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Original Article

In vitro activity of essential oils against *Pseudomonas aeruginosa* isolated from infected hip implants

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Abstract

Introduction: Essential oils have been used since ancient times and are known for their anti-inflammatory, anti-depressive, antiseptic, antifungal and antimicrobial properties.

Methodology: in this study the antimicrobial activity of two essential oils from *Melaleuca alternifolia* and *Thymus vulgaris–red thyme geraniol* was tested against 16 multidrug-resistant *P. aeruginosa* strains from infected hip implants as well as the "*in vitro"* cytotoxic activity on normal human Wong-Kilbourne derivative (WKD) cells.

Results: *Thymus vulgaris–red thyme geraniol* showed lower antimicrobial activity when compared to *Melaleuca alternifolia*. All tested oils were cytotoxic at concentrations lower than 0.12%.

Conclusion: Increase in drug resistance and lack of new antibiotics may encourage the development of natural treatments together with higher concern on environmental issues and natural lifestyle.

Key words: Antimicrobials; biopharmaceuticals; hip implants; infection; Pseudomonas spp.

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Introduction

The therapeutic potential of essential oils (EOs) has not been fully estimated yet, leaving several features regarding 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 remarkable number of different compounds.

For centuries, traditional medicine and aromatherapy have relied on of EOs properties and aromatic molecules are currently still appreciated as therapeutic agents. These natural compounds exert significant biological and pharmacological effects, mainly due to their lipophilic nature.

During the Middle Ages, indigenous herbs such as rosemary, sage, mint, and lavender were used. As early as the sixteenth century, some oils known as chemical oils, applied in the care of the body and mind were developed and at the same time several herbariums were published. Thanks to the scientific revolution, at the beginning of the nineteenth century, chemists were able to identify the different EOs components and develop the modern pharmaceutical industry. Despite the differences in EOs chemical composition, they share some general properties, such as antiseptic, antibacterial, antifungal, and antioxidant activities [1].

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 environment [1], with particular importance given to climate and seasons [1,3,4]. Phytotherapy has been set aside after the development of chemistry and the first synthetic drugs, to be rediscovered in the last decades as either an integrative method associated with conventional medicine or an alternative therapy.

Periprosthetic joint infection is one of the most devastating and costly complications following total joint arthroplasty. Given the increase of total joint arthroplasty being performed annually, the number of

complications necessitating revision surgery is growing and it is also associated with significant physical and psychological morbidity on patients [5].

The incidence of periprosthetic hip infections is estimated below 2%; despite a relatively low incidence of periprosthetic joint infections, the financial impact remains remarkable. The annual cost of infected revisions to U.S. hospitals increased from $320 million in 2001 to $566 million in 2009, and it is estimated that the cost will exceed $1.62 billion by 2020 [6].

Skin-concomitant bacteria, in particular gram-positive cocci, are the most typical isolates: *S. aureus*, enterococci and streptococci; coagulase-negative staphylococci and *S. aureus* are responsible for 50%–60% of the cases; moreover, recently, a rise of methicillin-resistant strains has been observed. Among gram-negative bacteria it is worth mentioning *P. aeruginosa* [7].

Bacteriologic analysis of swabs taken from surgical wounds at the end of surgery shows that 15% of surgical wounds were contaminated by skin flora.

The present study focused on antimicrobial activity of EOs, following previous investigations performed by the same research group on *P. aeruginosa* isolates collected from patients with infected hip replacement hospitalized at the Orthopedic Clinic of Sassari University [8-10].

Instead, the present study aimed at evaluating the effectiveness of minimum bactericidal concentration (MBC) of EOs derived from *Melaleuca alternifolia* and *Thymus vulgaris –red thyme geraniol* against multidrug-resistant *P. aeruginosa* isolates from hip implants. At the same time, cytotoxicity on human Wong-Kilbourne derivative (*WKD*) cells was assessed.

Methodology

Study population

The study included 16 patients with suspected hip implants infection. Samples were harvested by using intraoperative swabs directly in the operating room from infection sites in specific quantities needed to allow investigations. Antibiotic therapy must be suspended at least 2 weeks before harvest.

The study was conducted in complete agreement with the principles of the Declaration of Helsinki. Each participant received detailed information and signed a written informed consent before inclusion.

Sampling procedure for isolation of Pseudomonas spp.

Samples were immediately plated on blood agar (5% sheep), chocolate agar, Sabouraud dextrose agar, and thioglycollate broth. *Pseudomonas aeruginosa* was isolated in more than one culture medium without any contamination or mixed growth. The Vitek system (bioMérieux, La Balme les Grottes, Montalieu Vercieu, France) was used for phenotypic identification of all positive cultures. Antibiotic susceptibility was tested using the classic agar diffusion (Kirby-Bauer) method.

Plant Material

Melaleuca alternifolia and *Thymus vulgaris –red thyme geraniol* were collected from different areas on La Maddalena island in North-Eastern Sardinia, Italy (Coordinates 41°12′51.26″N 9°24′29.99″E). Voucher specimens were deposited at the Department of Biomedical Sciences, University of Sassari under a collective number (1239SSBM for *Melaleuca alternifolia* and 1240SSBM *Thymus vulgaris –red thyme geraniol*). Vegetal material collection and EO extraction were carried out in April during the morning. EO extraction was obtained by steam distillation with a distillation equipment of 3 litres of capacity. Three litres of water were used for steam distillation and the amount of leaves and herbaceous branches was 5 kg. The EO obtained was dried with anhydrous sodium chloride, filtered and stored in amber glass bottles in a refrigerator at 4°C until use.

EO chemical composition

Three replicates of each sample were analysed using a *Hewlett-Packard Model 5890A* GC equipped with a flame-ionization detector and fitted with a 60 m × 0.25 mm, thickness 0.25 μm *AT-5* fused SiO_2 capillary column (*Alltech, Milan, Italy*). 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 analysed 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 an *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 based on the interpretation of the EI-fragmentation of the molecules, Table 1 and Table 2.

EO antibacterial effect assessment using microdilution method

The EOs minimum bactericidal concentration (MBC) on 16 multidrug-resistant *P. aeruginosa* strains was analysed.

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 sub-cultured 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.

Table 1. Essential oil qualitative and semi-quantitative analysis *Melaleuca alternifolia*.

Compound[a]	LRI[b]	%[c]	Compound[a]	LRI[b]	%[c]
α-thujene	926	0.75	citronellyl acetate	1358	
α-pinene	933	2.41	neryl acetate	1368	
sabinene	973	0.09	cyclosativene	1369	0.81
1-octen-3-ol	979		α-copaene	1381	8.08
β-pinene	979	0.65	β-elemene	1396	10.00
β-myrcene	992	0.63	cyperene	1403	1.15
octan-3-ol	997		α-gurjunene	1412	0.51
α-phellandrene	1004	0.35	β-caryophyllene	1425	0.70
3-carene	1009		rotundene	1461	0.45
α-terpinene	1017	7.49	ishwarane	1472	26.64
p-cymene	1025	5.33	γ-muurolene	1481	2.88
limonene	1029	1.50	*ar*-curcumene	1487	1.86
1,8-cineole	1033	2.16	viridiflorene	1502	1.48
γ –terpinene	1059	18.39	δ-cadinene	1530	1.63
terpinolene	1088	3.13	monoterpene hydrocarbons	98.08	40.72
borneol	1166	0.66	monoterpene oxygenated:		47.06
terpinen-4-ol	1179	2.16	–alcohols		44.90
α-terpineol	1192	7.72	– esters		
δ- elemene	1341	0.85	– ethers		2.16
α-terpinyl acetate	1354		sesquiterpene hydrocarbons		5.73
α-cubebene	1355	0.64	sesquiterpenes oxygenated		
			total	**98.08**	**93.52**

[a] Compounds are listed in order of elution. [b] Linear retention index (LRI). [c] % Percentage of compound.

Table 2. Essential oil qualitative and semi-quantitative analysis *Thymus vulgaris –red thyme geraniol*.

Compound[a]	LRI[b]	%[c]	Compound[a]	LRI[b]	%[c]
p-cymene	1025	1.00	geranyl acetate	1387	23.61
limonane	1029	0.26	β-caryophyllene	1425	3.70
γ –terpinene	1059	2.71	caryophyllene oxide	1593	0.64
linalool	1103	42.06	monoterpene hydrocarbons		3.97
camphor	1144	0.47	monoterpene oxygenated:		87.42
borneol	1166	0.26	– alcohols		62.05
terpinen-4-ol	1179	0.53	– phenolics		1.29
α-terpineol	1192	0.44	– ketones		0.47
carveol	1231	0.85	– esters		23.61
geraniol	1265	17.91	– ethers		
geranial	1278		sesquiterpene hydrocarbons		3.70
bornyl acetate	1289		sesquiterpenes oxygenated		0.64
thymol	1295	1.29	**total**		**95,73**

[a] Compounds are listed in order of elution. [b] Linear retention index (LRI). [c] % Percentage of compound.

Cytotoxicity

EOs cytotoxicity was analysed on WKD cells. WKD cells were maintained in Roswell Park Memorial Institute-1640 (RPMI) 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. 1,5 $\cdot 10^5$/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 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.

Data analysis

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

Results

The results of the antimicrobial susceptibility test on *P. aeruginosa* strains are presented in Table 3.

All strains showed multiple antibiotic resistance to the 11 antibiotics tested, including

Table 3. Antimicrobial susceptibility of *P. aeruginosa* strains.

	1 [b]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Amikacin [a]	S	S	S	S	S	S	//	S	S	S	S	S	R	R	I	I
Amoxicillin / Clavulanic Acid	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ampicillin / 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
Cefpodoxime	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ceftazidime	S	S	S	S	S	S	//	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	//	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	//	S	//	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	//	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	//	S	R	I	R	R	R	R	R	S
Meropenem	S	S	S	S	S	S	//	S	S	R	S	R	S	R	I	S
Piperacillin / Tazobactam	S	S	S	S	R	S	//	S	S	S	S	S	S	R	S	S
Piperacillin	S	S	S	S	R	S	//	S	S	S	S	S	S	R	R	S
Tetracycline	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Tobramycin	S	S	S	S	S	S	//	S	S	S	R	S	R	R	R	S
Piperacillin / Tazobactam	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

R: resistant; I: intermediate; S: susceptible. [a] Antibiotics. [b] Number *P. aeruginosa* strains.

Table 4. Antimicrobial activity of EOs (% v/v) against 16 multidrug resistant *P. aeruginosa.*

Strains n°	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
EO: Thymus vulgaris – red thyme geraniol	8%	8%	16%	8%	16%	8%	16%	8%	16%	8%	8%	8%	8%	16%	8%	8%
EO: Melaleuca alternifolia	4%	4%	8%	8%	4%	4%	4%	8%	8%	4%	4%	4%	4%	4%	4%	8%

amoxicillin/clavulanic acid, ampicillin/sulbactam, ampicillin, cefpodoxime, ceftriaxone, cefuroxime, chloramphenicol, ertapenem, fosfomycin, tetracycline, and trimethoprim/sulfamethoxazole. On the other hand, all of them were susceptible to aminoglycosides, third-generation fluoroquinolones, third-generation cephalosporins and some carbapenems.

Data presented in Table 4 show that EO derived from *Thymus vulgaris –red thyme geraniol* has lower antimicrobial activity when compared to *Melaleuca alternifolia*. *Thymus vulgaris -red thyme geraniol* was effective on eleven strains of *P. aeruginosa* at the concentration of 8% and on the remaining five strains at 16%, while *Melaleuca alternifolia* was effective on the same number of *P. aeruginosa* strains at 4% and 8% concentration. This could be a promising basis for a potential therapeutic approach involving alternative natural and/or synergistic products to common antibiotic cements. EOs derived from *Melaleuca alternifolia* and *Thymus vulgaris –red thyme geraniol* showed cytotoxicity exceeding 50% at concentrations lower than 0.12%.

Discussion

Phytotherapy is one of the most antique practices used by humans to cure health problems; its traces have been found among ancient civilizations, including Egypt, Indus Valley, Greece, Chinese and Roman Empire [11]. Treating bacterial infections by antibiotics is 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 are exerted by their different compounds: EOs are made of complex mixtures including monoterpenes and sesquiterpenes: some of them have shown a strong antimicrobial activity against bacteria and fungi [12]. It is believed that the antimicrobial activity of EOs is not due to one specific mechanism, because they have different compositions and might thus damage microbial cells in different ways. However, not all these mechanisms work separately. 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 in EOs, as it lets them penetrating through the cell membrane by increasing permeability – a phenomenon resulting in the spillage of ions and molecules leading to cell death. In general, EOs cytotoxicity is due to a high concentration of phenolic compounds, such as eucalyptol, linalool or α-terpineol. They act through a mechanism most probably shared with other phenols, which usually involves the cell membrane. 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. 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 present in EOs may accumulate in the lipid layer and distort the lipid-protein interaction; 2) other type of interactions excluding the lipid component may occur in the proximity of protein hydrophobic portions, resulting in their destabilization [13]. Among non-phenolic EOs compounds, alkyl substituents (alkenyl groups rather than alkyls) have been reported to influence cytotoxic activity [14]. At present, we agree on 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 [15-17].

For what concerns primary total hip arthroplasty (THAs), it has been reported that the number of interventions of this kind performed in the United States continues to increase each year, as does the incidence of infectious complications. The changing profile of antibiotic resistant bacteria has made preventing and treating primary THA infections increasingly complex [18].

Besides, the skin flora represents a risk during intraoperative wound contamination, increasing the chance to develop postoperative infections after THA approximately 2-fold, despite contemporary aseptic and antiseptic prophylaxis measures.

Gram-negative bacteria are the cause of infection in 3%–6% of cases and are rather uncommon.

The microbial contamination of the prosthesis can occur during an operation through airborne contamination or direct inoculation during the handling of the prosthesis [19].

Alternatively, microorganisms can reach the prosthesis through the hematogenous route for a bacteremia or from an adjacent septic source. Prosthetic

infections are usually caused by the presence of microorganisms growing inside a structure called biofilm. The pathogenesis is determined by the interaction of the microorganisms and the biofilm production, from the inflammatory response of the host. The prosthesis is not affected by blood circulation, therefore the local penetration of antibiotic is impossible; consequently, on the surface of the prosthesis, an extracellular matrix with high concentration of water is formed, and the microorganisms shift from a planktonic phase to a sessile phase with low energy requirement.

Conclusion

Current and future research may have a strong impact on public health by offering useful indications towards optimization of the therapeutic use of some natural drugs. Specifically, the investigation of plant extracts, starting with the evaluation of the biological activities of a phytocomplex and further identification of the main active components, may provide advantages in assessing new substances to be used either alone or in combination with conventional drugs already used in clinical practice. In conclusion, we suggest that given the increasing lack of drugs and particularly of new antibiotic cements for prosthetic use, based on the results of the present study and other ongoing investigations, phytocomplex contained in essential oils might be adopted to overcome antibiotic resistance.

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