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Original Article

In vitro activity of hybrid lavender essential oils against multidrug resistant strains of *Pseudomonas aeruginosa*

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Abstract

Introduction: Lavender is an evergreen shrub native to Northern Africa and other mountainous Mediterranean regions. It grows throughout Southern Europe, the United States, and Australia. Lavender essential oil has been used since ancient times and is known for its anti-inflammatory, antidepressant, antiseptic, antifungal and antimicrobial properties.

Methodology: in this study, the antimicrobial activity of two Lavender essential oils (*Lavanda sumian* and *Lavanda grosso*) against 16 multidrug-resistant *P. aeruginosa* strains from clinical ocular samples taken from migrant patients has been investigated. The *in vitro* cytotoxic activity on human Wong-Kilbourne derivative (WKD) conjunctiva cells from healthy patients and nitric oxide synthase (NOS) activity on murine macrophage (J774.1A) were also evaluated.

Results: *L. sumian* showed lower antimicrobial activity when compared to *L. grosso*. Both lavender oils tested had no cytotoxic effect at very low concentrations, mostly *L. grosso*. The essential oils extracted from *L. sumian* and *L. grosso* significantly reduced NOS in a cell model.

Conclusion: Increase in drug resistance and lack of new antibiotics may encourage the development of natural antimicrobial treatments.

Key words: antimicrobials; biopharmaceuticals; cytotoxicity; infection; Pseudomonads.

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Introduction

Lavender belongs to the Lamiaceae family, which includes several evergreen species able to grow in different climate conditions. It is characterized by intensely fragrant flowers used in the production of perfumes, as well as for the extraction of essential oils (EOs) known for their beneficial effects on human health. For centuries, traditional medicine and aromatherapy have taken advantage of EOs properties and aromatic molecules are still appreciated nowadays as therapeutic agents. These natural compounds exert significant biological and pharmacological effects, mainly due to their lipophilic nature.

The therapeutic potential of lavender EOs has not fully been estimated yet, leaving many features regarding their pharmacological aspects to be discovered, even though numerous medicinal herbs have been used since ancient times. The reason for the current knowledge gap is due to the complex composition of EOs, which contain a striking number of different compounds.

Typology and quantity of components determine and characterize EOs properties. Variety and richness of compounds contribute to peculiar features of each EO [1], which can contain one predominant constituent or consist of many components in equilibrated concentrations. Even traces of some compounds can significantly influence EOs biological activity [2]. As for secondary metabolites, the chemical composition of EOs is strongly modulated by the environment [1], with particular importance given to climate and seasons [1,3,4]. Phytotherapy has been set aside with the development of chemistry and the first synthetic drugs, then rediscovered during recent decades as either an integrative method associated with conventional medicine or as an alternative therapy.

Following our previous investigations performed on *Pseudomonas aeruginosa* ocular isolates from

migrant patients affected by keratitis and endophthalmitis [5-7], the present study has focused on EOs antimicrobial activity.

Thus the minimum bactericidal concentration (MBC) of EOs derived from *Lavanda sumian* and *Lavanda grosso* has been evaluated against multidrug-resistant ocular isolates of *P. aeruginosa*. Moreover, cytotoxicity on human conjunctival Wong-Kilbourne derivative *(*WKD*)* cells and nitric oxide synthase (NOS) activity on murine macrophage (J774.1A) cells were also assessed, in view of a possible ocular application.

Methodology

Sixteen bacterial isolates of *P. aeruginosa* were used in the present study. All the bacterial strains were isolated by ocular swab from migrant patients of different nationality whith corneal infections resistant to standard antibiotic therapies from Ophthalmology Unit, Department of Surgery, Microsurgery and Medical Sciences, Sassari, Italy.

L. sumian and *L. grosso* essential oils used in the present study were produced, standardized and distributed by Exentiae (Exentiae s.r.l., Catania, Italy).

In order to identify and perform the antimicrobial susceptibility test on the 16 *P. aeruginosa* strains assessed, the MicroScan (Beckman Coulter. Inc., Brea, USA) MIC/Combo Panel was used. The Breakpoint Combo Panels use concentrations equivalent to the Breakpoint of Clinical and Laboratory Standards Institute (CLSI) following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretive criteria [8-9].

Minimum Bactericidal Concentration (MBC) values of the EOs on 16 multidrug-resistant *P. aeruginosa* strains was analyzed.

Samples were diluted in Luria Broth (LB) added with 0.5% Tween 80 at concentrations ranging from 16% to 0.0005% (v/v). The bacterial inoculum was performed at the concentration of 10^6 CFU/mL. An inoculum of 100 µL of microbial culture was added to 100 µL of each concentration of the different samples in 96-well plates and incubated at 37°C for 24 hours. Cultures that showed no visible turbidity were subcultured on the surface of a Plate Count Agar for colony counting. MBC was considered as the lowest concentration inhibiting 99% of bacterial growth. Each experiment was performed in duplicate and repeated three times.

Three replicates of each EOs were analyzed using a Hewlett-Packard Model 5890A Gas Chromatograph equipped with a flame-ionization detector and fitted with a 60 m × 0.25 mm, thickness 0.25 µm *AT-5* fused SiO2 capillary column (Alltech, Nicholasville, USA). Injection port and detector temperature were 280° C. The column temperature was programmed from 50 to 135° C at 5°C/minute (1 minute), 5 °C/minute to 225 °C (5 minutes), 5°C/minute to 260°C, and held for 10 minutes. The samples (0.1 µL each), generally analyzed without dilution (using 2,6-dimethylphenol as internal standard), were injected using a split/splitless automatic injector *HP 7673* and using He as carrier gas. The quantification of each compound was expressed as absolute weight percentage using internal standard and response factors. The detector response factors (*RF*s) were determined for key components relative to 2,6-dimethylphenol and assigned to other components on the basis of functional group and/or structural similarity.

MS analyses were carried out with a Gas Chromatograph Agilent Technologies model 7820A connected with a MS detector 5977E MSD (Agilent, Santa Clara, USA), by using the same conditions and column described above. The column was connected to the ion source of the mass spectrometer. Mass units were monitored from 10 to 900 at 70 eV. The identification of compounds was based on the comparison of their retention times with those of authentic samples and/or by comparison of their mass spectra with those of published data (Nist Library Mass spectra) or on the interpretation of the EI-fragmentation of the molecules.

In a second experiment, EOs cytotoxicity was analyzed on WKD cells. WKD cells were maintained in Roswell Park Memorial Institute-1640 (RPMI) medium (Sigma-Aldrich, Saint Louis, USA) supplemented with 10% foetal bovine serum and 100 U/mL penicillin/streptomycin and incubated at 37° C in 5% CO_2 air atmosphere. $1{,}5\times10^5$/mL cells were further seeded in 96-well plates and incubated overnight at 37°C, 5% CO_2. EOs dilutions (from 16% to 0.0005% V/V) were prepared in culture medium with the addition of Tween 80 (0.5%) and assessed for 10 minutes. The cytotoxicity assay (*In vitro* toxicology assay kit MTT based, Sigma-Aldrich, Milan, Italy) was performed according to the manufacturer's instructions. Wells were washed twice with PBS and 100 µL of culture medium without serum plus 1/10 MTT solution (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)/PBS was added. After 4 hours of incubation, M-8910 MTT solubilisation solution - 10% Triton X-100 plus 0,1N HCl in anhydrous isopropanol was added. The quantity of formazan (presumably directly proportional to the number of viable cells) was

Table 1. Antimicrobial activity of lavender EOs (% v/v) against 16 multidrug resistant *P.aeruginosa.*

Strains number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
lavanda SUMIAN Exentiae s.r.l.	16%	16%	16%	8%	16%	8%	16%	16%	16%	16%	16%	8%	8%	16%	8%	8%
lavanda GROSSO Exentiae s.r.l.	16%	16%	16%	16%	16%	8%	8%	8%	8%	8%	8%	8%	8%	8%	8%	8%

measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer. The percentage of viability was calculated according to the following formula: (OD [570 nm] sample assessed/(OD [570 nm] negative control) = R; R × 100 = % cells viability. If the percentage was ≥ 50%, the oil was considered to have no cytotoxicity.

A murine macrophage cell line (J774.1A) was cultured in RPMI-1640 medium (Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin and incubated at 37° C in 5% CO_2 air atmosphere. 5×10^5/mL cells were further seeded in 96-well plates and incubated at 37°C, 5% CO_2 for 24 hours. Some wells were pretreated with *L. sumian* and *L. grosso* EOs (dilutions from 4% to 0,0075 % V/V) for 2 hours. The cells were induced with mouse γ- interferon (γ- INF) (50 ng/mL) and Lipopolysaccaride (LPS) (2μg/mL) for 20 hours. At the end of the incubation NOS activity was detected by using the commercial kit Nitric Oxide Synthase Detection System Fluorimetric (Sigma–Aldrich, Milan, Italy)) according to the manufacturer's instructions.

Subsequently, cells were seeded on coverslips for observation under a scanning electron microscope (SEM). Each sample was tested for three concentrations of lavender EO: non cytotoxic, superior and inferior. The cells, to be observed by SEM, were fixed in 2% glutaraldehyde solution in 0.1 M cacodylate buffer (1 hour). After three washes, 5 minutes each in the same buffer, the samples were dehydrated through graded alcohol solutions, air-dried with hexamethyldisilazane

Table 2 Antimicrobial susceptibility of *P. aeruginosa* strains.

Strains number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Amikacin	S	S	S	S	S	S	ND	S	S	S	S	S	R	R	I	I
Amox/ Kclav	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Amp/ Sulbactam	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ampicillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Aztreonam	I	I	I	I	I	I	ND	I	I	R	I	R	I	R	I	I
Cefepime	S	S	S	S	S	S	ND	S	S	S	S	S	R	R	R	S
Cefpodoxime	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ceftazidime	S	S	S	S	S	S	ND	S	S	S	S	S	R	R	R	S
Ceftriaxone	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefuroxime	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ciprofloxacin	S	S	I	S	S	S	ND	S	R	S	R	R	R	R	R	S
Chloramphenicol	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Colistin	S	S	S	S	ND	S	ND	S	S	S	S	S	S	S	S	S
Doripenem	S	S	S	S	S	S	R	S	S	I	S	I	I	R	I	S
Ertapenem	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Fosfomycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Gentamycin	S	S	S	S	S	S	ND	S	S	S	R	S	R	R	R	R
Imipenem	S	S	S	S	S	S	R	S	S	R	S	R	S	R	I	S
Levofloxacin	S	S	S	I	S	S	ND	S	R	I	R	R	R	R	R	S
Meropenem	S	S	S	S	S	S	ND	S	S	R	S	R	S	R	I	S
Norfloxacin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ofloxacin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Pip/Tazo	S	S	S	S	R	S	ND	S	S	S	S	S	S	R	S	S
Piperacillin	S	S	S	S	R	S	ND	S	S	S	S	S	S	R	R	S
Tetracyclin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Tobramycin	S	S	S	S	S	S	ND	S	S	S	R	S	R	R	R	S
Trime/ Sulfa	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Trimethoprim	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

R: Resistant; I: Intermediate resistance; S: Sensible; ND: Not Determined.

for 10 minutes, examined and photographed in low vacuum using SEM FEI Quanta 200.

Data are expressed as means ± standard deviations. Significant differences between treatments were analyzed by one-way analysis of variance and Kruskal–Wallis test at $p < 0.0001$ (Prism 7 software).

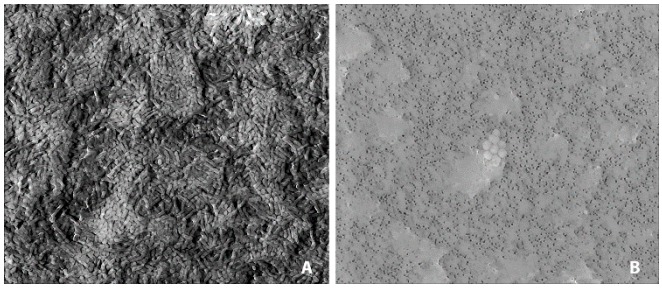
Approval from the Ethics Committee/Institutional Review Board of the Department of Surgery, Microsurgery, and Medical Sciences, University of Sassari, Sassari, Italy, was obtained and the study was conducted in complete agreement with the principles of the Declaration of Helsinki. Each participant received detailed information and provided written informed consent before inclusion.

Results

The EO derived from *L. sumian* has lower antimicrobial activity when compared to *L. grosso*. *L. Sumian* was ineffective on eleven *P. aeruginosa* strains at the concentration of 16%, while *L. grosso* was effective on the same number of *P. aeruginosa* at the concentration of 8% Table 1. This might be a promising basis for a potential therapeutic approach involving natural products alternative to and/or synergic with conventional medicine in the treatment of some chronic and acute pathologies.

All strains showed multiple antibiotic resistance to 14 antibiotics tested, including amoxicillin/clavulanic acid, ampicillin/sulbactam, ampicillin, cefpodoxime, ceftriaxone, cefuroxime, chloramphenicol, ertapenem, fosfomycin, norfloxacin, ofloxacin, tetracycline, trimethoprim and trimethoprim/sulfamethoxazole. On the other hand, all of them were susceptible to aminoglycosides, third-generation fluoroquinolones, third-generation cephalosporins and some carbapenems. The results of the MicroScan MIC/Combo Panel are presented in Table 2.

Figure 1. Scanning Electron Microscopy micrographs of *P. aeruginosa* (sample number one) post treatment with *L. grosso* EO at 16% concentration (V/V): **(A)** *P. aeruginosa* control sample number 1; **(B)** *P. aeruginosa* sample number 1, post-treatment with EO of *L. grosso* 16% (v/v)



EO derived from *L. sumian* showed cytotoxicity exceeding 50% at concentrations higher than 0.015% (IC_{50} 34 mg/mL). Conversely, for EO derived from *L. grosso* a cytotoxic effect was found for concentrations exceeding 0.0005% (IC_{50} 4,3 mg/mL). The EOs of *L. sumian* and *L. grosso* significantly reduced NOS activity in macrophage cells only at concentrations exceeding 8,5mg/mL and it was concentration dependent. No significant differences were found between *L. sumian* and *L. grosso*. Major compounds of hybrid lavender EO are presented in Table 3. The greatest inhibitory activity was observed at 34 mg/mL and 8,5 mg/mL concentrations.

Figure 1 shows the effect of exposure of *P. aeruginosa* strain number 1 to *L. grosso* EO at a concentration of 137 mg/mL. Control showed a cluster of well-preserved cells, characterized by intact cell walls (Figure 1 A). Changes in bacterial cells morphology were observed with the presence of few rounded bacterial cells compared to the control (Figure

Table 3. Major compounds of hybrid lavender EOs are listed in order of MS detector.

Compound	*Lavanda Sumian*	*Lavanda Grosso*
Eucaliptol	9,89[a]	0,66[a]
1,6 Octadien – 3 - ol	32,03	27,27
2 - Bornanone	9,81	9,34
endo- Borneol	3,68	6,77
Terpinen – 4-ol	4,16	5,19
alpha - Terpineol	0,49	1,08
(R) – Lavandulyl acetate	2,85	4,10
Caryophyllene	1,67	2,61
1,3,6 - Octatriene	25,79	34,58
1,6 - Cyclodecadiene	0,47	0,83
Naphthalene	0,35	0,64
alpha - Bisabolol	0,28	0,42
D - limonene	1,35	Tr
(E) Beta - Famesene	Tr	1,86

[a] RA%, Relative area percentage; Tr: traces.

1 B). Results suggested that *L. sumian and L. grosso* Eos might reduce the oxidative stress induced by LPS and γINF and this reduction might be related to phenolic compounds.

Discussion

These result should be confirmed *in vivo*, in particular for *L. grosso*, which appeared non cytotoxic at the lowest concentration tested (0.0005%). The chemical structure of single EOs components determines the cytotoxic way of action, as confirmed by the phenolic hydroxyl group. On the other hand, the position relative to the hydroxyl group does not seem to influence significantly the degree of antimicrobial activity. The different degree of cytotoxicity may be due to a distinct chemical composition of EOs. Given the elevated number of various chemical groups found in EOs, it is plausible that their cytotoxic activity may not be attributed to a single mechanism but rather to several pathways. Hydrophobicity is an important chemical feature of EOs, which let them penetrate through the cell membrane by increasing permeability – a phenomenon resulting in the spillage of ions and molecules leading to cell death. In general, cytotoxicity of the EOs is caused by a high concentration of phenolic compounds, such as eucalyptol, linalool or α-terpineol.

Treating bacterial infections by antibiotics is the gold standard but their indiscriminate use has led to an alarming antibiotic resistance among microorganisms. A new approach could be a synergistic therapy with traditional antibiotics and Eos with antimicrobial properties. Antibacterial and cytotoxic activities of Eos can be attributed to their different constituents. EOs contain complex mixtures including monoterpenes and sesquiterpenes: some of them have been shown to have antimicrobial activity against bacteria and fungi [8,9-11]. It is believed that EOs antimicrobial activity is not related to one specific mechanism, because they have different compositions and, therefore, may damage microbial cells in different ways. However, not all these mechanisms work separately. They act through a mechanism most probably shared with other phenols, which usually involves the cell membrane.

P. aeruginosa has been repeatedly identified as the most frequently isolated pathogen in all culture-positive cases of contact lens–related microbial infection of the cornea. [12-13]

Current therapeutic interventions involve anti-Pseudomonal antibiotics, *e.g.* aminoglycosides or fluoroquinolones, and prevention relies on reducing *P. aeruginosa* contamination of contact lenses and lens cases using contact lens care (disinfection) solutions. [14].

Molecules composing EOs target cell proteins embedded in cytoplasmic membrane. ATP synthases are known to mediate the active transport of ions and molecules thanks to their position within the cytoplasmic membrane and association with lipid molecules. Two mechanisms of cytotoxic activity have been hypothesized: 1) lipophilic hydrocarbons contained in EOs may accumulate in the lipid layer and distort the lipid-protein interaction; 2) other types of interactions excluding lipid component may occur in the proximity of protein hydrophobic portions, resulting in their destabilization [15]. Among non-phenolic EOs compounds, alkyl substituents (alkenyl groups rather than alkyls) have been reported to influence cytotoxic activity [16]. The present study is in agreement with the above mentioned considerations regarding the activity and efficacy of cytotoxic mechanisms through which EOs act on human cells. Moreover, very low EOs concentrations are necessary to achieve a non-cytotoxic effect [17]. Many compounds of plant origin have potent antioxidant activities. As expected, the Lavender essential oil presented antioxidant activity and this ability was concentration-dependent. Pretreatment of LPS and γINF induced cells with Lavender EOs significantly reduced NOS activity, primarily due to the presence of phenolic compounds as main components, which are responsible for the antioxidant properties [18-20]. Actually, at higher concentrations NOS activity was reduced to control levels: this effect could be interpreted by different mechanisms such as the radical scavenging activity or an inhibition of oxidative stress generation. However, further studies are warranted to confirm EOs antioxidant activity.

Conclusion

The investigation on plant extracts, starting with the evaluation of the biological activities of phytocomplexes with further identification of the main active components, may provide advantages for the assessment of new substances to be used either singularly or in combination with conventional drugs already used in clinical practice. In conclusion, there is an agreement on the fact that a progressive reduction of effective antimicrobial drugs and the associated increase of multidrug resistance, together with a scarce involvement of the pharmaceutical industry in the distribution of orphan drugs, should encourage the development of new treatments.

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