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Primary Mode of Action of *Cistus ladaniferus* L. Essential Oil Active Fractions on *Staphylococcus aureus* Strain

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Abstract

The purpose of this study was to investigate the primary mode of action of *Cistus ladaniferus* essential oil active fractions on *Staphylococcus aureus* strain ATCC6538P (CIP 53.156). The mode of inhibition of the active fractions was assessed by determining the minimum inhibitory concentration (MIC). The effects of time on cell integrity were determined by time-kill, bacteriolysis and loss of 260 and 280-nm-absorbing material assays. Measurement of intra- and extracellular ATP was used to evaluate the energy remaining in the cells after treatment. A bacteriostatic and a bactericidal mode of inhibition were established respectively for acetate and alcohol fractions at their MIC. No intracellular material leakage and no lysis occurred after treatments with these fractions. In both cases, we observed a decrease of the ATP level within *S. aureus* cells whilst there was no proportional increase outside the cells. However, the effects induced by alcohols are more pronounced than those provoked by acetates. Indeed, marked structural changes were observed by transmission electron microscopy (TEM). The septal material of cells undergoing division became thicker and stained more lightly. The proportion of septa is also markedly increased and defective with respect to placement. These observations suggest a blocking in cell division, probably caused by the inhibition of ATPase or a disturbance in proton motive force by the hydrophobic molecules viridiflorol and ledol, mainly present in alcohol fraction.

Keywords

Staphylococcus aureus, *Cistus ladaniferus* Essential Oil, MIC, ATP, Cell Division Blocking, TEM

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1. Introduction

Microbial pathogens are a major cause of a significant number of food-borne illnesses worldwide. Enterotoxin-producing *Staphylococcus aureus* is one of the most common food-borne diseases in many countries [1]. *S. aureus* colonization in humans can lead to local and systemic infections that range from minor skin infections to severe illnesses such as septicemia, toxic shock, endocarditis and pneumonia [2]. To face serious *S. aureus* infections, the search for new antibacterial agents has therefore become essential.

Since ancient times, plants have provided some of the most important therapeutic molecules to the traditional and folk medicine. Many drugs (*i.e.*, aspirin, morphine, taxol...) derived from plant secondary metabolism have been applied for the treatment and/or prevention of various diseases. These secondary metabolites are concentrated in oil-bearing bags from which are extracted essential oils, which constitute an important resource of novel therapeutic compounds.

Essential oils are complex natural mixtures of volatile secondary metabolites, isolated from plants by steam- or hydro-distillation and by cold expression. They consist of naturally occurring molecules showing various biological properties, including anti-oxidative, anti-tumoral, anti-inflammatory and antimicrobial properties. Essential oils are known to display activity against a wide range of bacteria, including antibiotic resistant species [3].

Cistus ladaniferus (Cistaceae) is an odorous shrub, up to 2 m high, widespread all around the occidental Mediterranean Sea (Portugal, Spain, southern France, Italy, Algeria and Morocco). It exudes an oleoresin (*labdanum*) with an amber-like scent, appreciated in flavour and fragrance industries. The composition of the essential oil isolated from either the aerial parts (*C. ladaniferus* oil) or from oleoresin (*labdanum* oil) has been substantially investigated and the various reports on this topic have been reviewed by Lawrence [4]. *C. ladaniferus* essential oil is extremely complex, as illustrated by the work of Weyersthal *et al.* [5], who identified 186 compounds in a *labdanum* oil sample from southern France.

In an ongoing project, which aims to identify new antibacterials from plants growing in Corsica, we already highlighted the effectiveness of various essential oils and their main components. Concerning *C. ladaniferus* essential oils, obtained from cultivated shrubs introduced from Spain, the chemical composition have been reported by Mariotti *et al.* [6]. The composition of oil samples isolated from individual plants varied substantially from sample to sample: α -pinene (2.1% - 47.4%), camphene (0.6% - 11.1%), *trans*-pinocarveol (4.4% - 10.9%), viridiflorol (4.4% - 22.6%), ledol (1.5% - 6.7%). In a previous work [7], done with the *C. ladaniferus* chemotype α -pinene (52.6%), viridiflorol (6.7%), camphene (4.9%), *trans*-pinocarveol (3.2%) and ledol (2.2%), we showed that the essential oil and its chromatographic fractions were able to inhibit the growth of several bacteria, including *S. aureus*. The aim of the present contribution was to go further into the effects generated by these products on *S. aureus* cells and to provide insights on their mechanism of action.

2. Material and Methods

2.1. Essential Oil and Fractions

C. ladaniferus essential oil was purchased from “Huiles Essentielles et Hydrolats de Corse” (Ajaccio, Corsica, France). The commercial oil was obtained by vapor distillation, with an industrial apparatus, from air-dried aerial parts of cultivated *C. ladaniferus*. The hydrocarbonated, oxygenated, acetate and alcohol fractions were chromatographically obtained and analyzed by GC-MS as described before [7].

2.2. Bacterial Strains and Growth Conditions

The *in vitro* antibacterial activity of *C. ladaniferus* oil and its fractions was tested against the gram-positive *Staphylococcus aureus* ATCC6538P (CIP 53.156) strain. The strain was routinely grown at 37°C on Mueller-Hinton 2 agar (MHA, Oxoid).

2.3. Disc Diffusion Assays

The agar diffusion method was used for the determination of antibacterial activities [8]. *Inocula* were prepared by diluting overnight cultures in Mueller-Hinton broth (MHB, Oxoid) medium to approximately 10^6 CFU·mL⁻¹.

Filter paper discs (Whatman disc, 6 mm diameter) were impregnated with 15 μL of the tested products and placed onto the inoculated Petri dishes containing Mueller-Hinton 2 Agar (MHA, Oxoid). After keeping at room temperature for 1 h, plates were incubated at 37°C for 24 h. Following incubation, diameters of inhibition zones were measured (mm) and recorded as the mean \pm standard deviation (SD). Each test was performed in triplicate on at least three separate experiments.

2.4. Minimum Inhibitory Concentration Assays

The minimum inhibitory concentration (MIC) assays were performed by a rapid INT (*p*-iodonitrotétrazolium chloride, Sigma-Aldrich) colorimetric assay [7]. The tested products were serially twofold diluted in dimethylsulfoxide (DMSO, Sigma-Aldrich). The DMSO was previously tested for antibacterial activity and no detrimental effect on bacterial growth has been observed at the concentration used. The solutions obtained were then added (10 μL) to a 96-well microplate containing 200 μL of MHB inoculated with 10^6 CFU. The microplates were incubated at 37°C for 18 h. The MICs of the samples were then detected following addition (50 μL) of INT (0.2 $\mu\text{g}\cdot\text{mL}^{-1}$). Viable bacteria reduced the yellow dye to pink. The MIC is defined as the lowest sample concentration that prevents this change and results in the complete inhibition of bacterial growth. All determinations were performed in triplicate and a negative control, consisting of MHB with DMSO (5%, v/v), was systematically included.

2.5. Time-Kill Studies

Time-kill procedures were performed according to the method described by Klepser *et al.* [9] and modified by Viljoen *et al.* [10]. Activities of the tested products used at their MIC were evaluated against *S. aureus* by measuring the reduction in the number of CFU per milliliter over 7 h. The tested products were serially twofold diluted in DMSO and added (10 μL) to a 96-well microplate containing 190 μL of MHB inoculated with 10^6 CFU. DMSO was added to both tests and control at a final concentration of 0.01% (v/v). The microplates were incubated with agitation at 37°C and samples (100 μL), taken in duplicate every hour, 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.

2.6. Bacteriolysis

The bacteriolysis assays were carried out according to the standard method described by Carson *et al.* [11]. A bacterial suspension was prepared by inoculating two colonies of *S. aureus* from overnight cultures on MHA 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 $\cdot\text{mL}^{-1}$). The tested products were added to the bacterial suspension at concentrations equivalent to the MIC. PBS-T was added to the control suspension. The suspensions obtained were mixed for 20 s with a Vortex mixer. Samples (100 μL) were taken in duplicate every hour from 0 h to 7 h, serially 10-fold diluted, and the OD_{620} was measured immediately (Jasco UVVisco UV-1200 spectrophotometer). Each test was performed on three independent experiments. The results were expressed as a ratio (in percent) of the OD_{620} at each time point *versus* the OD_{620} at 0 min.

2.7. Loss of Cytoplasmic Material

The measurement of the release of 260-nm and 280-nm absorbing materials from *S. aureus* cells was carried out in aliquots of 2 mL of the bacterial *inocula* in sterile peptone water (0.1 g/100 mL) added to the tested products at their MIC at 37°C. At 0, 30, 60, 90 and 120 min time intervals of treatment, cells were centrifuged at 1465 g and the absorbance of the obtained supernatant was measured at 260 and 280 nm using a spectrophotometer (Jasco UVVisco UV-1200). Control flasks without fraction were tested similarly or in presence of cetyl trimethyl ammonium bromide (CTAB) to allow the measurement of total cytoplasmic material (260 or 280 nm) in these conditions. Each test was performed on three independent experiments. The results were expressed as a ratio (in

percent) of the OD₂₆₀ or OD₂₈₀ at each time point *versus* the OD₂₆₀ or OD₂₈₀ of CTAB control experiment.

2.8. Transmission Electron Microscopy (TEM)

A suspension of *S. aureus* in exponential phase of growth was prepared by inoculating then 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 (10⁷ CFU·mL⁻¹). *S. aureus* cells were treated with the MIC of each product for 1 h. Control stood for 1 h in MHB-Tween 80 (0.1%, v/v). After centrifugation at 1077 g 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 postfixed 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 then examined with a transmission electron microscope (HITACHI H-7650) at an accelerating voltage of 80 kV.

2.9. Measurement of Intra- and Extra-Cellular Adenosine 5'-Triphosphate (ATP) Concentrations

To determine the efficiency of the tested products on membrane integrity, the intra- and extracellular ATP concentrations were measured as described by Gill and Holley [12]. The overnight cultures of *S. aureus* were centrifuged for 20 min at 4,000 g and the supernatants were removed. The cell pellets were washed three times with 20 mM of phosphate potassium buffer (PPB, pH 7.0) and then cells were collected by centrifugation under the same conditions. A cell suspension (10⁷ CFU·mL⁻¹) was prepared with PPB (20 mM; pH 7.0) and glucose (50 mM). Then, controls (30 mM of carbonyl cyanide-m-chlorophenylhydrazone (CCCP) or polymyxine B (PMB) and DMSO) or each product used at their MIC were added to the cell solution. Samples were maintained at room temperature for 1 or 20 min and incubated in ice immediately to prevent ATP loss until measurement. The ATP concentration was immediately determined using a fluorimeter (Jasco UVVisco) after the addition of 100 µL of luciferin-luciferase reagent (Sigma-Aldrich) to 30 µL of sample in 40 µL of PPB (extracellular ATP) or PPB-CTAB (0.15%) (intracellular ATP). Each test was performed on three independent experiments. The results were expressed as a ratio (in percent) of the relative fluorescence unit (RFU) at each time point *versus* the RFU of DMSO control experiment.

Statistical analyses were performed using R statistical software (www.R-project.org). Multiple mean comparisons were performed with the Kruskal-Wallis test at the 95% confidence level.

3. Results

3.1. Susceptibility Tests of *C. ladaniferus* Essential Oil Fractions on *S. aureus* Strain

The inhibition zones and the MICs determined for *C. ladaniferus* essential oil and its fractions are presented in **Table 1**. Hydrocarbonated fraction is not active against *S. aureus* (6.70 mm, > 50 g·L⁻¹). The essential oil (52.00 mm, 0.8 g·L⁻¹) prevents the growth of the selected strain. Moreover, this activity may be attributed to the oxygenated fraction (35.50 mm, 0.10 g·L⁻¹) and its two sub-fractions *i.e* acetate (30.00 mm, 1.25 × 10⁻² g·L⁻¹) and alcohol (50.00 mm, 1.50 g·L⁻¹) compounds. Thus, we focused on these active fractions for the next experiments.

3.2. Time-Kill Studies of the Active Fractions on *S. aureus* Strain

The 7-h time-kill curves are shown in **Figure 1**. We can observed a detailed progression of the bacteriostatic activity of acetate fraction and bactericidal activity of oxygenated and alcohol fractions, used at a concentration equivalent to their MIC. The bactericidal end-point (99.9% or ≥ 3 log₁₀ of inhibition) is obtained in 7 h for oxygenated fraction and in less than one hour for alcohol fraction.

3.3. Cell Integrity Studies

S. aureus cells untreated by active fractions correspond to the negative control of bacteriolysis. The absorbance at 620 nm of negative control does not vary significantly until 120 minutes. Bacteriolysis assays (**Figure 2**) show the cell integrity after a two hours treatment of oxygenated, acetate or alcohol fraction used at the MIC.

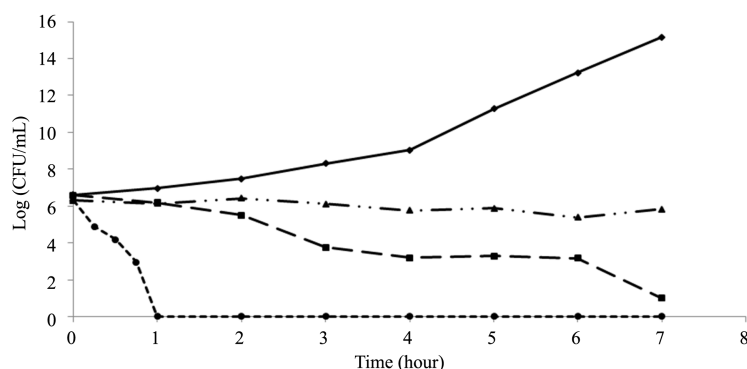


Figure 1. Time-kill curves of *S. aureus* cultures untreated (—●—) and treated with oxygenated (—■—) or acetate (—▲—) or alcohol (—◆—) fraction of *Cistus ladaniferus* essential oil.

Table 1. Inhibition zone and Minimal Inhibitory Concentration (MIC) of *Cistus ladaniferus* essential oil and its fractions on *S. aureus* strain.

	Inhibition zone (mm) \pm STD	MIC (g·L ⁻¹)
Essential oil	52.00 \pm 3.48	0.80
Hydrocarbonated fraction	6.70 \pm 0.47	>50.00
Oxygenated fraction	35.50 \pm 4.81	0.10
Acetate fraction	30.00 \pm 2.00	1.25×10^{-2}
Alcohol fraction	50.00 \pm 5.25	1.50

We can notice no or a very slight reduction of the bacterial cells percentage (around 100% of the original) throughout the experiment.

3.4. Loss of Cytoplasmic Material

In order to confirm the absence of cell lysis, loss of cytoplasmic material experiments are performed with the active fractions during the same period. **Table 2** indicates the percentage of 260-nm and 280-nm absorbing cytoplasmic material released in the extracellular medium. As expected for the cells control, no leakage occurs after 2 hours of treatment. In the same way, percentages observed for *S. aureus* cells in presence of oxygenated, acetate or alcohol fraction are weak (less than 1%) during the entire experiment at 260 nm and 280 nm.

3.5. Measurement of Intra- and Extra-Cellular Adenosine 5'-Triphosphate (ATP) Concentration

The effect of active fractions on extra- and intracellular ATP concentrations of *S. aureus* cells is presented in **Figure 3**. The quantity of intracellular ATP significantly ($p < 0.05$) decreases when compared to the DMSO control cells in presence of CCCP after 1 minute (78%) or 20 minutes (93.5%) of treatment. The percentage of intracellular ATP of cells treated for 1 minute with oxygenated, acetate or alcohol fraction is respectively 12%, 48% or 20%. In the same way, a 20 minutes treatment with the oxygenated, acetate or alcohol fraction leads respectively to a percentage of intracellular ATP in cells of 0%, 49% or 0%. As expected, the percentage of ATP released in the extracellular medium range from 0% to 4% after a 1 or 20 minutes treatment with DMSO or CCCP. In the assay with oxygenated, acetate or alcohol fraction, quite the same scale of values is observed.

3.6. Transmission Electron Microscopy

TEM was used to characterize the effects of acetate and alcohol fractions on *S. aureus* cells ultrastructure. Mi-

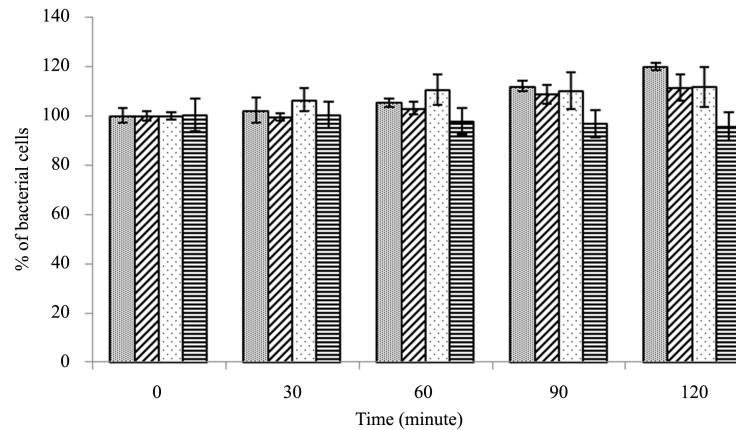


Figure 2. Cell integrity of *S. aureus* strain untreated (□) and after treatment by oxygenated (▨) or acetate (▤) or alcohol (▥) fraction of *C. ladaniferus* essential oil.

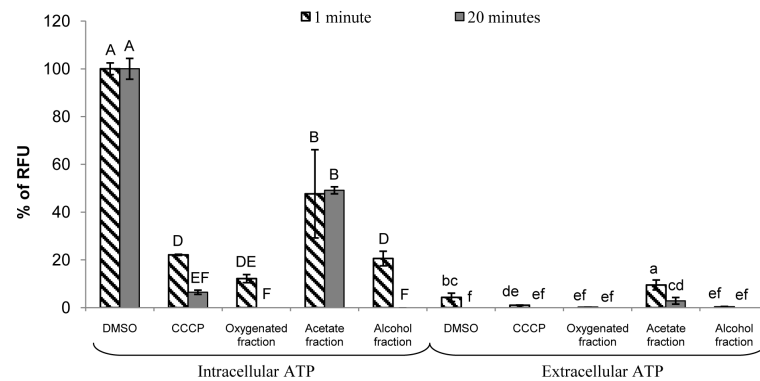


Figure 3. Effects on the inner membrane of *S. aureus* by measure of intra- and extracellular ATP in presence of active fractions of *C. ladaniferus* essential oil and controls (DMSO and CCCP). Uppercase letters indicate significant differences between experimental conditions for intracellular ATP (treatment \times exposure time) according to a Kruskal-Wallis test at the 95% confidence level. Lowercase letters indicate significant differences between experimental conditions for extracellular ATP (treatment \times exposure time) according to a Kruskal-Wallis test at the 95% confidence level.

Table 2. Percentage of 260-nm and 280-nm absorbing material leakage in the supernatants of *S. aureus* culture treated with active fractions of *C. ladaniferus* essential oil for 2 hours.

	Percentage of material leakage at 260-nm	Percentage of material leakage at 280-nm
Control	0.00 \pm 0.00	0.00 \pm 0.00
Oxygenated fraction	0.18 \pm 0.33	0.00 \pm 0.44
Acetate fraction	0.09 \pm 0.08	0.58 \pm 0.00
Alcohol fraction	0.04 \pm 0.15	0.00 \pm 0.95

crographies of untreated cells (control) and cells treated with the two fractions used at their MIC are presented in **Figure 4**. The cell wall and the plasmic membrane of *S. aureus* control cells are regular. The cytoplasmic contents are homogeneous (**Figure 4(a)** and **Figure 4(b)**). The cellular division is also visible in **Figure 4(a)**, where the septum is being formed. Control cells and cells exposed to the acetate fraction present a similar ultrastructural aspect (data not shown). On the other hand, the septum of *S. aureus* cells treated with the alcohol fraction

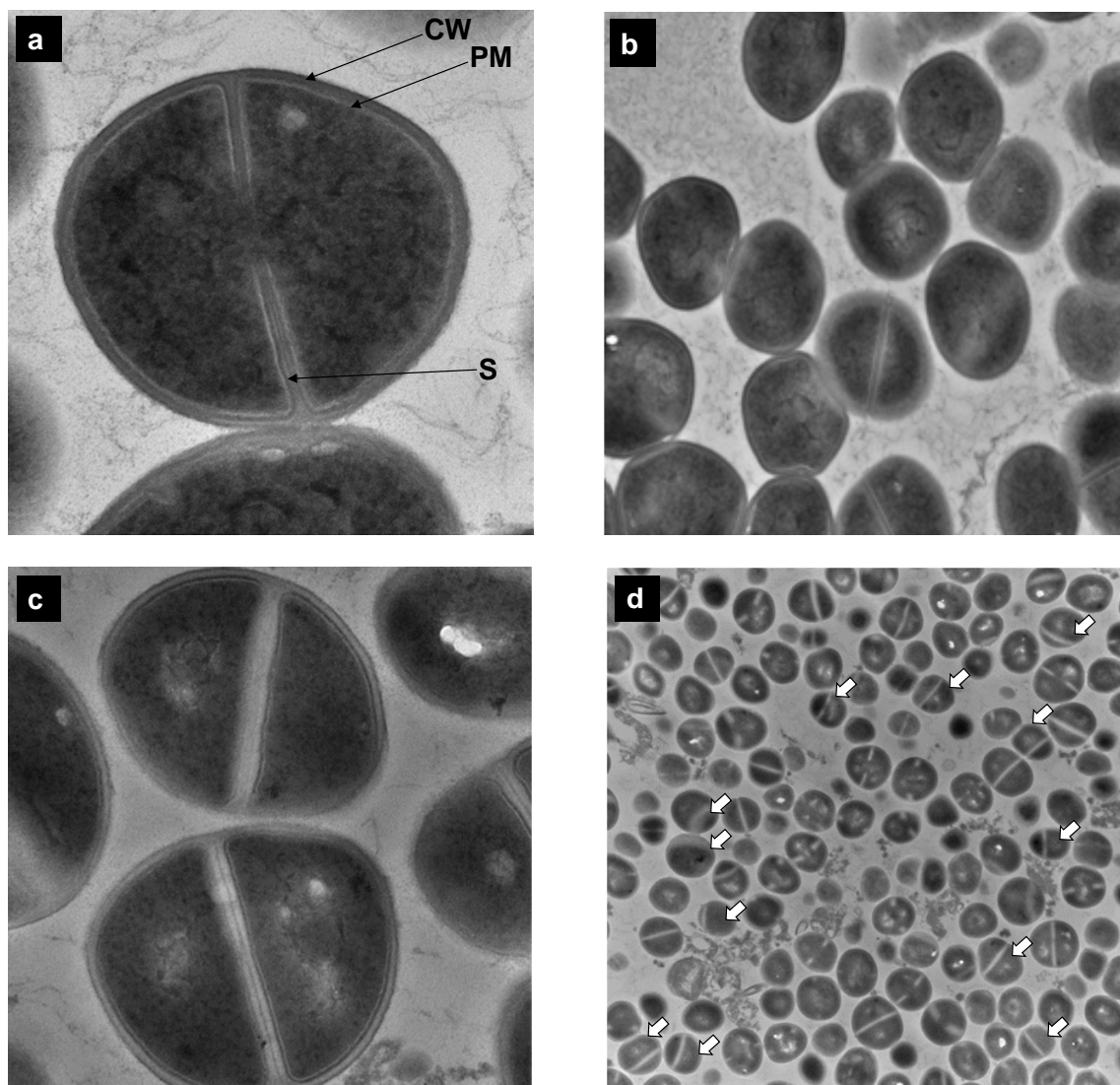


Figure 4. Transmission electron micrographies of *S. aureus* cells stained with uranyl acetate and lead citrate. (a) control cells $\times 120,000$; (b) control cells $\times 40,000$; (c): *S. aureus* cells treated with the alcohol fraction of *Cistus ladaniferus* essential oil at the MIC $\times 80,000$; (d): *S. aureus* cells treated with the alcohol fraction of *Cistus ladaniferus* essential oil at the MIC $\times 15,000$. CW: cell wall; PM: plasmic membrane; S: septum.

appears thicker and brighter (**Figure 4(c)**). In addition, the amount of septa is more important in the treated cells (**Figure 4(d)**) compared to control cells (**Figure 4(b)**). Some of *S. aureus* cells exposed to the alcohol fraction treatment also exhibit defects in septal positioning (**Figure 4(d)**, white arrows).

4. Discussion

In a previous work [7], we have shown that the composition of *C. ladaniferus* essential oil is largely dominated by α -pinene, an hydrocarbonated compound known to exert no or weak activity against several bacterial strains [13]. Actually, hydrocarbonated compounds are generally described as ineffective to inhibit bacterial growth [14]. The lack of α -pinene activity could thus explain the inefficiency of the hydrocarbonated fraction of *C. ladaniferus* essential oil. So, the involvement of oxygenated compounds in the antibacterial effects should be considered. Indeed, the oxygenated fraction and, more precisely its acetate or alcohol contents, reduce the viability of *S. aureus* cells at their MIC. The acetate fraction exhibits a bacteriostatic effect on the bacterial growth. In fact, no cell lysis or drastic cell wall damage seems to occur during the treatment with this fraction since no

intracellular material is released in the extracellular medium. However, a 50% reduction of intracellular ATP, which is stable in time, is observed which could be related to the bacteriostatic effect obtained on *S. aureus* strain. Acetate compounds seem to interfere with ATP synthesis by disturbing the ATPase efficiency or the proton motive force. Thus, no drastic change in bacterial ultrastructure is observed by MET but the decrease of ATP quantity in the intracellular medium prevents the cellular division and therefore the exponential growth of cells. Such a mode of action of acetate compounds has never been described in literature to our knowledge even if antibacterial, antifungal and phytotoxic activities [15] [16] of bornyl acetate, the major constituent of this fraction, have been reported.

The alcohol fraction exerts a quick and intense bactericidal activity. According to the literature, alcohols are described as the most active compounds of essential oils [14] and are known to exert bactericidal rather than bacteriostatic effect [17]. Cell death induced by alcohol fraction does not seem to be caused by a lytic effect. This is also supported by our electron micrographies on which *S. aureus* cells remain intact. However, after 1 h of treatment the septal material of cells undergoing division became thicker and more lightly stained, suggesting decreased levels of electron dense cell wall. The proportion of septa is also markedly increased in the treated cells compared to control cells and defective with respect to placement. These observations suggest a blocking in cell division. Campbell *et al.* [18] reported similar effects for *S. aureus* cells treated with targocil, an antibiotic that induces cell wall stress by inhibiting a late step in wall teichoic acid synthesis. Blocking of this biosynthetic pathway is lethal for bacterial cells and this could explain the rapid bactericidal effect induced by alcohol fraction in 1 h of treatment. On the other hand, according to Brown *et al.* [19] and Swoboda *et al.* [20], the biosynthesis of teichoic acid is an energy-dependant process. Thus, the total vanishing of ATP in the treated cells intracellular medium could be responsible of the disturbance in cell wall formation during division. Ultee *et al.* [21] have previously demonstrated that the level of ATP within *Bacillus cereus* cell treated with carvacrol, a naturally compound mainly present in thyme and oregano essential oils, decreased whilst there was no proportional increase outside the cell. Indeed, carvacrol significantly depleted the intracellular ATP pool to values close to 0 within 7 minutes. The authors therefore presumed that the rate of ATP synthesis was reduced or that the rate of ATP hydrolysis was increased. Furthermore, Gill and Holley [22] highlighted the inhibitory effect of carvacrol, eugenol and cinnamaldehyde on the membrane bound ATPase activity of *Escherichia coli* and *Listeria monocytogenes*.

The effects induced by these compounds appear to be related to their mode of action and specifically their integration in the bacterial cell membrane. Indeed, an important characteristic of essential oils and their components is their hydrophobicity, which enables them to partition into the bacterial lipidic bilayer. Such small hydrophobic molecules can thus provoke non-specific inhibition of membrane bound or embedded proteins, such as the F1F0 ATPase, which is involved in ATP generation and cellular pH regulation, or enzymes that are involved in the energy regulation process [23]-[25]. Also, changes in protein conformation as a consequence of hydrophobic interactions impair cell survival. The major constituents of the alcohol fraction are the stereoisomers viridiflorol and ledol. The hydrocarbon skeleton of these molecules is formed by the association of C3, C5 and C7 rings, which represent an important hydrophobic moiety. So, the chemical structure of these two main components may explain the whole effects observed on bacterial cells after treatment with alcohol fraction. Comparatively, the hydrophobic character of bornyl acetate, the major constituent of the acetate fraction, is less pronounced because of the presence of only C3 and C6 rings. This may also give reasons for the antibacterial activity described for the acetate fraction, which is expressed at a lesser extent relative to the alcohol fraction. Thereby, alcohols induce a bactericidal effect toward *S. aureus* cells while acetates exert a bacteriostatic action. So, when alcohols and acetates are combined within the oxygenated fraction, it is not surprising to obtain an additive effect rather than a synergism.

5. Conclusion

In conclusion, this work illustrates the rapid and intense antistaphylococcal efficiency of the alcohol fraction of *C. ladaniferus* essential oil. For this reason, an in-depth study is required to fully understand the way by which viridiflorol and ledol interact with the bacterial membrane and the consequences they bring out at the cellular level. To determine their hydrophobic interactions with membrane bound ATPase, it would be informative to study their effects upon the kinetics of purified enzymes.

Conflict of Interest

There is no conflict of interest.

References

- [1] Argudín, M.A., Mendoza, M.C., González-Hevia, M.A., Bances, M., Guerra, B. and Rodicio, M.R. (2012) Genotypes, Exotoxin Gene Content, and Antimicrobial Resistance of *Staphylococcus aureus* Strains Recovered from Foods and Food Handlers. *Applied and Environmental Microbiology*, **78**, 2930-2935. <http://dx.doi.org/10.1128/AEM.07487-11>
- [2] Paterson, G.K., Larsen, A.R., Robb, A., Edward, G.E., Pennycott, T.W., Foster, G., Mot, D., Hermans, K., Baert, K., Peacock, S.J., Parkhill, J., Zadocks, R.N. and Holmes, M.A. (2012) The Newly Described *mecA* Homologue, *mecALGA251*, Is Present in Methicillin-Resistant *Staphylococcus aureus* Isolates from a Diverse Range of Host Species. *The Journal of Antimicrobial Chemotherapy*, **67**, 2809-2813. <http://dx.doi.org/10.1093/jac/dks329>
- [3] Andrade, B.F.M.T., Barbosa, L.N., da Silva Probst, I. and Fernandes Júnior, A. (2014) Antimicrobial Activity of Essential Oils. *Journal of Essential Oil Research*, **26**, 34-40. <http://dx.doi.org/10.1080/10412905.2013.860409>
- [4] Lawrence, B.M. (1999) Progress in Essentials Oils. *Perfumer and Flavorist*, **24**, 41-50.
- [5] Weyerstahl, P., Marschall, H., Weirauch, M., Thefeld, K. and Surburg, H. (1998) Constituents of Commercial Labdanum Oil. *Flavour and Fragrance Journal*, **13**, 295-318. [http://dx.doi.org/10.1002/\(SICI\)1099-1026\(1998090\)13:5<295::AID-FFJ751>3.0.CO;2-I](http://dx.doi.org/10.1002/(SICI)1099-1026(1998090)13:5<295::AID-FFJ751>3.0.CO;2-I)
- [6] Mariotti, J.P., Tomi, F., Casanova, J., Costa, J. and Bernardini, A.F. (1997) Composition of the Essential Oil of *Cistus ladaniferus* L. Cultivated in Corsica (France). *Flavour and Fragrance Journal*, **12**, 147-151. [http://dx.doi.org/10.1002/\(SICI\)1099-1026\(199705\)12:3<147::AID-FFJ631>3.0.CO;2-Q](http://dx.doi.org/10.1002/(SICI)1099-1026(199705)12:3<147::AID-FFJ631>3.0.CO;2-Q)
- [7] Guinoiseau, E., Lorenzi, V., Luciani, A., Tomi, F., Casanova, J. and Berti, L. (2011) Susceptibility of the Multi-Drug Resistant Strain of *Enterobacter aerogenes* EA289 to the Terpene Alcohols from *Cistus ladaniferus* Essential Oil. *Natural Product Communications*, **6**, 1159-1162.
- [8] National Committee for Clinical Laboratory Standards. (1997) Performance Standards for Antimicrobial Disk Susceptibility Test. 6th Edition, Approved Standard M2-A6, Wayne.
- [9] Klepser, M., Ernst, E.J., Russell, E.L., Ernst, M.E. and Pfaller, M.A. (1998) Influence of Test Conditions on Antifungal Time-Kill Curves Results: Proposal for Standardized Methods. *Antimicrobial Agents and Chemotherapy*, **42**, 1207-1212.
- [10] Viljoen, A., Van Vuuren, S., Ernst, E., Klepser, M., Demirci, B., Baser, H. and Van Wyk, B.E. (2003) Osmitopsis Asteriscoides (Asteraceae)—The Antimicrobial Activity and Essential Oil Composition of a Cape-Dutch Remedy. *Journal of Ethnopharmacology*, **88**, 137-143. [http://dx.doi.org/10.1016/S0378-8741\(03\)00191-0](http://dx.doi.org/10.1016/S0378-8741(03)00191-0)
- [11] Carson, C.F., Mee, B.J. and Riley, T.V. (2002) Mechanism of Action of *Melaleuca alternifolia* (Tea Tree) Oil on *Staphylococcus aureus* Determined Par Time-Kill, Lysis, Leakage and Salt Tolerance Assays and Electron Microscopy. *Antimicrobial Agents and Chemotherapy*, **46**, 1914-1920. <http://dx.doi.org/10.1128/AAC.46.6.1914-1920.2002>
- [12] Gill, A.O. and Holley, R.A. (2006) Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* Cellular Membranes by Plant Oil Aromatics. *International Journal of Food Microbiology*, **108**, 1-9. <http://dx.doi.org/10.1016/j.ijfoodmicro.2005.10.009>
- [13] Santoyo, S., Caverio, S., Jaime, L., Ibanez, E., Senorans, F.J. and Reglero, G. (2005) Chemical Composition and Antimicrobial Activity of Essential Oil Obtained via Supercritical Fluid Extraction. *Journal of Food Protection*, **68**, 790-795.
- [14] Kalemba, D. and Kunicka, A. (2003) Antibacterial and Antifungal Properties of Essential Oils. *Current Medicinal Chemistry*, **10**, 813-809. <http://dx.doi.org/10.2174/0929867033457719>
- [15] Chikhouné, A., Hazzit, M., Kerbouche, L., Baaliouamer, A. and Aissat, K. (2013) *Tetralinis articulata* (Vahl) Masters Essential Oils: Chemical Composition and Biological Activities. *Journal of Essential Oil Research*, **25**, 300-307. <http://dx.doi.org/10.1080/10412905.2013.774625>
- [16] Ahluwalia, V., Sisodia, R., Walia, S., Sati, O.P., Kumar, J. and Kundu, A. (2014) Chemical Analysis of Essential Oils of *Eupatorium adenophorum* and Their Antimicrobial, Antioxidant and Phytotoxic Properties. *Journal of Pesticide Science*, **87**, 341-349. <http://dx.doi.org/10.1007/s10340-013-0542-6>
- [17] Dorman, H.J. and Deans, S.G. (2000) Antimicrobial Agents from Plants: Antibacterial Activity of Plant Volatile Oils. *Journal of Applied Microbiology*, **88**, 308-316. <http://dx.doi.org/10.1046/j.1365-2672.2000.00969.x>
- [18] Campbell, J., Singh, A.K., Swoboda, J.G., Gilmore, M.S., Wilkinson, B.J. and Walker, S. (2012) An Antibiotic That Inhibits a Late Step in Wall Teichoic Acid Biosynthesis Induces the Cell Wall Stress Stimulon in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, **56**, 1810-1820. <http://dx.doi.org/10.1128/AAC.05938-11>

- [19] Brown, S., Zhang, Y.H. and Walker, S. (2008) A Revised Pathway Proposed for *Staphylococcus aureus* Wall Teichoic Acid Biosynthesis on *in Vitro* Reconstitution of the Intracellular Steps. *Chemical Biology*, **15**, 12-21. <http://dx.doi.org/10.1016/j.chembiol.2007.11.011>
- [20] Swoboda, J.G., Campbell, J., Meredith, T.C. and Walker, S. (2010) Wall Teichoic Acid Function, Biosynthesis and Inhibition. *European Journal of Chemical Biology*, **11**, 35-45.
- [21] Ultee, A., Kets, E.P.W. and Smid, E.J. (1999) Mechanisms of Action of Carvacrol on the Food-Borne Pathogen *Bacillus cereus*. *Applied and Environmental Microbiology*, **65**, 4606-4610.
- [22] Gill, A.O. and Holley, R.A. (2006) Inhibition of Membrane Bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by Plant Oil Aromatics. *International Journal of Food Microbiology*, **111**, 170-174. <http://dx.doi.org/10.1016/j.ijfoodmicro.2006.04.046>
- [23] Sikkema, J., De Bont, J.A.M. and Poolman, B. (1995) Mechanisms of Membrane Toxicity of Hydrocarbons. *Microbiological Reviews*, **59**, 201-222.
- [24] Shabala, L., Budde, L., Ross, B., Siegmundfeldt, T., Jakobsen, H. and McMeekin, M. (2002) Responses of *Listeria monocytogenes* to Acid Stress and Glucose Availability Revealed by a Novel Combination of Fluorescence Microscopy and Microelectrode Ion-Selective Technics. *Applied and Environmental Microbiology*, **68**, 1794-1802. <http://dx.doi.org/10.1128/AEM.68.4.1794-1802.2002>
- [25] Burt, S. (2004) Essential Oils: Their Antibacterial Properties and Potential Applications in Foods—A Review. *International Journal of Food Microbiology*, **94**, 223-253. <http://dx.doi.org/10.1016/j.ijfoodmicro.2004.03.022>