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Cellular effects induced by *Inula graveolens* and *Santolina corsica* essential oils on *Staphylococcus aureus*

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Abstract This study was conducted to evaluate the antibacterial activity of *Inula graveolens* and *Santolina corsica* essential oils on *Staphylococcus aureus* and investigate their effects at the cellular level. The mode of inhibition of both essential oils against *S. aureus* ATCC 6538P (CIP 53.156) was assessed by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The effects of time and treatment dose on cell viability were determined by time-kill and bacteriolysis assays. Marked structural changes were observed by transmission electron microscopy (TEM). A bactericidal mode of inhibition was established for both essential oils, which rapidly reduced the cell viability of *S. aureus* at their MIC (5 mg·ml⁻¹). No lysis occurred after treatments with the MIC and eight times the MIC of each essential oil. Invaginations of the plasmic membrane with thickenings of the cell wall as well as an aggregation of the cytoplasmic contents were observed in *S. aureus* cells treated with the MIC of both essential oils. These results suggest that the cytoplasmic membrane and the cell wall are

involved in the toxic action of *Inula graveolens* and *Santolina corsica* essential oils.

Introduction

Staphylococcus aureus is a commensal organism commonly cited as a major hospital-acquired pathogen and the leading cause of nosocomial infections [1]. The spectacular adaptive capacity of this Gram-positive pathogen resulted in emergence and worldwide spread of lineages that acquired resistance to the majority of available antimicrobial agents. Among some of the most problematic clinically relevant strains at present, the methicillin resistant *S. aureus* (MRSA) ranks as one of the most difficult bacteria to treat in patients and eradicate from hospital environments. Recent reports of *S. aureus* isolates with intermediate or complete resistance to vancomycin portend a chemotherapeutic era in which effective bactericidal antibiotics against this organism may no longer be readily available [2–5]. The search for new agents has therefore become indispensable.

Essential oils are complex natural mixtures of volatile secondary metabolites isolated from plants by hydrodistillation. They are known to possess numerous biological properties including antioxidative, antitumoral, anti-inflammatory, antifungal and antibacterial activities [6–13]. Although in vitro antibacterial activity and in vivo efficacy of essential oils have been reported [14], less is known about their mechanism of action. Nevertheless, potential sites of their antibacterial action have been defined, e.g. they can act by damaging the cell wall, the plasma membrane, the membrane proteins or decreasing the proton motive force. They can also cause an aggregation of the cytoplasm or a leakage of the ions and the cell contents [13].

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In an ongoing project, which aims to identify new antibacterials from plants growing in Corsica, effectiveness of various essential oils and their main components has been demonstrated [15–17]. The specificity of their composition, mainly due to their acute chemical variability, seems to explain their high antibacterial activity [15–19]. The objective of this study was to go further into the effects of Corsican essential oils on bacterial cells. In this context, we evaluated the antibacterial activity of *Inula graveolens* and *Santolina corsica* essential oils against *S. aureus* and provided insights into their primary mode of action.

Material and methods

Bacterial strain and growth conditions

The test strain used in this work was *Staphylococcus aureus* ATCC 6538P (CIP 53.156). *S. aureus* ATCC 6538P was shown to be the most appropriate test microorganism because of its susceptibility to all β -lactams antibiotics, including methicillin. The cultures of *S. aureus* ATCC 6538P were obtained from the "Institut Pasteur Collection" (Paris, France), preserved frozen in cryovials at -80°C and routinely grown at 37°C on Mueller-Hinton agar (MHA, bioMérieux).

Essential oils

Inula graveolens essential oil was purchased at "Huiles essentielles et Hydrolats de Corse" (Mandriolu, Corsica). The main components quantified by GC (RI), GC-MS and ^{13}C NMR were as previously described [19]: bornyl acetate (43.3%), borneol (26.2%) and camphene (7%). *Santolina corsica* essential oil was isolated from the aerial parts of plants by hydrodistillation. The main components quantified by GC (RI), GC-MS and ^{13}C NMR were as previously described [15]: myrcene (34.6%), santolina triene (13.5%) β -phellandrene (11.7%) and lylratol (6.1%).

Susceptibility testing

The MIC and MBC assays were performed by standard broth dilution methods [20, 21]. In brief, serial twofold dilutions of each essential oil in Mueller-Hinton broth (MHB, Oxoid) were prepared in sterilized test tubes over the range 0.03–8% (v/v). To enhance oil distribution, Tween 80 detergent (Polyoxyethylene sorbitan monofluarate, Sigma) was included in all assays at a final concentration of 0.01% (v/v). The inoculum to be tested (13 μL , 10^8 CFU/ml) was added to each of the test tubes, which were incubated at 37°C for 24 h and finally centrifuged at 5000 g for 5 min at 20°C . The MIC was

then determined from the first test tube devoid of bacterial growth. All determinations were performed in triplicate and a control consisting of MHB with 0.01% (v/v) Tween 80 was included. After determination of the MIC, the samples showing no bacterial growth were streaked on MHA agar plates, which were incubated for 24 h at 37°C and finally examined for 99.9% killing.

Time-kill studies

Time-kill procedures were performed according to the method described by Klepser et al. [22] and modified by Viljoen et al. [23]. Activities of essential oils against *S. aureus* were evaluated by measuring the reduction in the number of CFU per milliliter over 2 h. An initial inoculum of 5×10^6 CFU·ml $^{-1}$ was prepared and 1 ml was added to 9 mL of MHB containing either the essential oils and Tween 80 (tests) or Tween 80 only (control). Essential oils were used at a final concentration equal to their MIC. Tween 80 was added to both tests and control at a final concentration of 0.01% (v/v). The test tubes were incubated with agitation at 37°C and samples (100 μl), taken in duplicate at 0, 10, 30, 60, 90 and 120 min, were serially 10-fold diluted, plated onto MHA and the total viable counts were determined after overnight incubation at 37°C . The limit of quantification by this method is 10^2 CFU·ml $^{-1}$.

Bacteriolysis

The bacteriolysis assays were carried out according to the standard method described by Carson et al. [24]. A bacterial suspension was prepared by inoculating two colonies of *S. aureus* from overnight cultures on blood agar into 400 ml of MHB, which was incubated at 37°C for 18 h with shaking. After incubation, the bacteria were separated from the growth medium by centrifugation at 10,000 g for 12 min at 4°C , washed twice with phosphate-buffered saline (PBS, pH 7.4), and resuspended in PBS supplemented with 0.01% Tween 80 (PBS-T, v/v). The bacterial suspension was adjusted so that the optical density at 620 nm (OD_{620}) of a 1 in 100 dilution was 0.310 ($\sim 3 \times 10^{10}$ CFU·ml $^{-1}$). Essential oils were added to the bacterial suspension at concentrations equivalent to the MIC and eight times the MIC at a ratio of 1:9. PBS-T was added to the control suspension. The suspensions obtained were mixed for 20 s with a Vortex mixer. Samples (10 μl) were taken in duplicate at 0, 0.5, 30, 60, 90 and 120 min, serially 10-fold diluted, and the OD_{620} was measured immediately (Analytik Jena Specord 205). Each test was performed on three independent experiments. The results were expressed as a ratio of the OD_{620} at each time point versus the OD_{620} at 0 min (in percent).

Transmission Electron Microscopy (TEM)

A suspension of *S. aureus* in exponential phase of growth was prepared by inoculating and incubating 80 ml of MHB at 37°C for 24 h with shaking. The bacterial suspension was adjusted so that the optical density at 620 nm (OD₆₂₀) of a 1 in 100 dilution in MHB was 0.200 (10⁷CFU·ml⁻¹). *S. aureus* cells were treated with the MIC of each essential oil for 5 min. Control stood for 5 min in MHB-Tween 80 (0.1 %, v/v). After centrifugation at 3,000 rpm for 10 min, the pellets were first fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature and then post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature. The post-fixed microbial pellets were processed in graded ethyl alcohol, propylene oxide, Spurr resin and cured for 24 h at 45°C. Ultrathin sections were stained with uranyl acetate followed by lead citrate and were examined with a transmission electron microscope (HITACHI H-600 TEM) at an accelerating voltage of 75 kV.

Results

The antibacterial activities of *I. graveolens* and *S. corsica* essential oils on *S. aureus* are presented in Table 1. The MIC values of both essential oils are 5 mg·ml⁻¹. The MBCs of *I. graveolens* and *S. corsica* essential oils are, respectively, 10 mg·ml⁻¹ and 8 mg·ml⁻¹. The MBC/MIC ratios of both essential oils are lower than 4.

Killing profiles of the two essential oils against *S. aureus* are presented in Fig. 1. Preliminary treatments of *S. aureus* with *I. graveolens* and *S. corsica* essential oils, used at a concentration equivalent to their MIC (5 mg·ml⁻¹), have shown that the bactericidal end-point (99.9% or ≥ 3log₁₀ of inhibition [25]) was obtained after 2 h and 4 h, respectively (data not shown). The 2-h time-kill curves show a detailed progression of the bactericidal activities. The *I. graveolens* essential oil at the MIC acts at once. During the first 10 min, the number of CFUs fell by 1log₁₀ and the detection threshold (10²CFU·ml⁻¹) is reached in 1 h. The bactericidal effect of *S. corsica* essential oil at the MIC is

Table 1 MICs, MBCs and MBC/MIC ratios of *I. graveolens* and *S. corsica* essential oils on *S. aureus* strain

Essential oils	MIC (mg·ml ⁻¹)	MBC (mg·ml ⁻¹)	MBC / MIC
<i>Inula graveolens</i>	5	10	2
<i>Santolina corsica</i>	5	8	1.6

MIC minimum inhibitory concentration, MBC minimum bactericidal concentration

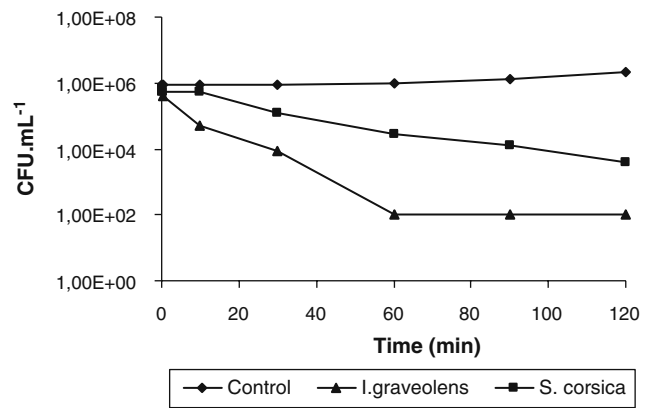


Fig. 1 Effects of *S. corsica* and *I. graveolens* essential oils (MIC, 5 mg·ml⁻¹) on growth of *S. aureus* over 2 hours

slower, i.e. the number of CFUs slows down gradually by 2log₁₀ in 120 min.

S. aureus cells untreated by essential oils correspond to the negative control of bacteriolysis. The absorbance at 620 nm (OD₆₂₀) of negative control does not vary significantly (mean OD₆₂₀ after 120 min corresponds to 93% of the initial absorbance). Figure 2 shows the OD₆₂₀ of *S. aureus* cells treated with *I. graveolens* and *S. corsica* essential oils. Treatment with *I. graveolens* essential oil at

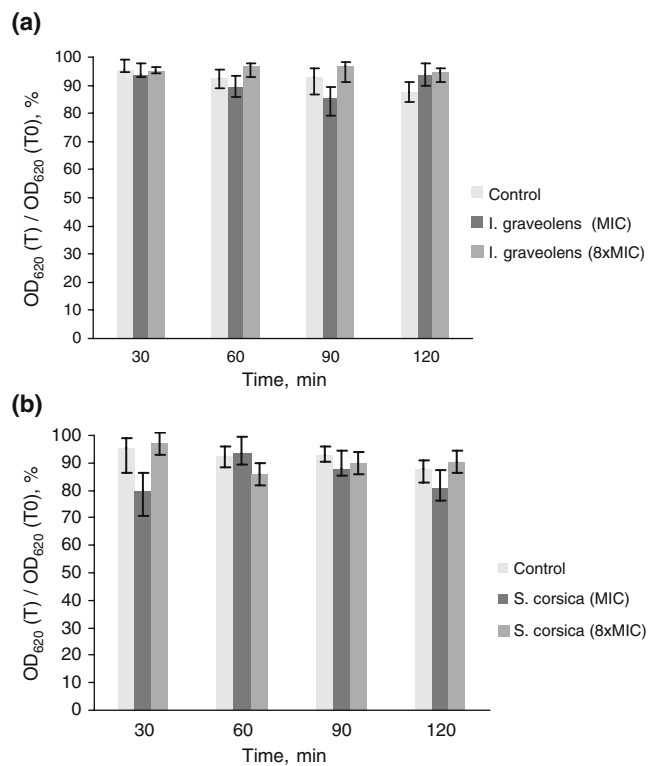


Fig. 2 Variations in time of the absorbance’s ratio at 620 nm of *S. aureus* control cells and after treatment with the MIC and eight times the MIC (8 MIC) of *I. graveolens* (a) and *S. corsica* (b) essential oils. Values given as means ± SEs for three replicates

the MIC and at eight times the MIC had no effect (OD_{620} range over 120 min, 91–97% of the original). Treatment with *S. corsica* essential oil at the MIC and at eight times the MIC slightly reduced the OD_{620} (OD_{620} range over 120 min, 86–91% of the original).

Transmission electron micrographies of *S. aureus* cells untreated (control) and treated with *I. graveolens* and *S. corsica* essential oils at their MIC are presented in Fig. 3.

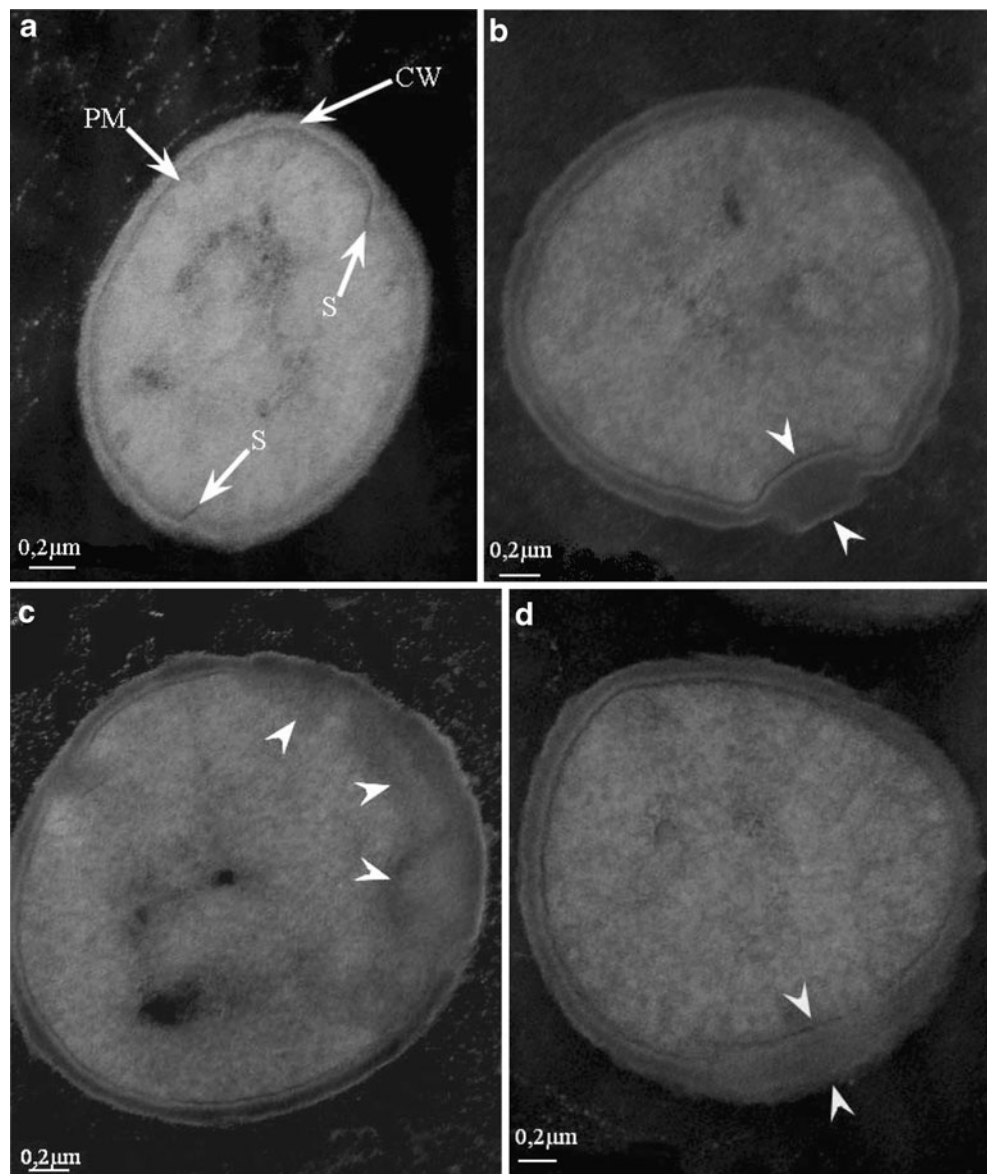
The cell wall and the plasmic membrane of *S. aureus* control cells are regular. The cytoplasmic contents are homogeneous. The start of cellular division is also visible in Fig. 3a where the septum of division begins to form. The plasmic membrane of *S. aureus* cells treated with *I. graveolens* essential oil appears to have one invagination (Fig. 3b) at the level of which the cell wall presents an unusual thickening. The same modifications are observed in

S. aureus cells treated with *S. corsica* essential oil (Fig. 3d). Under the action of both essential oils, cytoplasm of some cells also seem to be less dense and altered. In Fig. 3c, the cytoplasm seems to have lost its even distribution and intracellular materials are aggregated near the cell wall.

Discussion

In this study, the in vitro antibacterial activity of *I. graveolens* and *S. corsica* essential oils on *S. aureus* are reported. MIC values are close to MBC values, indicating that both essential oils display a bactericidal activity [26]. The oils considerably reduce the viability of *S. aureus* cells at their MIC and produce a lethal effect at concentrations higher than their MIC. Their bactericidal activity depends

Fig. 3 Transmission electron micrographies of *S. aureus* cells stained with uranyl acetate and lead citrate. **a** Control. **b** and **c** *S. aureus* cells treated with *I. graveolens* essential oil at the MIC. **d** *S. aureus* cells treated with *S. corsica* essential oil at the MIC. *PM* plasmic membrane, *CW* cell wall, *S* septum



however on the time of exposure. *I. graveolens* oil decreases cell viability within 2 h while 4 h are required for *S. corsica* oil.

Plants of *Inula* genus belong to the family *Compositae* [27] and are widely distributed in the Mediterranean area. The chemical composition of various *Inula* species, including *I. viscosa* and *I. graveolens*, has been described by Bohlmann et al. [28]. The essential oil of *I. graveolens*, used in this study, is constituted by a complex mixture of volatile molecules dominated by two oxygenated so that the activity of the whole oil is related to the presence of these two compounds, recognized as antibacterial agents [21, 28, 30]. Alcohols are known to possess bactericidal rather than bacteriostatic activity [29]. So, the high amount of borneol could explain the bactericidal effect induced by the whole oil on *S. aureus* cells. A bacteriostatic activity has however been mentioned by Debat et al. [31] for *S. aureus* cells treated with various *I. graveolens* extracts. In another study, Caldes et al. [32] reported that *I. graveolens* extracts, obtained by extraction with hot and cold water respectively and then lyophilization, were inactive against *S. aureus* and *S. faecium* cells. The process of extraction of plant products influences their chemical composition and this may also influence their antibacterial activity [13]. This could be an explanation for the different activities observed between the different extracts and the essential oil of *I. graveolens*.

The genus *Santolina* is represented by more than ten species in the Mediterranean area. *Santolina corsica* is an endemic species to Corsica and Sardinia [33]. The composition of essential oil from various species of *Santolina* has been widely studied [15]. The essential oil of *S. Corsica*, used in this work, is dominated by three monoterpenes hydrocarbons: myrcene (34.6%), santolina triene (13.5%) and β -phellandrene (11.7%). Since hydrocarbons are usually weakly active [13], the involvement of less abundant constituents in the antibacterial effect of the whole oil should be considered. Indeed, minor constituents might be involved in some type of antibacterial synergism with other active components of essential oils as evident by the work of Marino et al. [34].

Some antimicrobial agents induce severe membrane damage and provoke whole cell lysis [35–38] and this has been previously reported for oregano, rosewood and thyme essential oils [39]. However, the main components of these essential oils, including carvacrol, citronellol, geraniol and thymol [40], are not found in *I. graveolens* and *S. corsica* essential oils. Also, cell death induced by both essential oils does not seem to be caused by a lytic effect. This is also supported by our electron micrographies on which *S. aureus* cells remain unlysed. The structure of the plasmic membrane is however altered. Cells treated with both oils exhibit abnormal invaginations of their membrane. Carson et al. [24] reported similar effects for *S. aureus* cells treated

with *Melaleuca alternifolia* essential oil (tea tree oil [TTO]) and/or its main constituents. These agents do not provoke whole cell lysis but compromise the structural integrity of the plasmic membrane and induce a loss of the cytoplasmic contents [24, 41].

Cytoplasmic changes also occur under the action of *I. graveolens* and *S. corsica* essential oils. The cytoplasm of some cells is less dense and shows clumping of intracellular contents. Areas with aggregated material are observed close to the cell wall, which loses its uniformity and tends to become rough. Unusual thickenings are observed in the wall of *S. aureus* treated with both oils. This thickened appearance is more pronounced near the membrane invaginations. Similar damage has been previously observed on the wall of *Listeria innocua* cells treated with the antibacterial polypeptide nisin [42]. Calderón-Miranda et al. [43] also reported that *L. innocua* cells, exposed to 32 pulses of electric fields intensity of 30 kV/cm, exhibit an increase of the cell wall roughness. No disruption in either the cell wall or the plasmic membrane of *L. innocua* occurs. However, the cytoplasm is clumped and not evenly distributed as observed in our work.

The mechanism of essential oil action towards microorganisms is complex and not yet fully explained. The available literature data show that the primary site of the toxic action of essential oils is generally the plasmic membrane. This is directly related to the hydrophobicity of the terpenoid constituents of essential oils, which enables them to partition into the bacterial lipid bilayer, disturbing its structure and increasing its permeability to protons, ions and other cell constituents [14, 44–48]. In addition to membrane alterations, terpenoids might also cross the cell membrane, thus penetrating in the cell and interacting with critical intracellular sites [49]. They might have several invasive targets which could lead to the inhibition of bacterial pathogens such as *S. aureus*.

Taken together, our findings suggest that the bactericidal activity of *I. graveolens* and *S. corsica* essential oils resides in their ability to detrimentally affect the integrity of the plasmic membrane and the cell wall of *S. aureus*. Considering the multiple alterations produced and the large number of chemical constituents of essential oils, it is most likely that the effects produced by *I. graveolens* and *S. corsica* oils are not attributable to one specific mechanism. For this reason, an in-depth study is required to fully understand the way by which the two oils and their active constituents interact with the bacterial membrane and the consequences they bring out at the cellular level.

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