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Original Article

Essential oils in ocular pathology: an experimental study

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Abstract

Introduction: The antimicrobial activity of essential oils (EOs) has been known for ages; in particular, the EOs of *Melaleuca alternifolia*, *Thymus vulgaris*, *Mentha piperita*, and *Rosmarinus officinalis* have been used for the treatment of fungal and bacterial infections.
Methodology: This study focused on the *in vitro* cytotoxicity to normal human conjunctiva cells and antimicrobial activity of 20 EOs.
Results: The oils tested showed no cytotoxic effect at very low concentrations. *Rosmarinus officinalis*, *Melaleuca alternifolia*, and *Thymus vulgaris* L. red thyme geraniol sel oils had good antimicrobial activity against Gram-positive and Gram-negative strains.
Conclusions: The results of this study are of great interest and may have a major impact on public health, providing useful tips to optimize the therapeutic use of some natural drugs.

Key words: essential oils; cytotoxicity; bacteria.

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Introduction

Essential oils (EOs) have been used since ancient times for various purposes, including cosmetics and cuisine [1]. In particular, they were and still are used in the medical field because of their therapeutic properties. Their use was part of the ritual tradition of almost all cultures [2]; in the East, aromatic substances were often regarded as much more than just perfumes and were employed for both liturgical and therapeutic purposes, and the Ebers Papyrus records the use of many medicinal herbs, oils, and perfumes. 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, dedicated to 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 various components of the EOs and develop the modern pharmaceutical industry.

Despite the differences in the chemical composition of the EOs, they share some general properties, such as antiseptic, antibacterial, antifungal, and antioxidant activities [1-18].

EOs are very complex mixtures of secondary metabolites with specific chemical composition, which varies according to the species and the specific characteristics of the plant. The main EO components include monoterpene, diterpene, and sesquiterpene hydrocarbons, azulene, alcohols, aldehydes, and ketones [7-9].

Terpenes represent the most abundant class of metabolites (more than 22,000 compounds). They are involved in many cellular processes, such as photosynthesis, cell growth, reproduction, and cell defence.

Terpenes are also used as flavouring agents, fragrances, and drugs. The type and quantity of components determine the peculiar characteristics of each oil; furthermore, components present in minimal traces may influence their biological activity. EOs are liquid at room temperature, insoluble in water, and soluble in alcohol, ether, chloroform, and most organic solvents [19].

The antimicrobial activity of EOs has been well known for many years; in particular, the essential oils of *Melaleuca alternifolia*, *Thymus vulgaris*, *Mentha piperita*, and *Rosmarinus officinalis* have been used for the treatment of fungal and bacterial infections.

This study focused on the *in vitro* cytotoxicity to normal human conjunctiva cells and antimicrobial activity of 20 EOs.

Methodology

The cytotoxic effects of 20 standard components (Table 1) from EOs extracted by steam distillation on normal human conjunctiva cells (Wong-Kilbourne derivative [WKD] cells; European Collection of Cell Culture reference no. 93120839) were analyzed. WKD cells were grown in RPMI medium (Sigma-Aldrich SRL, Milan, Italy), supplemented with 10% fetal bovine serum and 100 U/mL penicillin and streptomycin, and incubated at 37°C and 5% CO_2 to a final concentration of 1.5 x 10^5/mL. Dilutions of EOs (from 16% to 0.004%) were prepared in culture medium supplemented with Tween 80 (0.5%). The wells were washed twice with phosphate-buffered saline (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 four hours of incubation, M-8910 MTT solubilizing solution was added, which comprised 10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol. The quantity of formazan was measured 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 x 100 = % cells viability. If the percentage was greater than 60%, the oil had no cytotoxicity, and if it was less than 60%, the oil was highly cytotoxic.

Table 1. Essential oils standard assessed

1	Camphor
2	Carvacrol
3	Trans-caryophyllene
4	*p*-cymene
5	Citronellol
6	Eugenol
7	Eucalyptol
8	Limonane
9	Menthol
10	Menthone
11	γ-Terpinene
12	Terpinolene
13	Thymol
14	Citronellal
15	Linalool
16	Linalilacetate
17	Terpinen-4-ol
18	α-pinene
19	Geraniol
20	Phellandrene

Furthermore, the cytotoxicity of two multi-dose, commercially available anti-glaucoma eye drops (Lumigan and Travatan, containing bimatoprost 0.1 mg/mL and travoprost 40 mcg/mL, respectively, as their active ingredients) and one multi-dose artificial tear solution (Optive Plus, containing carboxymethylcellulose 0.5%) on WKD cells was assessed. This was compared with the activity of the following highly cytotoxic essential oils: *p*-cymene, citronellol, eugenol,and eucalyptol.

Ninety-six-well plates containing WKD cells (1.5 x 10^5 cells/mL) grown in RPMI medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and streptomycin were incubated at 37°C in 5% CO_2.

The EOs (16% [v/v] to 0.004% [v/v]) were assessed for 10 minutes at room temperature; the cytotoxicity assay was performed as described above, following the manufacturer's instructions.

In a second experiment, the minimum bactericidal concentrations (MBCs) of 10 EOs that showed lower cytotoxicity in a previous experiment (data not shown) were evaluated. These included *Citrus aurantium* var. sweet, *Lavandulus angustifolia*, *Mentha piperita*, *Rosmarinus officinalis*, *Salvia sclarea*, *Melaleuca alternifolia*, *Thymus vulgaris* L. red thyme - geraniol bio, *Thymus vulgaris* L. red thyme - geraniol sel), *Thymus vulgaris* red thyme bio France, *Thymus vulgaris L.* red thyme sel), which had been used on three multidrug-resistant *Pseudomonas aeruginosa* strains isolated from eyes with very severe post-operative endophthalmitis in India [20-22], one *P. aeruginosa* strain from a patient with keratitis, isolated at the University of Sassari, Italy, and one *P. aeruginosa* reference strain (American type culture collection [ATCC] no. 27853).

The EOs were diluted in Luria broth (LB) supplemented with 0.5% Tween 80 at concentrations ranging from 16% to 0.125% (v/v). The inoculum of the bacteria was performed at a concentration of 10^6 CFU/mL. An inoculum of 100 µL of microbial culture was added to 100 µL of each concentration of EOs in 96-well plates (Techno Plastic Products AG, Trasadingen, Switzerland) 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 [23]. MBC was considered the lowest concentration that could inhibit 99% of bacterial growth. Each experiment was performed in duplicate and repeated three times.

Results

The results of cytotoxicity are summarized in Table 2. An important characteristic of EOs is their hydrophobicity, which allows them to penetrate through the cell membrane, thus increasing its permeability and causing leakage of ions and molecules from the cell which, at last, results in the cell death.

EOs with cytotoxic properties contain a high percentage of phenolic compounds, such as carvacrol, thymol, eugenol, and eucalyptol.

Previous research has demonstrated that the components of EOs act on cellular proteins in the cytoplasmic membrane [24].

Overall, EOs have no cytotoxic effect at very low concentrations. Given the high cytotoxicity of different standard components of EOs, their cytotoxicity was compared with the cytotoxic effect of three commercially available eye drops: two anti-glaucoma medications and one artificial tear solution.

Surprisingly enough, the tested eye drops showed *in vitro* cytotoxic activity against WKD cells, while the EOs standard no. 4, 5, 6, 7, at concentrations ranging from 0.0004% to 0.0078%, showed no cytotoxicity.

Interesting data were obtained with eucalyptol, which is cytotoxic to WKD cells only at a concentration of 16%, whereas it was non-toxic in

Table 2. Non cytotoxic concentration (%v/v) of essential oil on WKD.

Standard	(%v/v)	Standard	(%v/v)	Standard	(%v/v)	Standard	(%v/v)
1*	1.5	7	0.015	13*	3	19	0.031
2	0.0019	8	0.031	14	0.015	20	0.5
3	0.0078	9*	1.5	15	0.015		
4	0.0078	10	0.5	16	0.0039		
5	0.0039	11	0.25	17	0.015		
6	0.015	12	0.015	18	0.015		

*concentration µg/ml

Table 3a. Cytotoxicity of essential oils (a) and commercially available eyes-drop (b). All percentages of cell viability in bold indicate marked cytotoxicity on WKD cells.

Essential oil % (v/v)	*p*-cymene	Citronellol	Eugenol	Eucalyptol
16	**12.3**	**8.95**	**19.16**	20.83
8	**10.4**	**8.95**	**22.9**	66.6
4	**10.4**	**10.62**	**19.37**	75
2	**10.4**	**9.58**	**20.8**	97.9
1	**8.75**	**9.58**	**27.1**	87.5
0.5	**8.54**	**37.5**	**12.3**	95.8
0.25	**8.95**	64.6	**11.9**	97.9
0.12	**8.33**	87.5	**12.5**	100
0.062	**8.54**	**58.33**	**20.8**	60.41
0.0312	**8.75**	**56.25**	**50**	79.16
0.0156	**16.04**	**58.33**	64.6	79.16
0.078	60.41	**56.25**	81.2	77.1
0.0039	81.25	79.16	83.3	87.5
0.0019	85.41	79.16	93.75	89.58
0.0009	93.75	91.66	79.16	81.2
0.0004	89.6	91.66	87.5	89.58

Table 3b. Viability of eye drops

Eye drops	Viability
Lumigan	11.04%
Optive plus	38.5%
Travatan	54.16%

Table 4. MBC values of essential oils (%v/v)

	Pseudomonas aeruginosa	***Pseudomonas aeruginosa***	***Pseudomonas aeruginosa***	***Pseudomonas aeruginosa***	***Pseudomonas aeruginosa***
	India no. 1 4R	**India no. 18 2R**	**India no. 25 1R**	**CLINICAL STRAIN**	**ATCC 27853**
Citrus aurantium var. dulcis	> 16	> 16	> 16	> 16	> 16
Lavandulus angustifolia	0.5	> 16	16	16	16
Mentha piperita	< 0.12	16	16	16	16
Rosmarinus officinalis	1	16	8	8	4
Salvia sclarea	> 16	> 16	> 16	> 16	16
Melaleuca alternifolia	2	8	8	4	4
Thymus vulgaris L. red thyme geraniol bio	16	16	> 16	16	16
Thymus vulgaris red thyme geraniol sel	8	8	8	16	8
Thymus vulgaris red thyme bio - France	16	16	16	> 16	16
Thymus vulgaris red thyme sel	16	16	16	> 16	16

successive dilutions (Table 3a).

The eye drops that showed the highest cytotoxic activity were those containing bimatoprost as the active ingredient, with a viability of 11.04%, whereas Travatan showed a viability of 54.16%, close to the permitted limit, but always cytotoxic (Table 3b).

The lubricant Optive Plus was found to be cytotoxic (38.5% viability) to WKD cells. In this study, all the tests were carried out *in vitro*; therefore, all *in vivo* pharmacokinetic differences should be considered.

These experiments showed good antimicrobial activity of several essential oils, including *R. officinalis*, *M. alternifolia*, and *T. vulgaris* red thyme geraniol sel. MBC values are showed in Table 4.

The *Citrus aurantium* var. *dulcis* essential oil showed a MBC value > 16%.

Lavandulus angustifolia oils and *Mentha piperita* showed a MBC of 0.5 and 0.1 (% v/v), respectively, against *P. aeruginosa* (India no. 1), resistant to cefazolin, chloramphenicol, tetracycline, and aminoglycoside. *Rosmarinus officinalis* showed an excellent result; indeed, at 1% it inhibited the growth of *P. aeruginosa* (India no. 1), and at 4% it inhibited the growth of ATCC 27853 strain. *Citrus aurantium* var. *dulcis* showed a MBC of 16%.

Among the three essential oils assessed, *Melaleuca alternifolia* showed good antimicrobial activity, which seems to be associated with high concentrations of terpinen-4-ol.

Discussion

Natural products are a potential therapeutic approach and/or synergistic alternative to treat some chronic and acute diseases. In particular, researchers are trying to develop new substances and effective molecules against pain and inflammation, or with antimicrobial, anti-tumor, or immunomodulating activity. Many drugs are derived from natural substances, such as many antibiotics, cardiotonics, and chemotherapeutic agents.

Cytotoxicity to WKD cells of the 20 EOs tested and the three commercially available eye drops is to be confirmed in *in vivo* studies.

The results of the antibacterial activity of *Rosmarinus officinalis*, *Melaleuca alternifolia* and *Thymus vulgaris* – red thyme geraniol sel oils against *P. aeruginosa* strains is very important. This research may have a major impact on public health, providing useful tips to optimize the therapeutic use of some natural drugs. In particular, starting from the evaluation of the biological activity of the phytocomplex and identification of the main active components, ongoing research will provide useful bases for testing new substances either alone or in combination with other drugs already available in clinical practice.

The increase in drug resistance and the limited involvement of the big pharmaceutical industries in the development of new drugs are pushing to new treatments based on ease of prescription and low cost, as well as the idea of an optimal relationship with the environment.

Dedication

This work is dedicated to Prof. Cappuccinelli, in recognition of his achievement in the field of microbiology.

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