SUPPLEMENTARY MATERIAL

Properties and limits of some essential oils: chemical characterization, antimicrobial activity, interaction with antibiotics and cytotoxicity.

Francesca Scazzocchio^a, Stefania Garzoli^b, Cinzia Conti^a, Claudia Leone^a, Clio Renaioli^a, Federico Pepi^b, Letizia Angiolella^{a#}.

a)Department of Public Health and Infectious Diseases, University of Rome "Sapienza", Piazzale Aldo Moro 5, 00185 Roma, Italy

b)Department of Drugs Chemistry and Technology, University of Rome "Sapienza", Piazzale Aldo Moro 5, 00185 Roma, Italy

Corresponding author. Mailing address: E-mail: letizia.angiolella@uniroma1.it

Abstract

Because of the emergence of multidrug resistance bacteria and fungi, alternatives to conventional antimicrobial therapy are needed. This study aims to evaluate in vitro the antimicrobial activity of: *M. communis*, *C. sativum*, *P. capitatum*, *C. cuminum*, *O. basilicum*, *C. aurantium amara*, *C. winterianus*, *C. martini*, *S. sclarea*, *M. alternifolia*, *M. suaveolens* essential oils on bacteria and fungi, in relation to their chemical composition. The potential interaction of *M. alternifolia* (TTO), *C. sativum* (CDO) and *M. suaveolens* (EOMS) essential oils when used in combination with gentamicin and fluconazole has been evaluated. The results obtained showed a synergic effect on some bacteria and fungi, with FICI values ≤ 5 . The cytotoxicity of TTO, CDO and EOMS was investigated towards HeLa cells. Only EOMS did not result cytotoxic at the active concentrations on microorganisms. Further studies are necessary to obtain optimal ratios and dosing regimens for higher therapeutic efficacy and to decrease toxicological profiles

Key words: Bacteria, Fungi, Essential oils-Antibiotic interaction, Fluconazole, Gentamicin

Experimental

1. Organisms and growth conditions

The strains used in this study are: two clinical blood culture isolates *Staphylococcus epidermidis* MRSA, four clinical isolates *Klebsiellae pneumonia*, one *Acinetobacter baumani* and two *Candida albicans* CO23 and CORFLU (induced fluconazole resistance), all these clinical isolates showed

high resistance towards the antibiotics used for therapy and as control *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 24433. Bacteria and fungi are respectively grown in Trypticase Soy Broth and Sabouraud at an optimal growth temperature of 30- 37° C with aeration for 18–24 h.

2. Gas Chromatography-mass spectrometry (GC-MS) analysis of essential oils

The essential oils (EOs) of *Myrtus communis*, *Coriandrum sativum*, *Pelargonium capitatum*, *Cuminum cyminum*, *Ocimum basilicum*, *Citrus aurantium amara*, *Cymbopogon winterianus*, *Cymbopogon martini*, *Salvia sclarea* and *Melaleuca alternifolia* used in this study were kindly gifted by Talia (Rome, Italy, [http:// www.taliaessenze.com](http://www.taliaessenze.com)). All of them are commercial oils. *Mentha suaveolens* (EOMS patent WO 2011092655 A3) was produced by four-hour hydro distillation by Giardino degli aromi, (Ragusa, Italy). All EOs were analysed for chemical composition by GC/MS analysis (Perkin Elmer Clarus 500) and GC-FID equipped with a Restek Stabilwax fused-silica capillary column (60 m long, 0.25 mm i.d.), operating at 60°C for 5 min and then programmed to 220°C at rate of 5°C/min.; helium was used as carrier gas with 1 ml/min. Relative percentages of the components were calculated by electronic integration of the GC-FID peak areas.

The identification of the main compounds present in the essential oils was confirmed at first by GC injection of analytical standard sample comparing their retention times with those obtained in the essential oil analysis.

A second confirmation was performed by GC-MS comparing the mass spectra with those characteristic of the standard and, if not commercially available, with the literature NIST and NBS libraries.

3. Screening Antimicrobial Activity by Disk Diffusion Method

Screening of antimicrobial activity of EOs from *Myrtus communis*, *Coriandrum sativum*, *Pelargonium capitatum*, *Cuminum cyminum*, *Ocimum basilicum*, *Citrus aurantium amara*, *Cymbopogon winterianus*, *Cymbopogon martini*, *Salvia sclarea*, *Melaleuca alternifolia*, *Mentha suaveolens* was carried out by the disc diffusion method, used as a preliminary test to select the active essential oils.

The antimicrobial activity of EOs was evaluated against bacteria and fungi above reported according to the National Committee of Clinical Laboratory Standards (NCCLS 1997). Petri plates were prepared by pouring 20ml of Muller Hinton Agar or Sabouraud dextrose agar for bacteria and fungi respectively and allowed to solidify. An inoculum of 10 µl of 10⁷ CFU/ml bacterial or fungal

suspension, was poured and uniformly spread over the plates. 20 µl of each oil at concentration of 20, 10, 5 and 2.5 mg/ml were deposited on sterile paper disks (6mm diameter), placed in the centre of Petri plates. The Petri plates were then incubated for 18-24h at 30°C for fungi and 37°C for bacteria. The microbial growth inhibition zone diameter (disk included) was measured by a caliper and expressed in mm. The sensitivity to the different oils was classified by the diameter of the inhibition halos as follows: not sensitive for diameter less than 8 mm, sensitive for diameter 9-14 mm; very sensitive for diameter 15-19 and extremely sensitive for diameter larger than 20 mm in according to other reports (Djenane et al. 2011). Gentamicin (10 µg/disc) and fluconazole (25 µg/disc) were also included in the test as reference antimicrobials in order. All experiments were repeated independently at least three times.

4. Determination of Minimum Inhibitory Concentration.

A microdilution broth susceptibility assay for bacteria and fungi was used for the determination of Minimum Inhibitory Concentration (MIC) according to NCCLSM7-A3 and M27-A3 respectively (NCCLS 1993, 2008).

Mother solution of 100 mg/ml of *Coriandrum sativum* (CDO), *Melaleuca alternifolia* (TTO) and *Mentha suaveolens* (EOMS) essential oils were prepared in Muller Hinton Broth or RPMI 1640 supplemented with morpholinopropansulfonic acid (MOPS) buffer at pH 7 added with 0.02% Tween 80 to increase oil solubility. The dilutions, ranging from 0.012 to 25mg/ml of the essential oils, were prepared in 96 well plates. Wells containing media without essential oils were used as growth control. Fluconazole and Gentamicin 0.5 mg/ml solutions were prepared by dissolving the agents in endotoxin free water.

Freshly cultured microbial suspensions of bacteria and fungi were standardized to a cell density corresponding to 0.5 McFarland and 10 µl used as inoculum in each well. The plates were incubated for 18-24 h at 30 or 37° C respectively and microbial growth was visually assessed. The MIC was defined as the lowest essential oil concentration without visible growth.

5. Checkerboard Method Used to Evaluate the Synergic Action of EOs with Conventional Drugs

Twelve serial twofold dilutions of TTO, CDO, and EOMS were prepared following the same broth dilution method adopted to assess MIC. Dilution of the oils was prepared (25 to 0.0125mg/ml) together with a series of two fold serial dilutions of gentamicin (G) in the 128 to 0.125µg/ml and of fluconazole (FLC) in the 64 to 0.06-µg/ml range. All FLC and G dilutions were mixed with the appropriate concentration of oils as reported by White et al. (1996).

The analysis of the combination of the substances was carried out calculating the fractional inhibitory concentration (FIC). The FIC index (FICI) was calculated dividing the MIC of the combination of each EO and the reference antimicrobial by the MIC of EO or reference antimicrobial tested alone (Stringaro et al. 2014).

$$\text{FIC for oil} = \text{MIC of oil in combination with antimicrobial drug} / \text{MIC of oil alone}$$

$$\text{FIC of antimicrobial drug} = \text{MIC of antimicrobial drug in combination with oil} / \text{MIC of antimicrobial drug alone}$$

$$\text{FICI} = \text{FIC of oil} + \text{FIC of antimicrobial drug}.$$

When the FIC index of the combination is equal to or less than 0.5, the combination is termed as synergic; when FIC index falls between 0.5 and 2.0, it indicates additive or indifferent between the agents, and a value > 2 indicates antagonism between the two compounds (White et al. 1996).

6. Cells and Assay for Cellular Cytotoxicity

HeLa cells, an epithelial cell line of human origin, were grown at 37 °C using Eagle's Minimum Essential Medium (MEM) supplemented with 100 µg/ml of streptomycin and 100 U/ml of penicillin G and 8% heat-inactivated foetal calf serum (growth medium).

To evaluate the cytotoxicity of essential oils a tetrazolium-based (XTT) colorimetric assay was used (Scudiero et al. 1988). Briefly, HeLa cells were seeded in 96 well tissue culture plates (1×10^4 cells/well) in 100 µl of growth medium with or without compounds in two-fold dilutions, starting from 10 mg/ml. Triplicate wells were used for each oil concentration to be tested. The plates were incubated for 24 h at 37 °C in 5% CO₂-air. Then, 50 µl of XTT labelling mixture was added to each well (final XTT concentration 0.15 mg/ml) and the cells incubated for 4 h at 37 °C. The spectrophotometric absorbance of the samples was measured using an ELISA reader at 492 nm with a reference wavelength at 690 nm. Cytotoxicity was also scored microscopically as morphological alterations with respect to mock-treated cultures and the viability of cells was checked by neutral red uptake, a vital dye. The highest concentration of compound that did not produce any modification of absorbance at 492 nm, morphology and viability in 100% of the cells was the Maximum Not-Cytotoxic Concentration (MNCC).

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Table S1. Antimicrobial activity of Essential oils by diffusion test towards bacteria and fungi

Organism	EOs mg/ml	Inhibition zone diameter (mm) ^a										
		<i>Citrus arantium amara</i>	<i>Coriandrum sativum</i>	<i>Cuminum Cuminum</i>	<i>Cymbopogon martini</i>	<i>Cymbopogon winteranus</i>	<i>Pelargonium capitatum</i>	<i>Mentha suaveolans</i>	<i>Myrtuscommunis</i>	<i>Ocimumbasilicum</i>	<i>Salvia sclarea</i>	<i>Melaleuca alternifolia</i>
Gram positive												
<i>S.aureus Atcc25923</i>	20	ND	18.8±1.76	13.5±0.7	16±0.28	17±0.14	ND	16±0.35	18.4±6.36	18.5±0.7	15±2.82	25±1.41
	10	ND	16.6±4.73	0	14±0.77	11±1.0	ND	13±0.14	17±5.8	15±0.35	9±0.56	21±4.24
	5	ND	9.8±2.47	0	8±0	8±0	ND	10±0.14	0	11±0.41	0	20±7
	2.5	ND	0	0	7±0	7±0.7	ND	7±0.42	0	8±0	0	13±4.24
<i>S.epidermidis 01078637</i>	20	ND	23.6±0.56	14±0	16±0.35	16±0.21	ND	15±2.8	17±1.41	17±0.28	14±0.7	33.7±0.7
	10	ND	17.8±0.35	0	13±0.7	12±1.0	ND	12±1.41	12±1.7	15±0.7	8±0	28.9±6
	5	ND	0	0	7±0	7±0	ND	9±1.41	0	10±0.42	0	21.5±6.43
	2.5	ND	0	0	7±0	7±0	ND	6±0	0±0.7	8±0	0	10±0
<i>S.epidermidis 01058847</i>	20	ND	18.9±1.41	19±1.41	15±1.41	17±0.7	ND	15±1.41	21±1.41	18±0.7	14±0.7	27.6±0.70
	10	ND	12.7±1.2	0	14±0.7	12±0.7	ND	13±0	0	14±0.7	8±0	24±1.41
	5	ND	0	0	8±0	8±0.7	ND	9±1.41	0	11±0.41	0	21.6±0.84
	2.5	ND	0	0	7±0	7±0	ND	7±0	0	7±0	0	11±0.7
Gram-negative												
<i>E.coli Atcc 25922</i>	20	ND	23.5±1.41	ND	11.2±1.31	11±1.41	ND	15±1.41	ND	16±1.41	11.5±0.70	29.5±1.41
	10	ND	21.4±0.56	ND	9.8±0.28	9±0	ND	11±1.41	ND	10±0	8.6±0.98	15.2±0.35
	5	ND	8±0.70	ND	8.5±0	6±0	ND	9±1.41	ND	8±0	0	10.5±0.35
	2.5	ND	8±0	ND	8±0	6±0	ND	9±0	ND	8±0	0	8.5±0.35
<i>K.pneumoniae* 93872</i>	20	17±0	17±1.41	19.6±1.97	10.4±0.56	10.4±0.56	12±2.82	14±1.41	23±1.41	11.7±1.2	12.6±1.69	29.4±0.84
	10	10±0	15.8±1.7	18.7±1.13	9±0	9.8±0.84	10.8±1.83	11.4±1.7	16.2±1.7	10.6±1.48	8.3±1.83	19.5±1.41
	5	8±1.41	9±0	14±0	9±0	7.2±0.14	9±1.41	9.5±0.7	12±0	0	0	13.6±1.13
	2.5	8±1.41	9±0	10.8±0.42	8±0	6.2±0	7±0	9±0	10±0.7	0	0	9.3±0.98
<i>A.baumannii 93641</i>	20	15±1.41	29±1.41	14.1±0.14	12.2±0.28	12±1.41	15±1.41	ND	30±2.82	15±1.41	11±0	ND
	10	9±0	16.8±1.41	12.1±0.14	10.2±0.28	10.3±1.83	13.1±1.27	ND	26±1.41	13.1±1.14	10±0	ND
	5	9±0	8.4±1.97	10.7±0.98	8.5±0.70	9.8±0	10.3±0.42	ND	22±1.41	10.3±0.42	9±0	ND
	2.5	8±0	8.4±1.97	8.5±0.70	8.5±0.70	0	90	ND	21±1.41	9±0	8±0	ND
Fungi												
<i>C.albicans ATCC24433</i>	20	10±0.7	32±2.5	38±1.41	31±0.7	55±4.2	ND	26±1.41	28±2.82	18±0.7	16±1.41	55±5.65
	10	0	22±1.13	28±1.41	25±1.41	40±2.32	ND	25±0.70	16±1.41	15±1.2	14±1.41	41±3.4
	5	0	18±0.7	24±0.7	21±0.7	23±1.41	ND	25±1.41	14±0.7	15±0	11±0.7	38±1.41
	2.5	0	ND	23±0.28	8±0.28	12±0.70	ND	17±0.0	13±1.12	12±0.7	10±2.82	28±0.7
<i>C.albicans CO23</i>	20	0	35±2.8	37.5±2.8	32.5±0.7	48.5±2.8	15±1.41	20±0.56	30±2.82	20±0.7	18±1.41	60±5.65
	10	0	24±1.41	27.5±1.13	25±1.41	32±0.7	13±0.28	20±1.41	18.5±0.70	15±1.2	15±0.70	42.5±3.53
	5	0	20±0.7	25±0.28	22±1.13	30±1.41	10±0.28	20±0.70	15±0	15±0	13±0.70	40±2.82
	2.5	0	ND	25±0.70	10±0.28	10±0	9±0	18±0	13±0	15±0.7	12±2.82	37.5±0.70
<i>C.albicans CO23RFLU</i>	20	15±1.41	40±1.41	Nd	30±2.8	70±5.65	20±0.70	35±0.70	27.5±2.12	15±0.12	12±1.41	50±2.82
	10	10±0.70	32.5±0	30±1.41	25±1.41	55±2.82	20±1.41	30±1.41	15±1.41	15±0.7	12±1.41	40±1.41
	5	10±1.13	25±0.70	35±4.2	20±0.7	20±0.70	18±1.41	30±2.82	14±0	15±0	10±0	35±1.41
	2.5	0	20±1.13	33±1.13	0	14±1.41	13±0.28	15±0	13±1.41	10±0	10±0	20±0.70

** *K. pneumoniae* strain the most resistant clinical isolates. Diameter of zone inhibition (mm) and deviation standard (± SD); ND: not determined; Diameter :<8 mm not sensitive ; 9-14 mm sensitive;15-19 mm very sensitive; >20 mm extremely sensitive

Table S2. MICs of TTO, CDO, EOMS, Gentamicin and Fluconazole, towards bacteria and fungi

	Essential oils			Drugs
	TTO(mg/ml)	CDO(mg/ml)	EOMS (mg/ml)	Gentamicin (µg/ml)
Gram positive				
<i>S.aureus</i> Atcc 25923	25	6.25	0.19	1
<i>S.epidermidis</i> 010078637	12.5	6.25	ND	≥64
<i>S.epidermidis</i> 01058847	6.25	6.25	ND	≥64
Gram negative				
<i>E.coli</i> Atcc 25922	1.56	12.5	3.12	0.25
<i>K.pneumoniae</i> 93872	12.5	12.5	6.25	8
<i>K. pneumoniae</i> 12028678	3.12	3.12	6.25	4
<i>K.pneumoniae</i> 161641	3.12	3.12	3.12	2
<i>K.pneumoniae</i> 161884	3.12	6.25	3.12	2
<i>A.baumannii</i> 93641	25	25	ND	≥64
Fungi	Fluconazole (µg/ml)			
<i>C. albicans</i> ATCC 244344	3.12	6.25	0.78	0.5
<i>C.albicans</i> CO23	3.12	3.12	0.78	2
<i>C. albicans</i> CO23RFLU	3.12	3.12	0.78	≥64

ND: not determined