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PII: S0003-9861(23)00169-8

DOI: https://doi.org/10.1016/j.abb.2023.109670

Reference: YABBI 109670

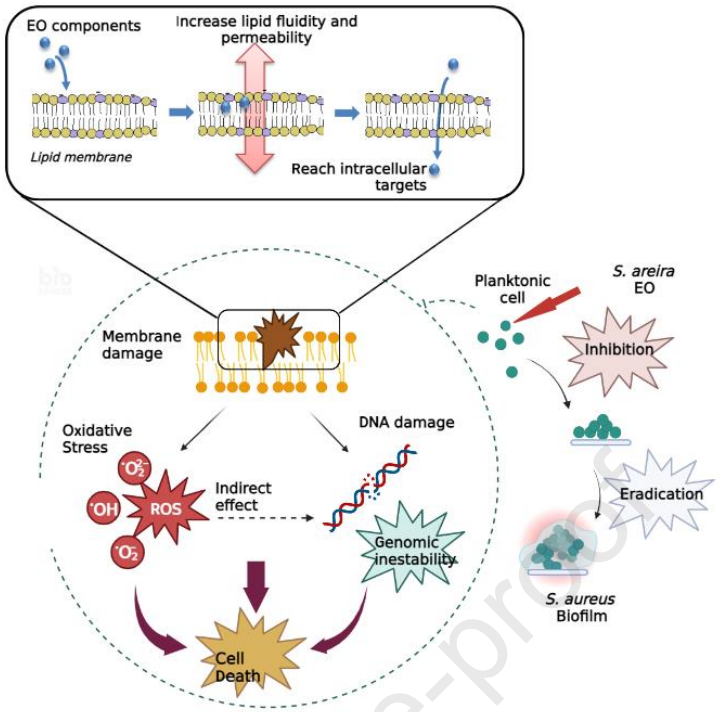
To appear in: *Archives of Biochemistry and Biophysics*

Received Date: 27 February 2023

Revised Date: 30 May 2023

Accepted Date: 12 June 2023

Please cite this article as: A.C. Cutro, M.S. Coria, A. Bordon, S.A. Rodriguez, A. Hollmann, Antimicrobial properties of the essential oil of *Schinus areira* (Aguaribay) against planktonic cells and biofilms of *S. aureus*, *Archives of Biochemistry and Biophysics* (2023), doi: https://doi.org/10.1016/j.abb.2023.109670.



Antimicrobial properties of the essential oil of *Schinus areira* (Aguaribay) against planktonic cells and biofilms of *S. aureus*.

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ABSTRACT

The essential oil (EO) of *Schinus areira* L. (Anacardiaceae) leaves has shown antibacterial activity against *Staphylococcus aureus.* In this study we aimed to unravel the mechanisms of its antibacterial action by using bacterial cells and model membranes. First, the integrity of *S. aureus* membrane was evaluated by fluorescence microscopy. It was observed an increase in the permeability of cells that was dependent on the EO concentration as well as the incubation time. For a deep evaluation of the action of the EO on the lipids, its effect on the membrane fluidity was evaluated on DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) : DMPG (1,2-dimyristoyl-sn-glycero-3-phospho-1'-rac-glycerol) (5:1) liposomes by dynamic scattering light and by using Laurdan doped liposomes. The results indicate that EO produces changes in lipid membrane packing, increasing the fluidity, reducing the cooperative cohesive interaction between phospholipids and increasing access of water or the insertion of some components of the EO to the interior of the membrane. In addition, the potential effect of EO on intracellular targets, as the increase of cytosolic reactive oxygen species (ROS) and DNA damage, were evaluated. The EO was capable of increasing the production of ROS as well as inducing a partial degradation of DNA. Finally, the effect of EO on *S. aureus* biofilm was tested. These assays showed that EO was able to inhibit the biofilm formation, and also eradicate preformed biofilms. The results show, that the EO seems to have several bacterial targets involved in the antibacterial activity, from the bacterial membrane to DNA. Furthermore, the antibacterial action affects not only planktonic cells but also biofilms; reinforcing the potential application for this EO.

KEYWORDS: natural compounds; membrane permeabilization; bacterial membrane; ROS; Biofilm

Introduction

The emergence of resistant and multi-resistant bacteria together with bacterial structures such as biofilms that are recalcitrant to antibiotic treatment has negative implications for human health, being responsible for clinically persistent and recurrent infections [1–3]. As a consequence, several studies have been carried out to find new antibacterial compounds that are able of inhibiting or eradicating bacterial growth and also have activity against resistant, multi-resistant bacteria [4–6].

The biofilm is the result of the grouping of bacterial cells adhered to a surface and embedded in an adhesive matrix formed by cellular excretions, such as polysaccharides, proteins, and nucleic acids that bind cells together, and the remains of dead cells. Therefore, the biofilm is not just cells trapped in a matrix but constitutes true dynamic, growing, and functional microbial communities [7]. Due to their slower growth rate compared with planktonic cells, the lower penetration power of antimicrobials, and gene expression that increases tolerance in a hostile environment, bacterial biofilms have great clinical relevance [4].

In particular, *Staphylococcus aureus*, a gram-positive coccus, is a versatile opportunistic pathogen and zoonotic pathogen that can cause many diseases in humans, from superficial skin infections to severe or even fatal invasive diseases [8]. Food contaminated with *S. aureus* may cause staphylococcal food poisoning [9], which is characterized by acute gastroenteritis with vomiting and diarrhea [10]. This bacteria can form biofilms on biotic and abiotic surfaces, including processing surfaces and equipment in dairy plants [11].

In this context, essential oils (EOs) represent a potential solution because they have been shown to have broad-spectrum antimicrobial activities against many foodborne pathogens, including gram-positive, gram-negative, and spoilage microorganisms [12]. The antimicrobial mechanism depends not only on the EO but also on the type of microorganism; since it is known that Gram-positive bacteria are more susceptible than Gram-negative bacteria [13,14]. The hydrophobic components of EOs can more easily enter and access cell targets such as bacterial membranes in gram-positive bacteria because they lack an outer lipopolysaccharide membrane system.

Previously, it has been reported that EO obtained from *Schinus areira* L. has shown antimicrobial activity against different bacteria, including *S. aureus* [15–19]. In particular, Celaya et al. have reported that EO extracted from the leaves and fruits of

S. areira, obtained from specimens collected in Jujuy (Argentina), showed antibacterial activity against the *S. aureus* methicillin-resistant strain [15]. Finally, in previous work, we demonstrated that EO obtained from leaves *S. areira* located in Santiago del Estero also showed antibacterial activity against *S. aureus,* showing bacteriostatic and bactericidal activity [16].

Even though there is extensive documentation on the antimicrobial properties of *S. areira* EO against *S. aureus,* among other bacteria, to our knowledge, studies focused on the possible mechanism of action as well as the bacterial targets of these EO are still scarce. It should be pointed out, that for future therapeutic applications of this EO, the knowledge about the cell target(s) is crucial to understanding which and how they are affected, and consequently, the survival of the pathogen in a food matrix, in living tissue or the host infection process can be impaired. Based on this, the present work aimed to evaluate the possible mechanism of action and get a deeper insight into the potential targets for the antimicrobial action of EO from the leaves of *S. areira* against *S. aureus*, evaluating the effect on the bacterial membrane and phospholipid bilayers, as well as possible intracellular targets such as the induction of oxidative stress and DNA damage. Finally, the effects of this EO on *S. aureus* biofilm formation and eradication were evaluated. This study has important implications for expanding the biotechnological potential of the *S. areira* EO, as it represents the first study that describes the possible mechanism of action of this EO along with its effects as an anti-biofilm agent.

Materials and Methods

Materials

The lipids used DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and DMPG (1,2-dimyristoyl-sn-glycero-3-phospho-1'-rac-glycerol) were both obtained from Avanti Polar lipids (USA). As a mimetic model of negatively charged membranes, a mixture of DMPC: DMPG (5: 1) was used [20]. The water used was ultra-pure, with a conductivity of (0.002 ± 0.001) µS / cm and pH= 5.0. The fluorescent probe H_2DCFDA and the organic solvents used (chloroform and methanol) were obtained from Merck Química SAIC (Argentina), all of the analytical grade, 5(6)-carboxyfluorescein (CF; Sigma, St. Louis, MO, USA). Vancomycin was from Klonal (Argentina). *S. areira* EO used was obtained and characterized in previous work [16], its chemical composition is listed in Table S1. For this work, the EO was solubilized in methanol and its stock solution was used in all the experiments. The minimum inhibitory concentration (MIC), i.e the lowest concentration of EO able to prevent visible growth of *S. aureus;* and the minimum bactericidal concentration (MBC), i.e the lowest concentration of EO required to kill *S. aureus*, were previously reported for this EO, with a MIC value of 64 µg/mL of EO and an MBC value of 256 µg/mL of EO [16].

Bacterial strains and growth conditions

Staphylococcus aureus ATCC 25923 used in this work was grown in Mueller Hinton (MH) obtained from Britania (Buenos Aires, Argentina) at 37 °C.

Fluorescence microscopy

The staining procedure was performed with solutions of SYTO-9 and propidium iodide (PI), to differentiate the live bacteria from those with membrane damage. Bacterial suspensions were prepared following manufacturing instructions of commercial BacLight bacterial viability kit L7007 obtained from Molecular Probes, Thermo Scientific (USA). [21]. Briefly, an overnight culture of *S. aureus* was inoculated in fresh MH media and incubated at 37 °C under agitation until reaching an OD_{600} of 0.3. Afterward, bacteria were incubated for 1 and 3 h with *S. areira* EO with a final concentration of 2 x MIC (128 µg/mL) or MBC (256 µg/mL). To remove the traces of the growth medium, cell suspensions were washed three times and

resuspended in PBS (10 mM, pH=7.40). Finally, both probes were added to achieve a final concentration of 6 µM of SYTO 9 and 30 µM of PI and incubated for 15 min with gentle agitation protected from light. Samples were imaged in an Olympus CKX 41 inverted fluorescence microscope coupled with an Olympus QColor3-RTV-R digital camera (Tokyo, Japan). The proportion of live and dead cells was determined by counting representative images taken from each condition repeated in triplicate using different batches of bacteria.

Preparation of unilamellar liposomes

Stock solutions of DMPC (10 mM) and DMPG (10 mM) were prepared, dissolving a proper quantity of solid lipids in chloroform. DMPC:DMPG (5:1) lipid films were prepared by slow evaporation of the organic solvent with a nitrogen stream in a glass tube with a conical base. The residual organic solvent was removed under a vacuum. To obtain a multilamellar vesicle suspension (MLVs), the dried film was hydrated in a medium containing PBS 20 mM, pH=7.40 at 40°C for 60 min. Finally, the LUV suspension was obtained by extrusion of MLV suspension previously obtained (pore diameter = 100 nm, Avanti Polar Lipids, USA) [22].

Phase transition temperature (Tm).

The effect of EO on the Tm of DMPC:DMPG LUV (850 µM) was determined by dynamic light scattering (DLS) measurements using the register of count rates (Count rates) as a function of temperature in the presence of increasing concentrations of E.O. added into the medium. From the data obtained the Tm and cooperativity were obtained by using the following equation (1):

$$Mean\ cout\ rare = rs1 + p1T + \frac{rs2 - rs1 + p2T - p1T}{1 + 10^{B\left(\frac{1}{T} - \frac{1}{Tm}\right)}} \qquad Eq.1$$

Where T is the temperature (°C), p_1 and p_2 correspond to the slopes of the straight lines at the beginning and the end of the plot, r_{s1} and r_{s2} are the respective count rate intercepting values at the y-axis [23]. From the fitted data, it was possible to determine the cooperativity (B) and the midpoint of the phase transition, which corresponds to T_m [23]. As a control methanol in the highest amount used was added, due to EO was solubilized in this solvent.

Laurdan fluorescence spectroscopy

For obtaining Laurdan doped liposomes, lipids were dissolved in chloroform to achieve a final concentration of 400 µM of DMPC:DMPG (5:1) and mixed with laurdan in ethanol to achieve a final concentration of 1 µM. Lipis film and extrusion was made as indicated above. The LUV suspension (400 µM of phospholipids) obtained was fractionated in aliquots of 700 µL and incubated for an hour with EO (40 µg/mL) and methanol (1.1 µL), respectively. After that, steady-state laurdan fluorescence emission and excitation were monitored with a fluorescence spectrometer CARY Eclipse, Agilent (USA). The temperature was controlled at 21 °C by a circulating water bath. Laurdan emission spectra were obtained in the range from 400 nm to 600 nm, using 350 nm excitation wavelengths, while the excitation spectra were obtained in the range from 300 nm to 430 nm, using 440 nm emission wavelengths. Blank spectra were obtained with unlabeled liposomes and were subtracted from the spectra of labeled liposomes. At the concentrations of vesicles and bandpass used, interference from scattered light was negligible. From the spectroscopic data, the laurdan generalized polarization was calculated by using the following equation adapted from Parasassi et al. [24]:

$$GP = \frac{I_{450} - I_{500}}{I_{450} + I_{500}} \qquad Eq.2$$

Leakage

For leakage experiments, MLVs were obtained as pointed out above by the dried film was hydrated in a medium containing PBS 20 mM, and CF 100mM pH=7.40 at 40°C for 60 min. Non-encapsulated CF was removed by washing the MLVs with PBS 20 mM three times, according to [25,26]. The fluorescence intensity resulting from the CF release was monitored with a fluorescence spectrometer CARY Eclipse, Agilent (USA) USA), with excitation and emission wavelengths of 492 nm and 517 nm, respectively. The extent of content release after the addition of 85 µg/ml of EO was expressed as a percentage according to equation 3. For 100% of leakage, Triton X-100 (5 % v/v solution) was added to promote total lysis.

$$Leakage\ (\%) = \frac{F - F_i}{F_t - F_i}\ x\ 100 \qquad Eq.3$$

where F is the fluorescence intensity of the sample after each incubation time, F_i is the initial fluorescence intensity of the sample, and F_t is the total fluorescence intensity of the sample after the Triton X-100 addition.

Reactive Oxygen species (ROS) assay

An overnight culture of *S. aureus* in MH broth at 37 ºC was diluted (1/10) in fresh MH broth and incubated at 37ºC, with agitation, to obtain an optical density OD_{600} = 0.30. Then, the H_2DCFDA probe was added up to a final concentration of 20 µM and incubated at 37 °C with agitation in darkness for 30 min. After that, the cell suspension was centrifuged at 5000 RPM for 5 min, and the pellet obtained was resuspended in PBS. This procedure was repeated two times. Finally, aliquots of 800 µL of the cellular suspension were mixed with different concentrations of *S. areira* EO (MIC, and 2 x MIC) and incubated for 1 h at 37 °C with agitation. Control experiments were conducted with the addition of methanol; and H_2O_2 up to a final concentration of 1% and 2 mM, respectively. Fluorescence emission spectra were registered with excitation at 485 nm and emission from 507 to 600 nm [27].

DNA Damage

An overnight culture of *S. aureus* in MH broth at 37 ºC was diluted (1/10) in fresh MH broth and incubated at 37ºC with agitation up to obtain an optical density OD_{600} = 0.30. From this cell suspension, 2 mL aliquots were taken and incubated for 24 h at 37 °C, in the absence or presence of EO at MBC concentration. Finally, the DNA isolation was performed by using a commercial kit DNA Puri Prep- B Kit, Inbio Highway (Argentina). The DNA concentration of each sample was determined by absorbance measurement at 260 nm and 280 nm in a NanoDrop 2000c UV-Vis spectrophotometer Thermo Scientific (USA).

For the electrophoresis, each sample was mixed with loading buffer (25 mg bromophenol blue, 10 mL Tris EDTA (TE) buffer 1X pH= 8.0 and 20 mL glycerol with pH value adjusted to 8.0 and 10 µL of ADN (90 ng/µL) aliquots were transferred into a 1% (w/v) agarose gel prepared using Tris-Acetate EDTA buffer (TAE) supplemented with 5 µL of GelGreen, Biotium (USA). Electrophoresis was then run for 45 min at 90 mV. The gels were visualized using the transilluminator BluePad Dual Blue/White Light, Bio-Helix (Taiwan).

Biofilm inhibition and eradication assays

For biofilm inhibition determination, the culture of *S. aureus* was grown overnight in MH broth, and aliquots of 75 µL were dispensed into each well of 96-well polystyrene flat-bottom microplates in the presence of 75 µL of EO solution (0.25 x MIC and 0.5 x MIC) and gentamicin (0.5 x MIC) prepared in sterile MH broth. After incubation of 72 h at 37 °C, the medium was discarded and each well was washed three times with phosphate-buffered saline PBS (20 mM, pH=7.40), dried, stained for 20 min with 0.5% (w/v) crystal violet, and washed with water. The stained biofilms were resuspended in 150 µL absolute ethanol. The optical density (OD) was measured at 595 nm by using a ELx808iu microplate reader BioTek, Agilent (USA). The negative control was MH broth only and the positive control contained cell cultures without treatment. All determinations were performed in triplicate [28]. A similar procedure was performed for the eradication trials with certain modifications, in this case, aliquots of 150 µL of bacteria cell culture were dispensed in each well and after 72 h of incubation at 37 °C, the planktonic cells were gently removed and the wells were washed three times with sterile buffer PBS 20 mM (pH= 7.40) and filled with 150 µL of dilutions of EOs (MIC, 2 x MIC and MBC) or gentamicin (MIC) in sterile MH and incubated for 24 h at 37 °C. After this period, the medium was discarded and each well was washed, dried, stained, and spectrophotometrically quantified as it was previously described for the inhibition assay. The negative control was MH broth only and the positive control contained cell cultures without treatment. All determinations were performed in triplicate [29–31].

Biofilm Fluorescence Microscopy

The *S. aureus* biofilms were developed in µ-Slide 8 Well high Ibidi (Germany) for 72 h at 37 °C as it was previously described for eradication biofilm protocol. After the incubation with the antibacterial agents, the biofilms were washed one time with 150 µL sterile buffer PBS 20 mM (pH= 7.40) and filled with 80 µL with sterile buffer PBS 20 mM (pH= 7.40), stained with SYTO 9 (6 µM) and IP (30 µM) (viability kit Live/Dead; LIVE/DEAD BacLight, Molecular Probes IncEugene, OR, USA) for 5 min in darkness; and visualized in an inverted fluorescence microscope as described above [21].

Statistical analysis

Analysis of variance (ANOVA) followed by Dunnett post-test for multiple comparisons and Student's t-test were carried out using the statistical software Graph Pad Prism. Data are shown as the mean value. Error bars on data presentation represent the standard error of the mean (SEM).

Results and Discussion

Effect of EO on bacterial membrane

In previous work, we demonstrated that the EO of *S. areira* exhibits antimicrobial activity against *S. aureus* and showed that the EO can interact with lipid membranes by using model lipid membranes [16]. In this sense, to unravel the possible mechanism of action in the present study we evaluated the effect of the *S. areira* EO on membrane permeability by using a differential staining method, composed of two fluorophores: SYTO-9 able to stain both: cells with damaged and undamaged membranes, and PI, only able to stain membrane-damage bacteria.

S. aureus cell suspensions were incubated at different times (0, 1, and 3 h) with two EO concentrations (2 x MIC and MBC). After staining with SYTO-9 and PI, microscopic images were obtained. As can be seen from the images, the amount of PI-stained cells increases with the EO concentration, as well as with the incubation time (Fig. 1).

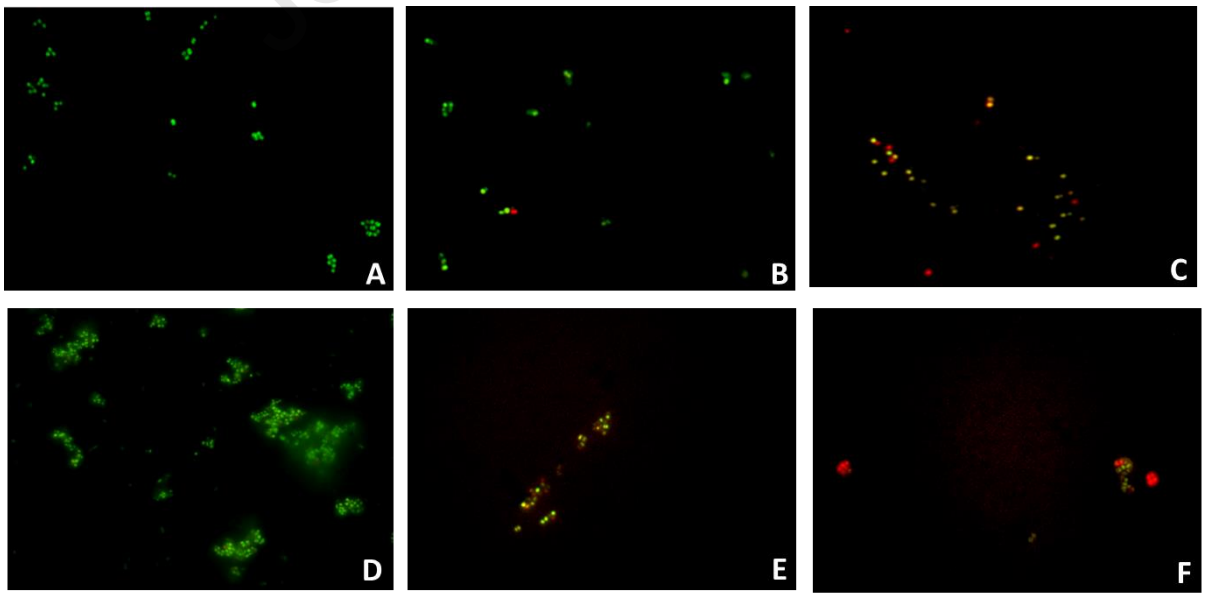

Figure 1. SYTO-9 : PI staining of *S. aureus* after 1 h of incubation without agent **(A),** and with 2 x MIC **(B)** or MBC **(C)** concentration of EO. SYTO-9 : PI staining of *S. aureus* after 3 h of incubation without agent **(D),** and with 2 x MIC **(E)** or MBC **(F).**

To obtain quantitative information, the percentage of PI stained cells (i.e. those with the membrane damaged) was estimated in each condition (Fig. 2). It was possible to observe a significant increase in membrane damage after 1 h of incubation at both EO concentrations tested, with a higher effect after 3 h of incubation.

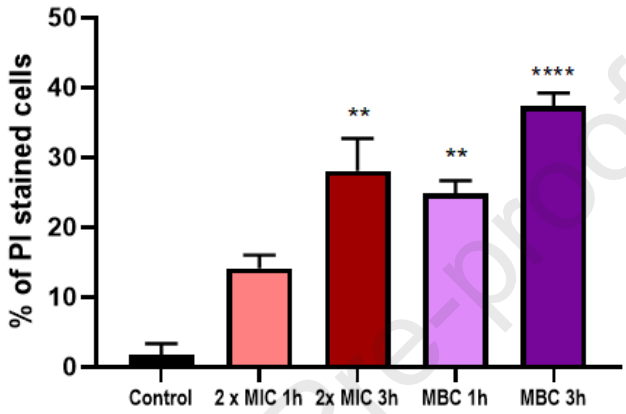

Figure 2: *S. aureus* PI stained cells count after 1 h of incubation with different concentrations of *S. areira* EO. The data represents an average of 5 independent measurements; the error bars indicate the standard deviation of the averaged values. ****, $p<0.0001$; **, $p<0.05$. One-way ANOVA followed by Dunnet's test for multiple comparisons vs. control.

These results implied that EO can induce damage in the *S. aureus* bacterial membrane, in good agreement with reports for other EO that pointed at the bacterial membrane as one of the main targets for its antibacterial action [12].

Effect of EO on model lipid membranes

The effects of this EO on model membranes composed of DMPC:DMPG (5:1), as a simplified model of the negatively charged bacterial membrane [22], were conducted to gain a better comprehension of the effect that EO produces in the lipid membrane. It should be pointed out, that in all experiments conducted with liposomes, the lipids were in high excess relative to the EO components.

First, the changes in the phase transition temperature (Tm) of the lipid membrane induced by EO were assessed by DLS. This technique evaluates modifications in the optical properties of lipids through the variation of the average count rate. Thus, by

plotting the mean count rates as a function of incubation temperature, it is possible to obtain the Tm and cooperativity of the process [32,33]. As it is shown in Fig. 3A, in all the conditions tested, a sigmoidal behavior was observed. However, as the concentration of EO increases, the slope becomes less steep. The Tm value of liposomes shifts from 24.3 ± 0.6 °C, for control liposomes to lower values as the concentration of EO tested increases (Fig. 3B), indicating a fluidification effect of the EO on the membrane; these results are in accordance with the literature [34,35]. Changes in Tm of lipid bilayers are used to probe the interaction between the acyl chains of lipids and exogenous substances. The change in this calorimetric parameter could be attributed to the packing of some components of the EO within the hydrophobic interior of the phospholipids array [36]. The physical state of the lipid bilayer depends on the strength of Van der Waals interactions between adjacent lipids [32], it seems that the interaction of some EO components with the lipids could contribute to the energy needed to disrupt the interactions, resulting in a more fluid state of the membrane. [37,38]

Besides the changes in the Tm, the cooperativity, which measures the sharpness of the phase transition, is very sensitive to the presence of any additive in the lipid bilayer [39]. In this sense, following the changes in the cooperative also gives information on the changes induced in the bilayer. As can be seen in Fig. 3C the cooperativity is affected at all the EO concentrations, even at the lowest, where there are no obvious changes in the Tm. Typically, pure lipid systems showed high cooperativity in the phase transitions, which results in the complete transition of phases with small temperature changes. The obtained results indicate a decrease in the phospholipid chain interactions by the action of the EO, which could diminish the lipid packing while increasing membrane lability (Fig. 3C).

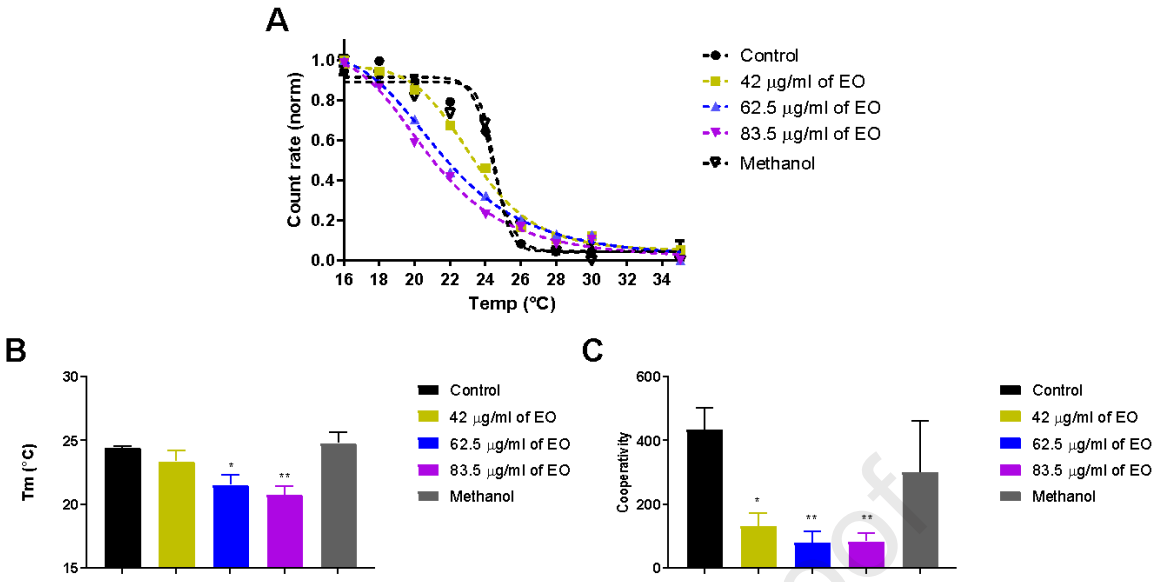

Figure 3: A) Experimental values of the counting rate as a function of temperature at different concentrations of *S. areira EO*. Values of Tm and cooperativity were estimated using the regression method, figures **B** and **C**, respectively. Data represent an average of 5 independent measurements; the error bars indicate the standard deviation of the averaged values. ***, p<0.0001; **, p<0.01; *, p<0.05. One-way ANOVA followed by Dunnet's test for multiple comparisons vs. control.

Secondly, by using Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene), a fluorescent probe widely used for sensing changes in membrane phase state, the effects of EO in the bilayer were assessed. Information about phase state changes of the membrane could be reported as fluorescence spectral changes which are attributed to dipolar relaxation phenomena, originated by the sensitivity of the probe to the polarity of its environment. The quantitation of the phases can be obtained using steady-state generalized polarization (GP) [24]. Thus, the effect of 40 µg/mL EO on liposomes (400 µM) was determined. The fluorescent spectra obtained showed a decrease in the laurdan fluorescence intensity of the excitation spectra at longer wavelengths, with the maximal change located at 389 nm, in the presence of EO in comparison with the control (Fig. 4A). In addition, the laurdan emission spectrum is decreased and red-shifted (maximum at 489 nm) by the action of EO in the bilayer in comparison with the control components, in good agreement with the Tm shift observed (Fig. 4B).

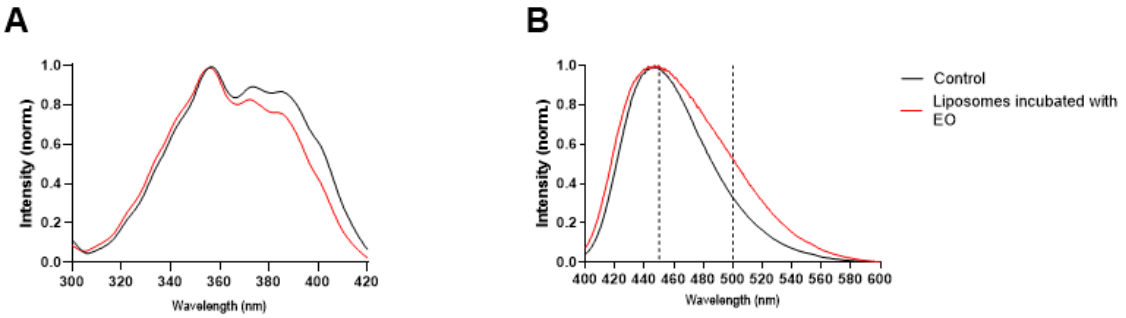

Figure 4: Effect on membrane polarity of EO by laurdan fluorescence spectroscopy of liposomes. Excitation **(A)** and emission **(B)** spectra of liposome suspensions loaded with laurdan incubated with and without EO. Lines in Graph B correspond to wavelengths used for GP values estimation.

Moreover, the GPs values were calculated from the experimental data. It could be noticed that both GP has a notable decrease in liposomes incubated with *S. areira* EO. Since the control value was 0.49 and 0.30 for EO incubated liposomes. It should be pointed out that the GP values of liposomes without EO are in agreement with values previously published [40].

This behavior indicates a change in the membrane polarity due to the increased access of water molecules, or the insertion of some components of the EO, into the membrane, which is consistent with a more fluid membrane, led by the interaction of the EO.

Finally, to evaluate if those structural modifications found on model membranes were able to promote an increase in membrane permeabilization, leakage experiments with CF were conducted. As can be seen in Fig. 5, after the addition of EO the fluorescence recorded increased, indicating a leakage of the fluorescent probe with a maximum leakage of 20.75 ± 4.1 %. These results are in good agreement with the fluorescent microscopy results that show the permeabilization of the bacterial membrane. A similar rate of leakage in model membranes was observed for the EO of *Melaleuca alternifolia* (tea tree oil) [41]

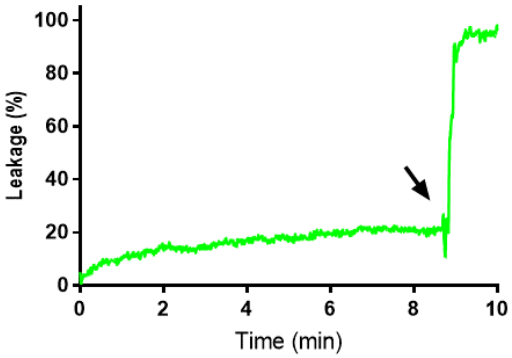

Figure 5: Leakage of liposomes of DMPC:DMPG (5:1) in the presence of EO, as a function of time. Liposome suspension contained 750 µM total phospholipid concentration and 85 µg / ml of EO. Arrow indicates the Triton X-100 addition to achieve 100% of leakage.

Overall, these results indicate that the interaction of EO with the membrane leads to structural modifications such as lateral diffusion, lateral expansibility, bilayer thickness and bending, as was previously described [42], that results in an increase in the membrane permeability. Those membrane perturbation effects were found in previous works for EO, different pure monoterpenes [36,39,43], and cyclic compounds that could effectively diffuse to lipid bilayer and increase membrane fluidity [44]. It should be pointed out that the composition of *S. areira* EO is rich in monoterpenes and two of the major compounds (1-epi-Cadinol and δ-Cadinene) are cyclic (Table S1).

Studies on intracellular targets

As the results shown above demonstrated an alteration of the bacterial membrane permeability induced by the EO, possible intracellular targets were evaluated. First, the induction of ROS was assessed by using *S. aureus* cells labeled with the probe H_2DCFDA and incubated with different concentrations of *S. areira* EO at 37 °C for 1 h. H_2DCFDA is a reagent able to cross the bacterial envelope, after diffusion in the cell, the acetyl groups in H_2DCFDA are cleaved by an intracellular esterase to produce the nonfluorescent compound that is oxidized rapidly to highly fluorescent 2',7'-dichlorodihydrofluorescein by the presence of ROS [27]. In this mode, the intensity of the detected fluorescence is proportional to the ROS levels within the cell. The result obtained showed that EO from *S. areira* was capable of increasing

the fluorescence, therefore generating oxidative stress at MIC and 2 x MIC concentrations on *S. aureus* cells, showing the maximal effect at the maximal EO concentration assays (Fig. 6 A and B).

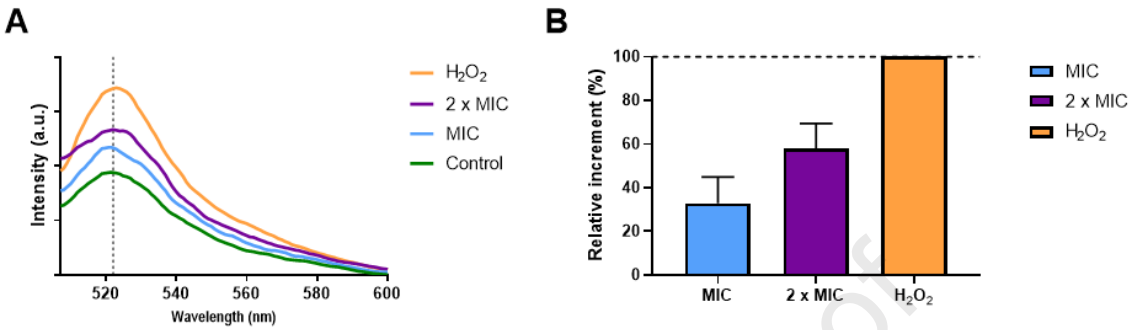

Figure 6: Fluorescence Spectroscopy of *S. aureus* incubated with H_2DCFDA and *S. areira* EO. A) Emission spectra were recorded, λ exc= 485 nm. Control experiments in the presence of methanol and H_2O_2 were carried out. **B)** Relative changes in fluorescence intensity were calculated considering 100 % of the change in the intensity observed in the presence of H_2O_2**.**

A similar, effect has been reported for another EO such as *Thymus vulgaris L* and its critical component thymol was capable of inducing high levels of intracellular ROS in *P. aeruginosa* related to the cytotoxicity and antibacterial effect observed [45]. In the same way, EO components such as eugenol at high concentrations also showed pro-oxidant effects [46].

One of the typical targets of the ROS is the DNA, it was widely reported that induction of ROS-mediated oxidative stress could result in the damage of several intracellular macromolecules, including DNA [47]. In this sense, the integrity of genomic DNA, after bacteria were incubated for 24 h in the presence of *S. areira* EO al MBC concentration, was evaluated by performing DNA isolation and visualized after running electrophoresis in agarose gel. DNA band corresponding to the control (i.e. without EO) was completely clear in comparison with the one corresponding to the EO-treated bacteria, where lower molecular weight DNA below the main high molecular weight band is observed, indicating a partial DNA degradation (Fig 6). These results could be ascribed to the ROS generated by the EO, but a direct interaction of some component of the EO with DNA cannot be discarded. In this sense, the direct incubation of DNA with EO was also tested, showing no changes in the DNA electrophoresis band (Fig. 7).

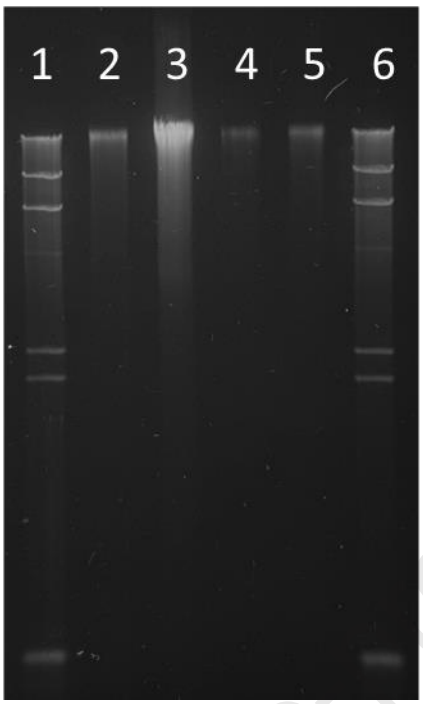

Figure 7: Image of DNA agarose gel electrophoresis. 1 and 6: Marker (Lambda/Hind III); 2: Control DNA of *S. aureus* after 24 h of incubation (90 ng) 3- *S. aureus* DNA (90 ng) after 24 h of EO incubation at MBC concentration in MH broth 4 and 5: DNA *S. aureus* (90 ng) incubated at 37 °C for 1 h with EO 5 mg/mL and 2.5 mg/mL, respectively.

It was previously reported that some EO can inactivate genetic material, causing DNA damage [48]. In this way, Ju et al. [49] confirmed that the mixture of eugenol and citral could lead to the degradation of the DNA of *Penicillium roqueforti*. In the same way, it was reported that Marjoram (*Origanum marjorana* L.) EO induces DNA degradation in *Klebsiela kneumonieae* [50]. Besides, at present, the specific mechanism of DNA damage caused by EO is still unclear and needs further study [48]. However, experiments conducted with certain components of other EO, such as thymol, reveal that this component intercalates in the DNA inducing damage. However, our results suggest that *S. areira* EO induces DNA damage throughout those ROS molecules instead of direct interaction with genomic DNA.

Studies on *S. aureus* biofilms

As was pointed out above, the effect of new antibacterial agents on biofilms is a key point to evaluate. Thus, the effect on the *S. aureus* biofilm formation was tested, and for that purpose, cells were incubated at EO sub-MIC concentrations, and after 72 h

the biofilms were quantified. It was noticed a significant dose-dependent reduction in the biofilm formation at all EO concentrations tested, reaching a maximum effect at 0.5 x MIC, with a reduction of the biofilm formation of about 75%, showing a similar effect to the gentamicin at 0.5 x MIC (Fig. 8A). Since control experiments at the same EO concentration tested did not show significant changes in bacterial growth curves (Fig. S1), this behavior seems to be related to a specific biofilm inhibition, such as Quorum Sensing, among others. It is important to note that many antibiotics could promote biofilm formation at sub-optimal conditions in an attempt by the bacteria to protect itself from antimicrobial activity [51]. Therefore, the fact that the EO was able to inhibit biofilm inhibition at sub-MIC concentrations reinforces its potential as an antimicrobial drug, especially toward gram-positive bacteria.

On the other hand, the capacity to eradicate a preformed biofilm was also analyzed. To accomplish that, *S. aureus* biofilms of 72 h were incubated with increasing EO concentrations, and control experiments in the presence of gentamicin (MIC) and the absence of antimicrobials (Control) were included. The EO showed eradication power that was dose-dependent, reaching the maximal effect at the higher concentration of EO tested (MBC) (Fig. 8B). A microscopic observation of the biofilm after being stained with SYTO-9 and PI confirms the effect of EO over the biofilm architecture showing a decrease in biofilm density in comparison with the control without any agent (Figure 9).

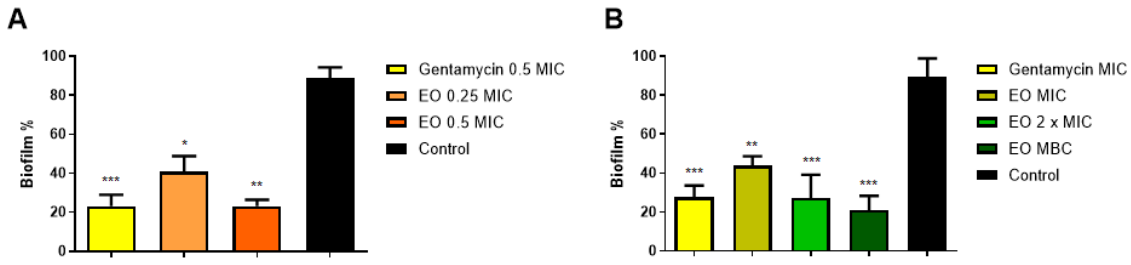

Figure 8: *S. areira* EO Inhibition on *S. aureus* biofilms. A) The total amount of biofilm biomass of *S. aureus* incubated during biofilm formation with sub-MIC concentrations of *S. areira* EO, gentamicin and with no antimicrobial compound (Control). **B)** The total amount of *S. aureus* biofilm biomass (72 h of formation) after the incubation for 24 at MIC, 2 x MIC and MBC concentrations of *S. areira* EO, gentamicin and with no antimicrobial compound (Control). The data represents an average of 3 independent measurements; the error bars indicate the standard deviation of the averaged values. ***, p<0.0001; **, p<0.01. *, p<0.05. One-way ANOVA followed by Dunnet's test for multiple comparisons vs. control.

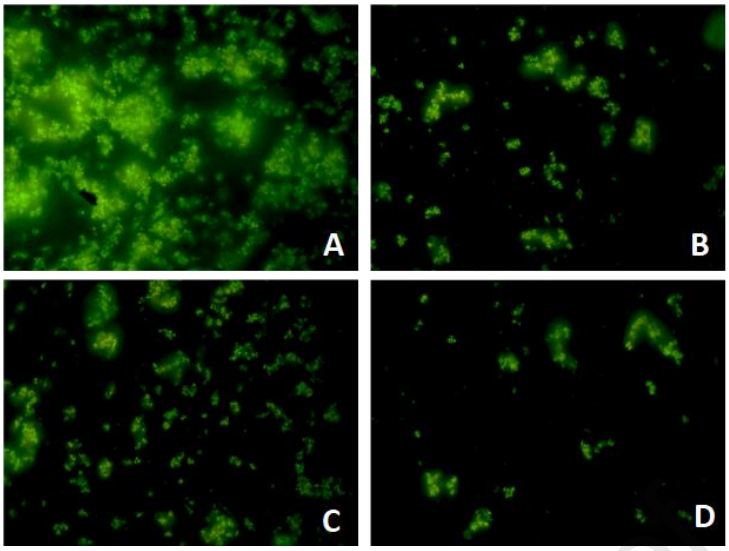

Figure 9: Fluorescence microscopic images of *S. aureus* Biofilms (72 h formation) after being incubated for 24 h with different incubation conditions, staining with SYTO 9 and PI: A) Positive Control (no antimicrobial compound); **B)** Gentamicin; **C)** 2 x MIC and **D)** MBC of *S. areira* EO.

This is the first work that showed that the EO of *S. areira* can inhibit *S. aureus* biofilms, as well as destroy preformed biofilms. In good agreement with other EO, such as oregano EO, that is efficient in eradicating *S. aureus* biofilms from sensible and resistant bacteria [52].

Conclusion

Recently, the use of some EOs as alternative antimicrobial and pharmaceutical agents has attracted considerable interest from scientists worldwide. However, a relevant point of antimicrobial activity is the mechanism of action, which can vary with the type of EO or the strain of the microorganism used [53]. In this way, the results obtained in this work, for the first time allow us to understand the mechanism of action of *S. areira* EO against *S. aureus* (Fig. 10). Should be reinforced here that EO is composed of several molecules, therefore beside that we describe the mechanism of whole EO the effects observed should be due to the interaction of one or more of these components of the whole EO. This EO was able to alter the bacterial permeability that is accompanied by reduced viability of *S. aureus*. Indeed, the interaction of this EO with the bacterial membrane affects its structure; leading to a reduction in the phospholipid package, which results in a more fluid membrane, which favors the increase in the membrane permeability, and affects its barrier function. In addition, EO is capable of inducing oxidative stress through ROS

generation and producing damage in the bacterial genomic DNA. Besides, the EO is capable of significantly inhibiting *S. aureus* biofilm formation at sub-MIC concentrations, at which no significant reduction in bacterial growth curves was detected. This could indicate that another potential target for action would be the *Quorum Sensing* system. Likewise, once the *S. aureus* biofilm is formed, the *S. areira* EO is capable of attacking a preformed biofilm, inducing a significant reduction at MIC, 2 x MIC, and MBC concentrations.

Overall, our results support the hypothesis that EO exerts its action through different cellular targets, which could imply a low potential for the development of microbial resistance, a great advantage compared to the single-target effects of conventional antibiotics [54].

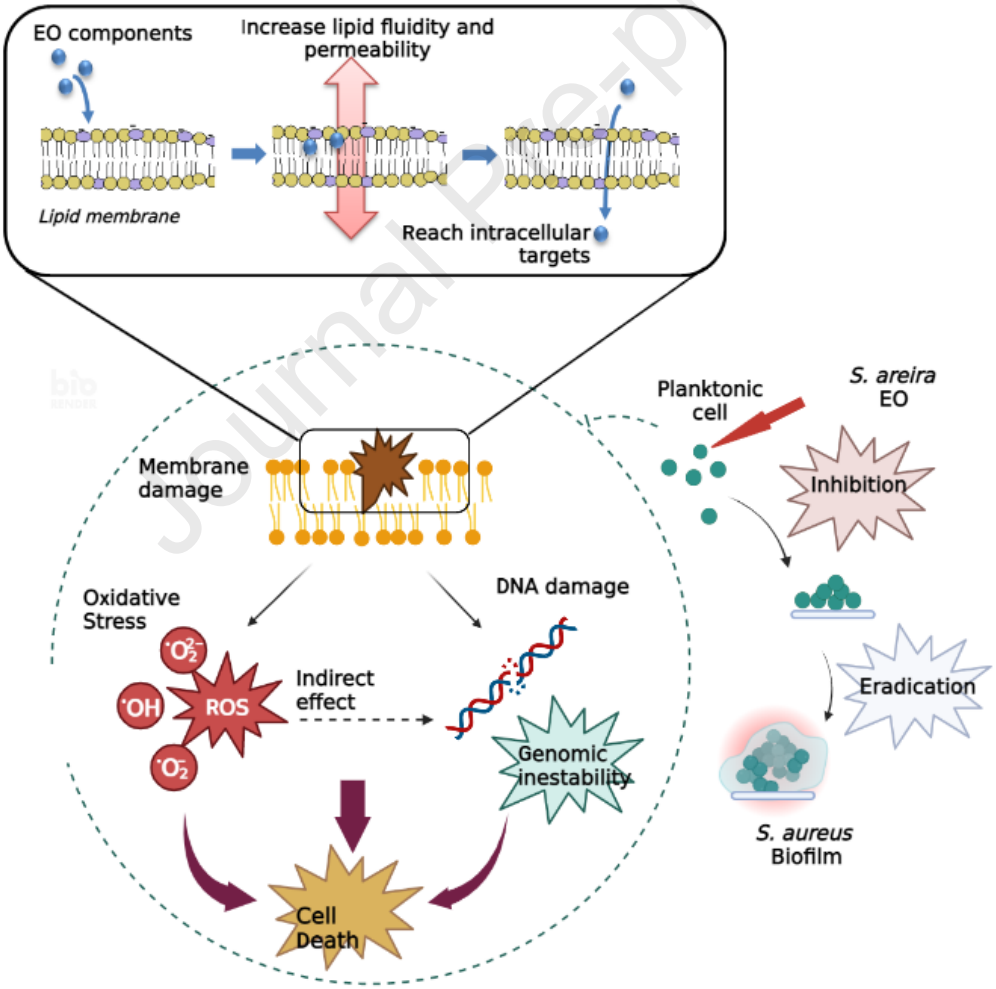

Figure 10: Schematic representation of *S. areira* cell targets on S. *aureus* and antibiofilm action

ACKNOWLEDGEMENTS AND FUNDING

The authors acknowledge the financial support of ANPCyT-FONCyT (PICT 2017-2349), CONICET (PIP 112202101 00756CO) and Universidad Nacional de Santiago del Estero (PI-UNSE 23A/250 and 23/A273-A-2022). AC, MSC, SR, and AH are members of the Research Career of CONICET. AB acknowledges a fellowship from CONICET.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

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Highlights

- EO of *S. areira* can alter the permeability of the *S. aureus* membrane.
- EO of *S. areira* is capable of inducing oxidative stress through ROS generation.
- EO of *S. areira* has different targets involved in the antibacterial activity, from the bacterial membrane to DNA.
- EO of *S. areira* was able to inhibit the biofilm formation, as well as eradicate preformed biofilms of *S. aureus*