



Air dispersed essential oils combined with standard sanitization procedures for environmental microbiota control in nosocomial hospitalization rooms



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ABSTRACT

Objective: Environmental bacterial contaminant microorganisms are an ongoing problem in hospitals. Essential oil vapours (EO) may help reducing this type of contamination. Aim of this study was to evaluate the efficacy of nebulized selected essential oils (EO) in reducing the microbial contamination in residential health care house rooms.

Design: The study was carried out in a two-story 112-bed tertiary care structure (approximately 1060 m²). Contamination in rooms and corridors was monitored for a total of $n = 5$ months, including a starting baseline sampling and one end-study point, and without combined treatment (standard sanitization alone). Contact slides were collected for microbiological analysis.

Results: Reductions in both bacterial and fungal contamination were observed between rooms cleaned using standard sanitization alone or in combination with essential oils nebulization (average 90% decrease for total count, $P < 0.01$; 90% for yeasts and molds, $P < 0.05$). Decreases of antibiotic (70%), mucolytic (100%), bronchodilators (100%), and steroidal (67%) and non-steroidal anti-inflammatory drugs (33%) prescriptions were observed, with no adverse effects on patients.

Conclusions: The selected EO composition is effective in reducing both the environmental microbial contamination and pharmaceutical drugs consumption in a nosocomial health care house. This study demonstrates that aerial EO diffusion combined with standard sanitization procedures, has great potential to reduce the microbial contamination in critical hospital environments such as hospitalization rooms.

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1. Introduction

Appropriate disinfection of environmentally contaminated hospital surfaces can eliminate or reduce the communicable diseases transmission mediated by environmental pathogens. Due to the presence of several multiresistant strains of pathogenic microbial species among which *Staphylococcus aureus*, *Clostridium difficile*, *Candida* sp., *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, the use of conventional environmental sanitization strategies (including the use of active oxygen releasers, hypochlorite, quater-

nary ammonium salts, etc.), may not show the appropriate efficacy to achieve their eradication.¹

Among the strategies alternative or complementary to those involving conventional sanitizing agents, the use of essential oils (EO) represents a new experimental frontier in terms of safety, efficacy and of patients compliance to antimicrobial treatments.

The EO antimicrobial capacity have been extensively evaluated by several in vitro studies, which established their inhibitory activity against pathogenic bacteria, fungi, molds and yeasts, using agar layer tests, as recently exhaustively reviewed by Lang and Buchbauer and by Seow et al.^{2,3}

By contrast, Inouye et al. investigated the antimicrobial activity of EOs against pathogens of the respiratory tract by exposure of the culture medium to gaseous components of EO from different botanical origins. These authors found that the growth of *Haemophilus influenzae*, *Streptococcus pyogenes*, *Streptococcus pneu-*

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Fig. 1. Hospital planimetry.

monie, *S. aureus*, and *Escherichia coli* was inhibited by a wide range of EO concentration in the range 1.56–1600 mg_{EO}/L_{air} (minimal inhibition dose, MID), depending on its botanical origin.⁴

On the same line, Hood et al. reported that the EO from *Lepidospermum petersonii* was able to inhibit in vitro the growth of the pathogenic fungus *Aspergillus fumigatus* and to eradicate the pathogen through aerial exposure in lungs of immunosuppressed and infected BALB/c mice.⁵

Recently, Laird et al. shown that a vaporized mixture of citrus EO is effective in reducing experimental contaminations from *Enterococcus* sp. and *S. aureus* on stainless surfaces,⁶ while Ali et al. showed the capacity of gas phase lemon grass EO components to suppress the mycelial growth and conidial germination of *Colletotrichum gloeosporioides*, the pathogenic agent responsible for anthracnose disease in papaya fruits.⁷

These results highlighted the potential of EO volatiles as potent preventive and therapeutically useful tool for the control of environmental pathogens.

However, to the best of our knowledge there are no literature reports on the evaluation of their efficacy in the control of relevant infective microbial agents in health care houses or hospitals.

Hence, aim of this work was to evaluate the efficacy of volatiles from selected essential oils, in combination with a standard cleaning protocol, in reducing the microbial contamination in the patient rooms of a residential health care house.

The mixture of essential oils was applied in the form of aerosol mixture with water, after nebulization using an ultrasound vapour generator to (i) avoid heating of active principle(s), and to (ii)

achieve the most uniform distribution of the terpenic antimicrobial agent(s) over the patient room surfaces. The impact of hospital rooms exposure to vaporized EO on the consumption of specific drugs classes have been also evaluated.

2. Materials and methods

2.1. Location and patients

The study was conducted at the “Don Ambrogio Cacciamatta” Foundation, a 114-bed residential health care house in Iseo, Italy. The 2-story building encompasses approximately 1060 m². More details about planimetry and patients’ number and distribution are reported in the paragraph Experimental design. Patients hospitalized in the control and treated rooms were both age and sex matched. Following the internal healthcare house procedures, at admission the subjects enrolled in the study were not affected and/or under treatment for any chronic or infective respiratory diseases, and randomly distributed between treatment and control rooms. Before entering hospitalization all patients or their legal tutors gave a written consent form.

2.2. Essential oil diffusion

The EO mixture was dispersed using commercially available ultrasound vaporizers (mod. Rondó, Gisa s.r.l, Romentino, Novara, Italy). The devices were filled daily with water (500 mL) and 100 µL of essential oils mixture (0.02% vol/vol), and let working for $t = 8$ h

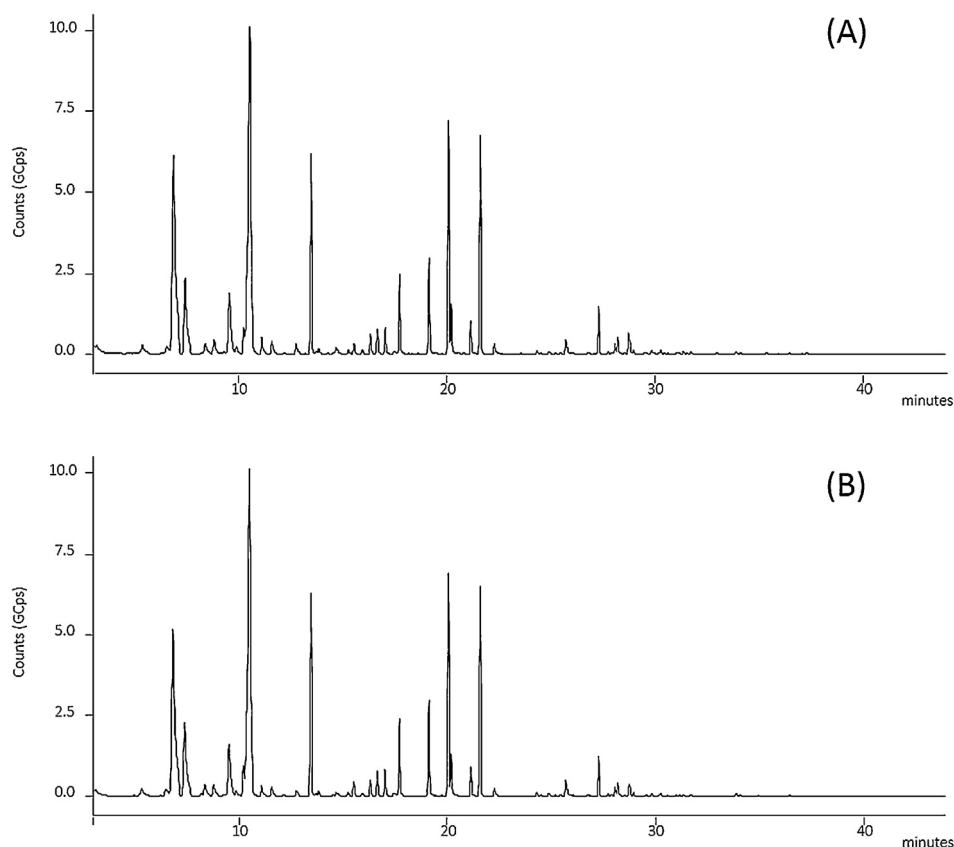


Fig. 2. GC–MS profile (A) of the original EO mixture diluted in ethylacetate and (B) of the components extracted from the nebulized EO/water aerosol. Major identified compounds: santene (5.37 min), α -pinene (6.87 min), camphene (7.43 min), β -pinene (8.40 min), β -myrcene (8.82 min), cyclofenchene (9.55 min), *p*-cymene (10.24 min), eucalyptol (10.54 min), β -ocymene (11.10 min), γ -terpinene (11.60 min), terpinolene (12.76 min), β -linalool (13.50 min), isomenthone (16.34 min), endo-borneol (16.68 min), *L*-terpinen-4-ol (17.05), α -terpineol (17.74 min), β -citronellol (19.16 min), linalyl acetate (20.10 min), *trans*-geraniol (20.22 min), citronellyl acetate (25.74 min), bornyl acetate (21.63 min), geranyl acetate (25.74 min), β -caryophyllene (27.33 min), α -muurolene (28.25 min), β -farnesene (28.76 min).

during the night, every day throughout all the study period. During daylight hours, room doors were kept open, allowing air diffusion through all the department environments (rooms and corridor).

2.3. GC–MS analysis

GC–MS analysis was performed on a Bruker Scion SQ equipped with a single quadrupole and a FactorFour Varian column (VF-5 ms, 30m; 0.25 mm i.d., film thickness 0.25 μ m). The oven temperature was initially set at 60 °C (hold time 3 min), with a gradient from 60 to 120 °C (8.0 °C/min, hold 1 min), then from 120 to 280 °C (4 °C/min, hold 1.5 min) and from 280 to 330 °C (10 °C/min, hold 2 min). The injector temperature was set to 250 °C, and column flow to 1.00 mL/min. Carrier gas was helium 5.5; ionization energy 70 eV; the split/splitless ratio was set to 1:30 after 40 s. Data acquisition was performed in full scan (m/z 50–1200) in EI from 3 to 60 min. EOs were diluted 1:1000 in ethylacetate and injection volume was 1 μ L.

Peaks were identified by comparing the retention times with those of authentic standard MS fragmentation patterns, or by matching with those stored in NIST (2011, vers. 2.0) mass spectral database library. The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as the mean of three injections for each oil, without correction factors.

For analysis, (A) 1 μ L of EO mixture was diluted to 1 mL with dichloromethane and 1 μ L of this solution injected into the gaschromatograph. (B) To evaluate the aerosol composition, part of the produced dispersion was collected under gentle reduced

pressure using an in house built device, bubbled into 10 mL of dichloromethane, and this step repeated until a sufficient amount of EO components was collected. This mixture was dehydrated by addition of anhydrous sodium sulphate, filtered on paper and 1 mL injected in the same conditions of (A).

2.4. Biological samples collection and analysis

Microbial counts were done using Hycheck™ “Total Count” and Hycheck™ “Yeast and Mold” contact slides (Beckton Dickinson, USA). Counts were done according to the manufacturer instructions.

Briefly, each slide was put in contact with the selected surfaces, immediately transferred at $T=4^{\circ}\text{C}$ to the incubator where they were kept for $t=120$ h for yeast and mold assay and $t=24$ h for total microbial count, both at $T=37^{\circ}\text{C}$, and the number of developed colonies visually counted and recorded. This standardized and conventional protocol allows the detection of the most common environmental microorganisms.

Total microbial assay selectivity: *Enterococcus faecalis* (Gram-positive), *E. coli* (Gram-negative), *Proteus vulgaris* (Gram-negative), *Salmonella typhimurium*, *S. aureus* (Gram-positive). Yeasts and molds assay selectivity: *Candida albicans*, *Aspergillus niger*, *Saccharomyces cerevisiae*. Measurements were done in triplicate and results expressed as mean $\pm$ standard deviation (colonies/cm²)

2.5. Sanitization

Anionic surfactants for tableware washing; anionic surfactants, nonionic and amphoteric surfactants, polyaminic resin,

antifermentation agents for hands washing (Gruppo Dac, Brescia, Italy). Environmental cleaning: two different products containing (i) phosphoric acid (15–25%), ethoxylated alkylalcohol (0–5%) non-ionic surfactants (5%) (Kaltol, Italchim, Bologna, Italy) and (ii) non-ionic surfactants (5–15%), cationic surfactants, 2-butoxyethanol after dilution of 50 g in 10 L of water (PSC Pavimenti, C.C.I.A.A., Bergamo, Italy).

The EO mixture composition was as follows: *Lavandula angustifolia* 24% (w/w), *Melaleuca cajuputi* 24% (w/w) (Cajeput), *Abies siberica* 20% (w/w), *Myrtus communis* 20% (w/w) and *Pelargonium graveolens* (Geranium bourbon) 12% (w/w). According to the Scientific Committee on Consumer Safety (SCSS) of the European Commission (EC) (Opinion on Fragrance allergens in cosmetic products, available online at http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/scss_o_102.pdf) the level of skin contact allergenic potential to humans for the EO of *L. angustifolia* (CAS number: 84776-65-8) and *P. graveolens* (CAS number 90082-51-2 and 8000-46-2) is: ++.

2.6. Experimental design

Nebulizers were placed in $n=2$ of the $n=8$ rooms located at the first floor of the residential health care house department. The second floor, with identical planimetry, was used as matching control environment and subjected to standard disinfection and cleaning procedures only.

Sampling was carried out every $n=30$ days for $n=5$ months (T_0 = baseline, T_1 = 30th day, T_2 = 60th day, T_3 = 90th day, T_4 = 120th day and T_5 = 150) in $n=5$ different random points within the same room (tables and cabinets surfaces) and in corridors (handrails). To evaluate the baseline bacterial charge, and to exclude potential, non-treatment derived antimicrobial effects respectively, at T_0 and during the last 30 days of monitoring (from T_4 to T_5) the vaporizers were not activated.

The EO mixture was diffused as above described, without any modification of the standard daily cleaning protocol comparing to that used in the control sector.

2.7. Statistics

Depending on type of comparison, statistical differences were evaluated by one-way ANOVA followed by T -tests on pairwise comparisons with the least square difference (LSD) post hoc adjustment for multiple comparisons, or by student's T -test for comparison of groups alone. Differences were considered significant when $P < 0.05$. Computations were performed using the R-commander GUI for R (v. 3.1.3).⁸

3. Results

3.1. Location and patients

The present study was conducted in the hospitalization department of a residential health care house. Control and treatment rooms were those placed at its first floor (Fig. 1).

Each set of $n=8$ two beds rooms were connected to the same corridor, and aerated independently from the others, and the diffusers placed in $n=2$ rooms.

All rooms guested the same patients (total number $n=32$) for the entire duration of the study, from November 2014 to March 2014.

3.2. Original and nebulized EO mixture composition

The GC–MS chromatograms reported in Fig. 2 show that the profile of the EO mixture components recovered from the nebu-

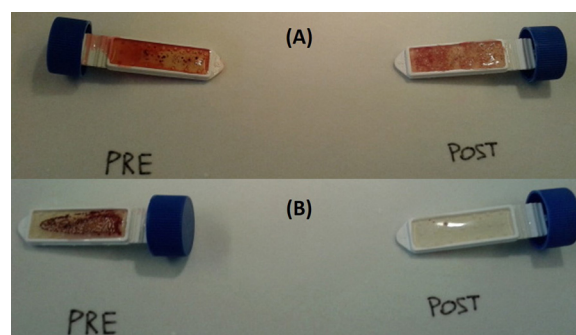


Fig. 3. Representative visual representation of bacterial colonies development on contact slides after exposure to Table surfaces, before (left) and after (right) sanitization with conventional disinfectants (a) or with conventional disinfectants in combination with vaporized EO (b).

lized EO (upper panel) was almost overlapping that of the same mixture directly diluted in solvent (lower panel). These results demonstrated that the ultrasound nebulization mode allowed incorporating the EO mixture components into the aerosol independently from their individual boiling points and polarity.

According to the SCSS of the EC, the skin contact allergens identified in EO mixture were almost all of weak concern (compound (CAS number) allergenic potential): α -pinene (CAS number: 80-56-8) ++, β -pinene (CAS number: 127-91-3) ++, terpinolene (CAS number: 586-62-9) +, camphor (CAS number: 76-22-2/464-49-3) + r.t., β -citronellol (CAS number: 106-22-9/1117-61-9/7540-51-4) ++, β -linalool (CAS number: 78-70-6) ++ (non-ox.), linalyl acetate (CAS number: 115-95-7) + (non-ox.) ++ (ox.), caryophyllene oxide (CAS number: 87-44-5) Non-ox.: +, ox.: + (r.t.: rarely tested; non-ox: non-oxidized, ox: oxidized).

Only the allergenic potential of *trans*-geraniol (CAS number: 106-24-1) is classified as of special concern (+++).

3.3. Total bacterial, fungi and yeast counts at baseline and postdisinfection

In Fig. 3 a representative photographic representation of the development of bacterial colonies on Hycheck™ “Total Count” contact slides is shown.

Already at visual inspection, it can be appreciated that the sanitization protocol involving conventional disinfectants alone, had no effect on the presence of bacterial colonies (Fig. 3a). Conversely, a dramatic decrease in bacterial growth was evident when the conventional disinfectants were used in combination with vaporized EO (Fig. 3b).

The baseline and postdisinfection total bacterial, fungi and yeast counts observed in the selected sampling sites (in room tables, cabinets and handrails in corridors), monitored before treatment beginning (day 0), during sample treatment (day 30th, 60th, 90th and 120th) and after one month from treatment suspension (from day 121th to day 150th) are reported in Fig. 4.

The highest bacterial contaminations were found on Table surfaces (tables: 100–200 colonies/cm²; cabinets: 10–30 colonies/cm²; handrail: 30–80 colonies/cm²).

Compared to rooms subjected to standard disinfection only, a stable reduction of microbial charge induced by the exposure to the vaporized EOS mixture was observed in the in room sampling sites until the fourth month of treatment (tables > 90%, $P < 0.001$; cabinets surfaces > 75%, $P < 0.001$, Fig. 4).

One month after suspension of the EO treatment, the values of microbial contamination in the treated rooms increased again to overlap those measured in control rooms, confirming that the

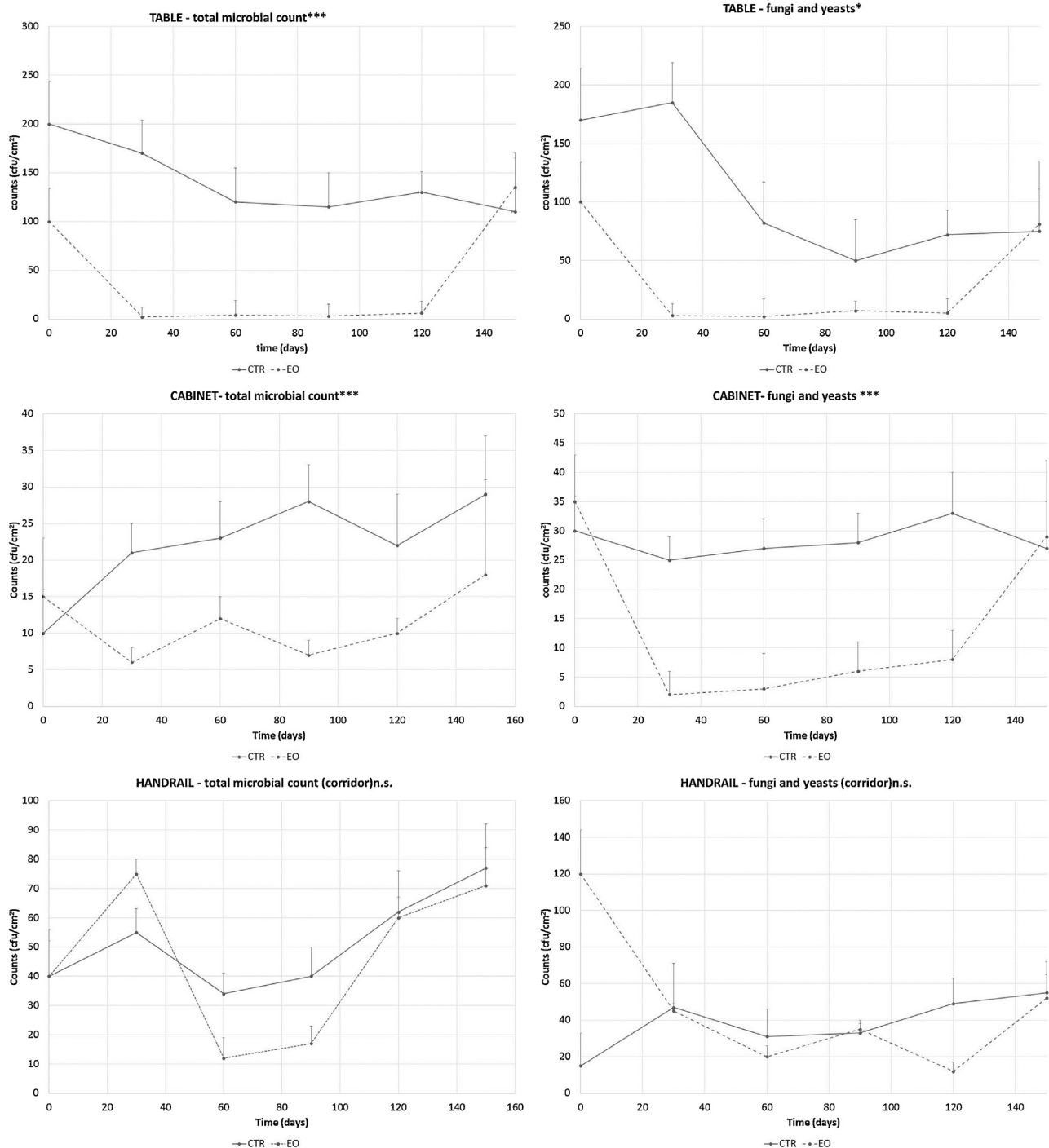


Fig. 4. Time dependent course of the microbial environmental populations observed throughout the study in control (CTR, continuous line) and treated (EO, dotted line) rooms. EO vaporization was suspended at the 120th day. Asterisks indicate the level of significance of differences between counts overall means in control (CTR) and EO treatment (T) groups (values monitored from 30th to 120th day). * $P < 0.05$, *** $P < 0.005$, n.s. not significant (Student's T-test).

observed antibacterial action was not due to external environmental factor.

By contrast, the bacterial population on the handrails of both treated and control corridors did not show any significant difference in the mean bacterial counts measured throughout the study. This indicated that, very likely, the lower concentration of the EO antimicrobial agents in corridor due to dilution in air reduced their capacity to inhibit the microbial growth.

3.4. Drugs for nosocomial related pathologies

In Table 1 are reported the data regarding medical prescriptions, and the relative total days of treatment, issued to treat the pathologies (mainly infections and airways/respiratory problems) arisen in the patients during their hospitalization ($n = 120$ days).

As expected, antibiotics were the most prescribed drug class, with a total number of $n = 26$ prescriptions generating a total of 102 days of pharmacological treatment. Antibiotics were followed by mucolytic drugs ($n = 14$ prescriptions, 84 days of treatment),

Table 1

Total number of pharmaceutical drugs prescriptions (Ps) issued for patients in control (CTR) and treated (EO) rooms throughout the study period. Antibiotics: sulphamethoxazol + trimetoprim (Bactrim®), amoxicillin, levofloxacin, ceftriaxone; mucolytics: acetylcysteine, bromexin; Bronchodilator: salmeterol xinafoate; NSAIDs: paracetamol, ketoprofen, ibuprofen; corticosteroids: fluticasone, betamethasone, desametasone.

Drug class	Group					
	CTR		EO treated		Δ (% EO vs CTR)	
	Ps	Days	Ps	Days	Ps	Days
Antibiotics	20	102	6	25	–70	–76
Mucolytics	14	84	0	0	–100	–100
Bronchodilators	7	52	0	0	–100	–100
NSAIDs	3	7	2	4	–33	–43
Corticosteroids	6	34	2	10	–77	–70
Total	50	275	10	39	–80	–86

bronchodilators ($n = 7$ prescriptions, 52 days of treatment), NSAIDs ($n = 3$ prescriptions, 7 days of treatment) and corticosteroids ($n = 6$ prescriptions, 3 days of treatment).

In all cases, a reduction in both number of prescriptions and days of treatment was observed for the patients in the EO treated branch of the department with an overall total decrease of 80% and 86% respectively.

4. Discussion

In this study, we evaluated the disinfectant efficacy of an EO mixture against microbial organisms present in the hospitalization department of a health care house.

For its dispersion into the room environment, we choose an ultrasound device due to its ability to produce a uniform, constant and more reproducible water/EO aerosol comparing to conventional spraying systems. The results reported in this study clearly showed that during the ultrasound mediated dispersion of the EO mixture, each of its component was incorporated in the aerosol according to its abundance only, and not depending from its polarity or boiling point, allowing a continuous administration of aerosol with constant composition. This situation was completely different from that expectable in case of the thermally induced vaporization, which leads to the gasification of more volatile components first, and then of those endowed with increasing boiling point (more polar and/or with higher molecular weight).

As sampling tool, we choose contact slides. These devices have been previously applied to monitor the contamination from methicillin and vancomycin-resistant microbial agents in hospital environments.⁹

Other previous reported applications of contact slides regarded, for example, the monitor of environmental microbial contamination in the International Space Station, or the estimation of the number of cultivable aerobic bacteria and gram-negative enteric bacilli in poultry feathers.^{10,11}

Among the environmental Gram positive and Gram negative bacteria detectable using the contact slides, there are several species involved in communicable diseases occurring in hospitals and health care hoses, in particular in those treating elder patients. *E. faecalis* has been associated to nosocomial infections and identified in teeth root during canal treatment, *E. coli* can be present due to fecal contamination, *P. vulgaris* from fecal contamination is a common cause of wound and urinary tract infections especially during long-term hospitalization, while *S. aureus* is a diffused cause of respiratory tract and skin infections.

S. typhimurium, although not generally considered as an environmental contaminant, has been identified as the cause of numerous *Salmonella* outbreaks in nosocomial hospitals.¹²

Regarding yeasts and molds assay selectivity among the detectable species of interest there are *C. albicans* (known to induce oral and urinary infections), *A. niger* (the cause of aspergillosis), and *S. cerevisiae* (sometimes involved in ulcerative colitis).

Our results show an evident correlation between infection events and inflammatory problems in patients, with the presence of environmental microorganisms.

The aerial exposure of room surfaces to the EO mixture components induced a dramatic reduction (in some cases near to full eradication) of their microbial contamination and, by consequence, a reduction of infection transmissions with concomitant decrease of drugs administration for their treatment.

The same reduction in drugs consumption was observed for all the patients dwelling the rooms in the sector treated with EO, and not only for those dwelling rooms with EO diffusers. This finding strongly suggested that the air dispersed EO can exert a direct protective effect on the patients' respiratory tract, not only mediated by the reduction of the bacterial populations.

The protective efficacy in patients dwelling rooms adjacent to those in which diffusers were working, compared to the lack of antibacterial action in the correspondent corridors, can be explained in terms of patient exposure to the cumulative dose of residual, air diluted EO absorbed by the patients through the physiological respiration process.

These results indicate that the antimicrobial effect can be transferred to distant rooms due to the air circulation occurring during the daily hospital activities (during which rooms doors remain open and patients and staff move within and between the different sectors), in spite of the loss of EO disinfection efficacy on contact surfaces due their dilution by environmental air circulation.

However, the clarification of this finding will require further future experimental work, as the microbial monitoring was conducted only in rooms where the diffusers were placed.

No adverse effects from exposure to the vaporized EO mixture were reported throughout all the study period. This observation was in accordance to the low allergenic potential of its individual components, of which only α -pinene (0.003%, ++), and linalyl acetate (0.002, +) exceeded the 0.001%, value set as maximal concentration for potential allergens in leave on cosmetic products. However, it should be taken into account that the diffusion of these volatile substances leads to maximal concentrations in air that can be easily calculated in the sub-part per billion range, while the actual concentrations are expected to be even lower as the mixture is slowly released over the $n = 8$ h of diffusers activation.

The main limitation of the present study due to its experimental design, is that no univocal mechanism of antimicrobial action can be proposed for the marked inhibitory effect of the EO mixture used, so that further work is needed to identify the mechanism(s) of action of its components.

However, the increasing number of detailed studies appearing in the recent scientific literature in the last years, help better understand the multifaceted activity of the numerous components present in EO with variable composition and concentrations.

Previous studies¹³ showed that the EO from *Helichrysum italicum* significantly reduces the multidrug resistance of *Enterobacter aerogenes*, *E. coli*, *P. aeruginosa*, and *A. baumannii*.¹³ Interestingly, the authors also found that combinations of the two most active fractions of the EO, with each other or with phenylalanine arginine β -naphthylamide, yielded synergistic activity. Isolated geraniol, one of the EO components, increased the efficacy of β -lactams, quinolones, and chloramphenicol.¹³

Rao et al., showed that carvacrol, a component of EO from several plant species, induces a robust transcriptional response in *S. cerevisiae*, closely resembling that of calcium stress and with genetic responses highly similar to those elicited by rapamycin.¹⁴

In addition, recently reported results demonstrated the capacity of carvacrol to generate stress in the endoplasmic reticulum of *C. albicans*. These key results suggested that EO components may exert antifungal activity through activation of specific signalling pathways downstream of cellular interaction rather than nonspecific modification of membrane properties, this latter the way EO were previously commonly believed to act.¹⁵

Finally, Malic et al., investigating the antimicrobial effects of EO against bacteria associated with urinary catheter infection and cultivated in planktonical form or as biofilms, found that biofilms were up to 8-fold more tolerant of the test agents.¹⁶ One component of the EO, eugenol, exhibited higher antimicrobial effects against both planktonic cells and biofilms comparing to terpinene, tea tree oil, and cineole.

In conclusion, the results reported in the present study highlight the utility of bioactive volatiles from essential oils in controlling and reducing the hospital contamination from environmental microbes, contributing to reduce infective events, and of related drugs administrations, in patients subjected to long-term hospitalisation periods and for this reason always at risk to enter in contact with resistant pathogens.

Conflict of interest

None declared.

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