenone (5.42%), camfene (4.67%) and carvacrol (4.71%). These results are in accordance with other literature data [14].

3.3. Qualitative screening for the antimicrobial activity of EO and EU

The results of the qualitative testing of the antimicrobial activity were analyzed following the algorithm suggested by Singh *et al.*, 2014, i.e: less than 8mm of growth inhibition zone (no antimicrobial activity), 9-14mm (susceptible), 15-19mm (very susceptible) and > 20mm (extremely susceptible) [15]. 75% of the *P. aeruginosa* strains proved to be susceptible to EO and 50% to EU, exhibiting growth inhibition zones between 10 and 14 mm. All *A. baumannii* strains were susceptible to EO and EU, with growth inhibition zones between 12 and 19 mm.

3.4. Quantitative assay of the antimicrobial activity of EO and EU

Both rosemary EO and EU exhibited good antimicrobial activity against the Gram-negative non-fermentative bacterial strains *P. aeruginosa* and *A. baumannii* as seen in figure 2.The MIC values for *Rosmarinus officinalis* E Oranged from 13.07 to 52.26 mg/mL, while for EU between 1.86 and 41.86 mg/mL. Synergistic relationships between EO/ EU: CIP have been proved, except for the *A. baumannii* 286 (MIC EU – 1.86 mg/ mL and MIC EU: CIP 8.86 mg/ mL, suggesting a possible antagonism of action between the two components), by calculating the fractional inhibitory concentration index (data not shown).

Table 2 – Compositionof *Rosmarinus officinalis* EO

No	Compound	Retention time (min)	% of relative aria
1	α-pinene	7.501	19.094
2	camfene	7.779	4.672
3	3-octanone	7.997	1.433
4	mircene	8.081	1.620
5	β-pinene	8.165	1.935
6	d-limonene	8.832	1.935
7	1,8-cineol	8.934	5.486
8	α-terpinene	9.234	0.516
9	terpinolene	9.683	0.690
10	d-linalool	9.748	1.274
11	crisantenone	10.291	0.592
12	cis-verbenol	10.664	0.619
13	L-camfor	10.796	5.713
14	borneol	11.145	3.238
15	4-terpineol	11.221	1.644
16	α-terpineol	11.398	0.956
17	3-ciclopenten-1-etanol	11.466	0.896
18	verbenone	11.685	5.418
19	5-caranol	12.356	1.099
20	bornyl acetate	12.852	4.962
21	carvacrol	12.914	4.707
22	butyl carbinol acetate	13.500	0.575
23	geranyl isovalerate	14.401	1.160
24	valeric acid 2 ethyl-hexyl esther	14.650	5.553
25	caryophyllene	15.477	1.706
26	2-methyl decane	16.127	2.338
27	hexadecane	17.533	1.737
28	γ-sitosterol	28.131	13.366
	Total		94.934

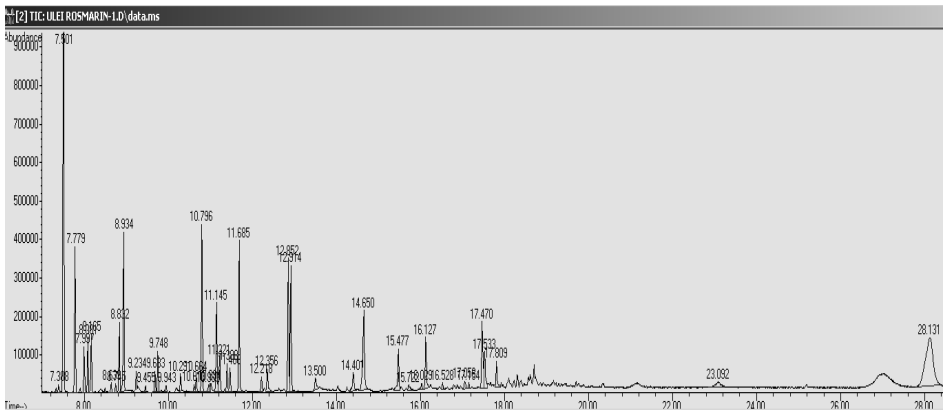

Figure 1 – Chromatogram of the *Rosmarinus officinalis* EO – separation details and retention times of the identified compounds

3.5. Investigation of the underlying mechanisms of the antimicrobial activity of EO and EU by flow cytometry

A promising approach in fighting the multidrug resistance issue by improving the clinical performance of the antibiotics is represented by efflux pump inhibitors (EPIs).The targeting of the efflux pump activity with an inhibitor could restore the microbial susceptibility to a certain antibiotic. Ideally such an inhibitor should facilitate the intracellular accumulation of an antibiotic and therefore reduce the minimum inhibitory concentration [16].It is already known that the antimicrobial/EPI synergy is a general mechanism of plant defense against Gram-negative bacteria and fungal infections [17]. It has been also shown that several *Berberis* species (*B. repens*, *B. aquifolia* and *B. fremontii*) synthesize an inhibitor of the *Staphylococcus aureus* NorA MEP, identified as 59-methoxyhydnocarpin (59-MHC).

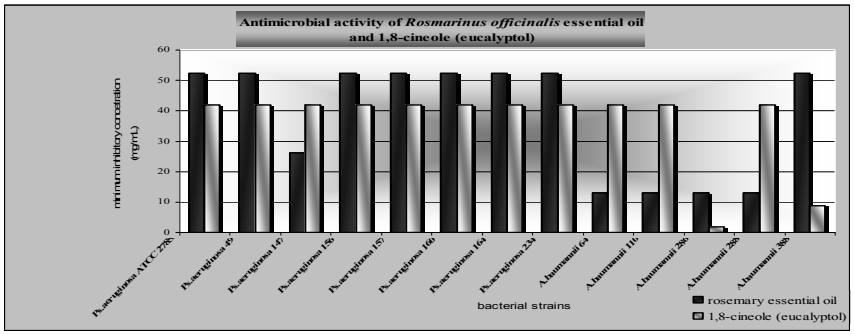

Figure 2 – Antimicrobial activity of *Rosmarinus officinalis* essential oil and 1,8-cineole (EU) against *P. aeruginosa* and *A. baumannii* strains

Considering these aspects, we have investigated the potential of rosemary EO and EU to modulate the efflux pumps activity in the selected MDR strains.

EB is used for detection and quantification of bacterial efflux pumps activity, and the intensity of the EB treated samples could be associated with the ratio between the net influx of EB due to the permeabilization of the bacterial cell wall and its extracellular elimination through the efflux pump activity [18]. Measuring the fluorescence intensity (FI) in samples stained with PI and EB and combining the results, the EPI activity as well as the cell coatings permeabilisation could be assessed as potential mechanisms of the proposed antimicrobial

combinations. Typical histograms of the fluorescence intensity (MFI) measurements median are presented in figure 3. In our experiments most of the tested variants induced a drastic decrease in the viable *P. aeruginosa* bacterial population, when tested at the MIC concentration (fig. 3b), but not of the *A. baumannii* strains, in whose case there were registered higher MFI values for the cells labeled with PI (fig. 3 c, d).

In our experiments, EU at subinhibitory concentrations corresponding to MIC/8 induced cell wall permeabilization demonstrated by an increased of PI MFI only in case of *P. aeruginosa* (fig. 4 a, c). Also, EO and EU in subinhibitory concentration exhibited both a cellular wall permeabilization and an EPI effect both on *P. aeruginosa* and *A. baumannii* strains (fig. 4 b, d). The increased PI uptake indicating a membrane damage was previously reported after treatment with EOs, such as clove, oregano, thyme, tea-tree oils, or cinnamon oil [19].

The combinations between EO/ EU and ciprofloxacin exhibited an EPI effect on the tested strains (fig. 5 a-d), with increased MFI values for the EB labeled samples comparing with the viable cells control. The EPI effect was more intensive for EU as compared to EO.

4. Conclusions

The results of the present study demonstrate that *Rosmarinus officinalis* essential oil and one of its majoritary compounds, 1,8-cineole (eucalyptol) exhibited very good antimicrobial activity against *P. aeruginosa* and *A. baumannii* MDR strains and a synergic activity with ciprofloxacin, a 2nd generation quinolone. The flow cytometry analysis demonstrated that the tested natural compounds act by inducing cellular wall permeabilization and efflux pumps inhibitory activity, suggesting their potential use for the restoration of antibiotics efficiency on resistant strains harboring an efflux mechanism. This study could be extended at molecular level in order to assess the vegetal extracts' influence on the expression of some antibiotic resistance genes.

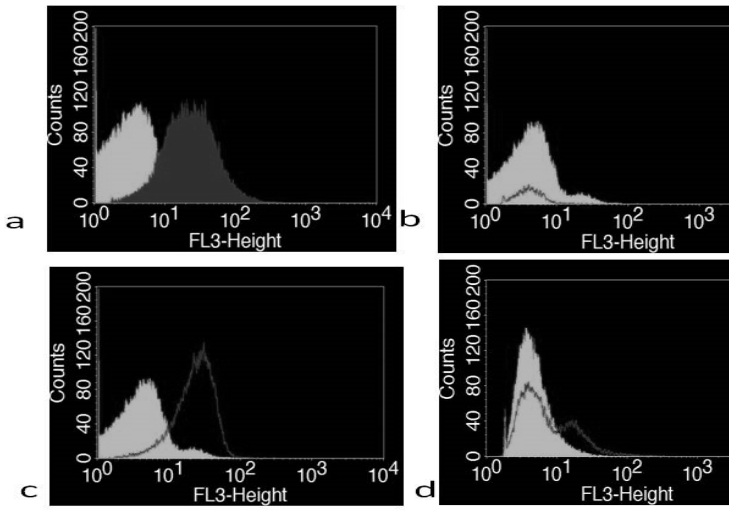

Figure 3 – a - overlay of the histogram for the median of the fluorescence intensity, viable cells control (green) and heat treated cells control (red); b, c, d - typical aspects of the histograms obtained at MIC concentration values for *P. aeruginosa* (b) and *A. baumannii* (c, d) strains

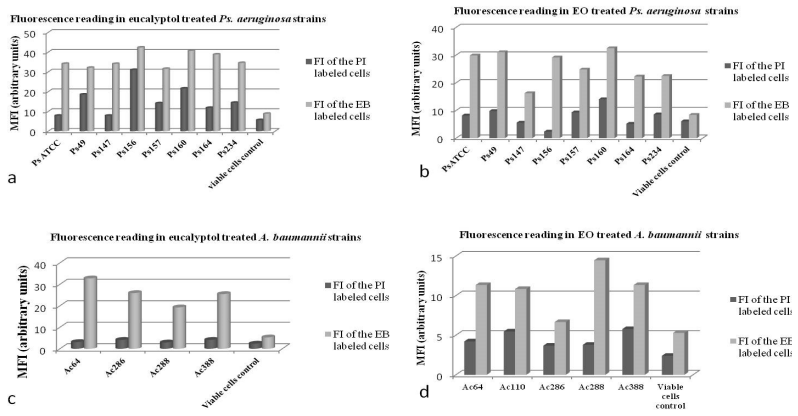

Figure 4 – a, b – median of fluorescence intensity measurements obtained for the *P. aeruginosa* samples treated with eucalyptol and rosmary EO respectively; c, d - median of fluorescence intensity measurements obtained for the *A. baumannii* samples treated with eucalyptol and rosmary EO respectively in subinhibitory concentrations, corresponding to MIC/8

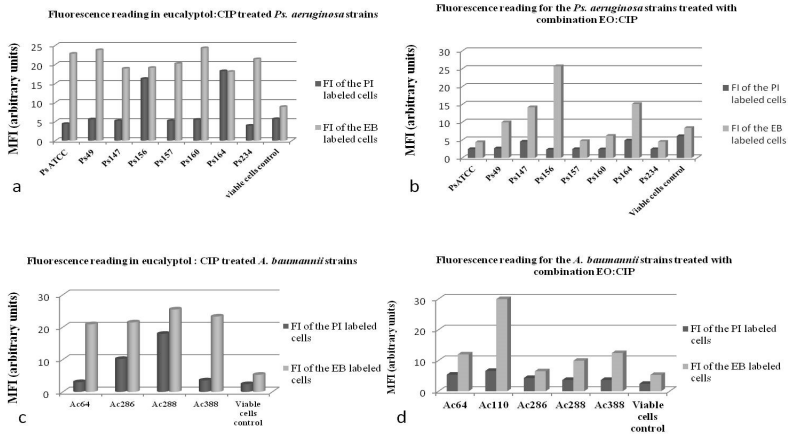

Figure 5 – a, b – median of fluorescence intensity measurements obtained for the *P. aeruginosa* samples treated with eucalyptol:ciprofloxacin combinations and rosmary EO:ciprofloxacin combinations, respectively; c, d - median of fluorescence intensity measurements obtained for the *A. baumannii* samples treated with eucalyptol:ciprofloxacin combinations and rosmary:EO ciprofloxacin combinations, respectively – all the results are normalised to the MFI of the ciprofloxacin treated cells

4. Acknowledgements

This work was supported by the strategic grant POSDRU/159/1.5/S/133391, project "Doctoral and Post-doctoral proGrams of excellence for highly qualified human resources training for research in the field of Life sciences, Environment and Earth Science" co-financed by the European Social Found within the Sectorial Operational ProGram Human Resources Development 2007-2013 and by the of the research project **PN-II-RU-TE- 2014-4-2037**.

References

1. L.B. RICE, Progress and Challenges in Implementing the Research on ESKAPE Pathogens, *Infect. Control Hosp. Epidemiol.*, 31 (S1), pp.S7-S10 (2010).
2. J.N. PENDLETON, S.P. GORMAN, B.F. GILMORE, Clinical Relevance of the ESKAPE Pathogens, *Expert Rev. AntiInfect. Ther.*, 11(3), pp.297-308 (2013).
3. O. STEFANOVIĆ, I.RADOJEVIĆ, S. VASIĆ, L. ČOMIĆ, Antibacterial Activity of Naturally Occurring Compounds from Selected Plants -Antimicrobial Agents, V. BOBBARALA, eds., In Tech, 2012, pp.1-19.
4. A. SILVÉRIO, M. LOPES, 2012, Antimicrobial Activity of Condiments - Antimicrobial Agents, V. BOBBARALA, eds., In Tech, 2012, pp.109-127.Clinical and Laboratory Standards Institute, ed.2015.
5. M.C. CHIFIRIUC, G. MIHĂESCU, V. LAZĂR, Medical microbiology and virology, The Bucharest University Press, 2011, pp.166-183.
6. G. MIHĂESCU, C.M. CHIFIRIUC, L.M. DIȚU, Antibiotics and antimicrobial chemotherapeutic agents, Romanian Academy Press, 2007.

7. D.L. PATERSON, R.A. BONOMO, Extended-Spectrum β-Lactamases: a Clinical Update, *Clinical Microbiology Reviews*, pp.657-686 (2005).
8. M.M. EHLERS, J.M. HUGHES, M.M. KOCK, Prevalence of Carbapenemases in *Acinetobacter baumannii* -Antibiotic Resistant Bacteria - A Continuous Challenge in the New Millennium, M. PANA, ed., In Tech, 2012, pp.213-234.
9. L. POIREL, R.A. BONNIN, P. NORDMANN, Genetic Basis of Antibiotic Resistance in Pathogenic *Acinetobacter* Species, *IUBMB Life*, 63(12), pp.1061–1067 (2011).
10. A. HOWARD, M. O'DONOGHUE, A. FEENEY, R.D. SLEATOR, *Acinetobacter baumannii* - Anemerging opportunistic pathogen, *Landes Bioscience*, Virulence 3:3, pp.243–250 (2012).
11. H. LI, Y.-L. LUO, B.J. WILLIAMS, T.S. BLACKWELL, C.-M. XIE, Structure and function of OprDprotein in *Pseudomonas aeruginosa*: From antibiotic resistance to novel therapies, *Int. J. Med. Microbiol.*, 302(2), pp.1-7 (2012).
12. M.L. OUZZARA, W. LOUAERA, A. ZEMANEA, A.-H. MENIAI, Comparison of the Performances of Hydrodistillation and Supercritical CO_2 Extraction Processes for Essential Oil Extraction from Rosemary (*Rosmarinus officinalis* L.),*Chemical Engineering Transactions*, 43: 1129-1134 (2015).
13. A.M. OJEDA-SANA, C.M. VAN BAREN, M.A.ELECHOSA, M.A.JUÁREZ, S. MORENO, New insights into antibacterial and antioxidant activities of rosemary essential oils and their main components, *Food Control*,31(1), pp.189 -195 (2013).
14. S. SINGH, S.S. DAS, G. SINGH, C. SCHUFF, M.P. DE LAMPASONA, C.A. CATALÁN, Composition, *in vitro* antioxidant and antimicrobial activities of essential oil and oleoresins obtained from Black Cumin seeds (*Nigella sativa* L.), *Biomed Res Int.*, 2014(ID 918209), pp.1-10 (2014).
15. V. APARNA, K. DINESHKUMAR, N. MOHANALAKSHMI, D. VELMURUGAN, W. HOPPER, Identification of Natural Compound Inhibitors for Multidrug Efflux Pumps of *Escherichia coli* and *Pseudomonas aeruginosa* Using In Silico High-Throughput Virtual Screening and In Vitro Validation, *Plos One*, 9 (7), pp.1-13 (2014).
16. Y C. FIAMEGOS, P.L. KASTRITIS, V. EXARCHOU, H. HAN, A.M.J.J. BONVIN, J. VERVOORT, K. LEWIS, M.R.HAMBLIN, G.P. TEGOS, Antimicrobial and Efflux Pump Inhibitory Activity of Caffeoylquinic Acids from *Artemisia absinthium* against Gram-Positive Pathogenic Bacteria, *Plos One*,6(4): e18127, pp.1-10 (2011).
17. M. MARTINS, M.P. McCUSKER, M. VIVEIROS, I. COUTO, S. FANNING, J.-M. PAGÈS, L. AMARAL, A Simple Method for Assessment of MDR Bacteria for Over-Expressed Efflux Pumps, *Open Microbiol. J.*, 7, pp.72-82 (2013).
18. K.A. HAMMER, C.F. CARSON, Antibacterial and Antifungal Activities of Essential Oils - Lipids and Essential Oils as Antimicrobial Agents, H. THORMAR, ed., John Wiley & Sons, Ltd., 2011, pp. 256-293