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Growth inhibition and morphological alterations of *Staphylococcus aureus* caused by the essential oil of *Aloysia triphylla*

[Inhibición del crecimiento y alteraciones morfológicas de *Staphylococcus aureus* causadas por el aceite esencial de *Aloysia triphylla*]

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Abstract: *Aloysia triphylla* essential oil (EO) has antimicrobial activity on *Staphylococcus aureus* which is a pathogen responsible for severe infections and food contamination. The target of EO is mainly in the cytoplasmic membrane. In this work the mechanisms of action of the EO of *A. triphylla* on *S. aureus* were investigated. *A. triphylla* was collected from La Paz. The oil was analyzed by GC-MS. The antimicrobial effects were evaluated by MIC, MBC, killing time and TEM. MIC values were 23 µg/mL for 6.3 x 104 CFU/mL, 92 µg/mL for 5.71 x 105 CFU/mL and 180 µg/mL for 9 x 106 CFU/mL. The MBC was 5920 µg/mL for all cellular concentrations and it was necessary more time to kill bigger cell populations. Multilamellar and mesosome-like structures on the membrane were seen by TEM. *A. triphylla* oil is an antibacterial compound against *S. aureus* which main mechanism of action seems to be the cytoplasmic membrane disruption.

Keywords: *Aloysia triphylla*, essential oil, antimicrobial activity, *Staphylococcus aureus*, killing time, electronic microscopy.

Resumen: El aceite esencial (AE) de *Aloysia triphylla* posee actividad antimicrobiana contra *Staphylococcus aureus*, patógeno responsable de infecciones nosocomiales e alimenticias. El blanco de acción de los AE es la membrana citoplasmática. El mecanismo de acción del AE de *A. triphylla* sobre *S. aureus* fue investigado. El AE de *A. triphylla* (La Paz, Argentina) fue analizado por CG-EM. Se evaluó el efecto antimicrobiano por CIM, CBM, tiempo de muerte y MET. Los valores de CIM fueron 23 µg/mL para 6.3 x 104 UFC/mL, 92 µg/mL para 5.71 x 105 UFC/mL y 180 µg/mL para 9 x 106 UFC/mL. La CBM fue 5920 µg mL para todas las concentraciones celulares estudiadas. El tiempo de muerte fue determinado; necesitando mayor tiempo para matar una población celular elevada. Fueron observados por MET estructuras multilamerales y semejantes a mesosomas. El AE de *A. triphylla* es un potencial compuesto antibacteriano contra *S. aureus* donde su principal mecanismo de acción es por disrupción de la membrana citoplasmática.

Palabras clave: *Aloysia triphylla*, aceites esenciales, actividad antimicrobiana, *Staphylococcus aureus*, tiempo de muerte, microscopia electrónica.

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INTRODUCTION

The search of natural antimicrobials for the treatment of infectious diseases and for food preservation is having an increasing attention. Researchers have become interested in biologically active compounds isolated from natural sources for the elimination of pathogenic microorganisms, especially those microorganisms that have built resistance to antibiotics (Gachkar *et al.,* 2007). The aromatic plants have the ability to produce secondary metabolites such as essential oils. These oils are variable mixtures of terpenoids, specifically monoterpenes [C10] and sesquiterpenes [C15], although diterpenes [C20] and a variety of low molecular weight aliphatic hydrocarbons may also be present (Dorman & Deans, 2000, Demo & Oliva, 2008). These essential oils become considerably attracting as antimicrobial agents due to their biological activities, which have been widely registered in the literature. Much effort has been made to identify novel compounds with antibacterial activity and to analyze their mechanisms of action. Their site of action seems to be the phospolipid bilayer of the membrane which is involved in many cell biochemical mechanisms including electron transport inhibition, protein translocation, phosphorylation steps and other enzyme dependant reactions (Dorman & Deans, 2000; Carson *et al.,* 2002; Turgis *et al.,* 2009).

Aloysia triphylla (L`Her) Britton, (*Aloysia citriodora* Palau) popularly known as "cedrón", is a member of the Verbenaceae family. It is a perennial aromatic plant and grows widely in North and South America and also in northeast, northwest and central regions of Argentine. It is cultivated from Mexico till the South region of the continent. In the international herbal market it is recognized due to the sensory and medicinal properties of its essential oils (Gil *et al.,* 2007). There are several scientific studies that support the use of products obtained from *A. triphylla*. By this way, good antimicrobial activity of the methanolic and ethanolic extracts could be descripted, as well as the antimicrobial activity of the essential oil against *Candida albicans*, Gram-negative and Gram-positive bacteria (Sartoratto *et al.,* 2004; Demo *et al.,* 2005; Oskay *et al.,* 2005; Demo & Oliva, 2008; Akroum *et al.,* 2009). In the last group is found *Staphylococcus aureus*, which is one of the main pathogen responsible for nosocomial infections. The appearance of *S. aureus* strains resistant to antibiotics represents a difficult problem to solve (Inoue *et al.,* 2005; Silva & Fernandes Jr, 2010). The objective of this work was to make an approach of the mechanisms of action of *A. triphylla* essential oil on *S. aureus,* analyzing the killing time and visualizing the essential oil damage by transmission electronic microscopy.

MATERIAL AND METHODS

Plant material and essential oil obtention

The plant material was obtained from plants growing in farms or plantations located in La Paz, province of Córdoba, in Argentina. Dried leaves (70 g) of the plant were used for each hydrodestillation in a Clevenger-like apparatus. The essential oil obtained was dried with anhydrous sodium sulphate and stored in the freeze until analysis (De Feo *et al.,* 1998). The essential oil was analyzed with GC-MS.

Gas chromatography

The essential oil composition was analyzed with a Shimadzu GC-R1A gas chromatograph equipped with a fused silica column (30 m x 0.25 mm) coated with CBP-1. The temperature of the column was programmed from 60 ºC to 240 ºC at 4º C/min. The injector and detector temperatures were at 270 ºC. The gas carrier was He, at a flow rate of 1 mL/min. Peak areas were measured by electronic integration. The relative amounts of the individual components are based on the peak areas obtained, without FID response factor correction. Programmed temperature retention index of the compounds were determined relative to n-alkanes. GC analysis was still performed using a column Supelcowax-10 with the same conditions as described above (Zunino *et al.,* 1998).

Gas Chromatography-Mass Spectrometry

GC-MS analyses were performed on a Perkin Elmer Q-910 using a 30 m x 0.25 mm capillary column coated with CBP-1. The temperature of the column and the injector were the same as those from GC. The carrier gas was He, at a flow rate of 1ml/min. Mass spectra were recorded at 70 eV. The oil components were identified by comparison of their retention indices, mass spectra with those of authentic samples, by peak enrichment, with published data, mass spectra library of National Institute of Standards and Technology (NIST 3.0) and our mass spectra library which contains references mass spectra and retention indices of volatile compounds. GC-MS analysis was

still performed using a column Supelcowax 10 with the same conditions as describe above (Adams, 1989).

Microorganisms

The biological activity of the essential oil was tested against the gram positive bacterium *Staphylococcus aureus* ATCC 25923.

Culture methods

Tubes containing Müeller-Hinton Broth (MHB) (Britania) were prepared at pH 7, inoculated with the microorganism and incubated overnight (18 h) at 37 ºC Optical densities were measured at 620 nm in a spectrometer. Cell densities were estimated from standard curves and confirmed by the viable plate count on Tripteine Soy Agar (TSA) (Britania). The microorganism cell concentration necessary to cause reduction of resazurin within 2 h was determined. For this, serial 10 fold dilutions of the overnight culture were prepared in MHB. Aliquots (170 µL) of the inoculumms were dispensed into microtitre containing 20 µL of dimethyl sulfoxide (DMSO) and 10 µL of resazurin solution. The microtitre was incubated for 2 h at 37 ºC. The appropriate dilution to work was that one unable to reduce resazurin (blue), this means ≈ 1 log cycle lower than the cell density required to reduce resazurin (usually 10^6 CFU/mL). The plate count method was made to this dilution. Resazurin is a redox indicator that is blue in its oxidized form and pink in its reduced form. (Mann & Markham, 1998)

Determination of the Minimum Inhibitory Concentration (MIC) of Aloysia triphylla essential oil

The antimicrobial activity of the essential oil was determined by the Broth Microdilution Method described by Mann & Markham, (1998) Serial two fold dilutions of the essential oil were prepared in DMSO by vortexing it at room-temperature. A sterile 96-well microtitre tray was set up with the dilution of bacteria as follows: column 1-10, 170 µL inoculum plus 20 µL of essential oil dilution; column 11, 170 µL inoculum plus 20 µL of diluent (positive control = pink); column 12, assay medium (MHB) plus 20 µL of diluent (negative control = blue). The microtitre was incubated at 37 ºC for 3.5 h. After incubation 10 µL of resazurin solution was added to all the wells. After a second incubation of 2 h at 37 ºC, wells were assessed visually for colour change, with the hightest dilution remaining blue indicating the MIC. Benzatinic Penicillin G (2.400.000 UI) was used as positive control. (Mann & Markham, 1998)

Determination of the Minimum Bactericidal Concentration (MBC) of Aloysia triphylla essential oil

The MBC was determined as follows: 100 µL of the dilution belonging to the MIC and the previous dilutions were inoculated in Mueller-Hinton Agar (MHA) and incubated at 37 ºC for 24 h. The MBC was considered as the last dilution without cellular growth (Finengold & Baron, 1992).

Inoculum effect of Aloysia triphylla essential oil on MIC and MBC

In order to see the influence that the inoculum has on MIC and MBC, the MIC and the MBC of *A. triphylla* were determined using final inoculumms concentrations of 10^4, 10^5 and 10^6 CFU/mL, with the methods previously described.

Killing-Time assays

Cells of *S. aureus* from an overnight culture in Tripteine Soy Broth (TSB) were centrifuged at 4500 rpm for 15 min. The cell pellet was resuspended with phosphate-buffered saline (PBS; pH 7.0) and the essential oil was added at concentrations ranges from 2.9 to 56.2 mg/mL. During all the time of the experience, the suspensions were mixed in a shaker at 120 rpm, 37 ºC. The samples were removed at different time intervals for the determinations of viable cell, during 8h. For this, aliquots of each sample were plated on Tripteine Soy Agar (TSA). The plates were then incubated at 37 ºC for 24 h and the presence or absence of growth was estimated. Killing curves were constructed by plotting numbers of viable cells against time. All assays were performed in triplicate (Carson *et al*., 2002).

Transmission Electronic Microscopy (TEM)

Cells of *S. aureus* from an overnight culture in Tripteine Soy Broth (TSB) were treated at the MBC of essential oil (5.9 mg/mL) and centrifugated at 4500 rpm for 15 min. The cell pellet was resuspended with phosphate-buffered saline (PBS; pH 7.0) and kept until the TEM was performed. For the TEM, the bacterial suspension was fixed in glutaraldheyde (2.5%) in buffer S-collidine (0.2 M, pH: 7.4) for 3 h at 4 ºC. It was washed twice with S-collidine and refixed in osmium tetroxide (1%) for an hour at room

temperature. It was washed twice with S-collidine and dehydrated with acetone (30%, 50%, 70%, 90% and 100% for 5 min). Pre-inclusion was made in epoxi resine EMbed 812 1:1 in 100% acetone during all the night at room temperature. Inclusion was made with EMbed 812 at 56 ºC, during 24 h. (Cristofolini *et al.*, 2009).

RESULTS

Chemical composition of the essential oil of *Aloysia triphylla*

The chemical composition of the essential oil from *Aloysia triphylla* collected from La Paz (Argentina) was investigated by means of gas chromatographic techniques. The essential oil average yield was 0.4% (w/v) and the main components found were: geranial (21,3%), neral (18,7%), limonene (6,9%), caryophyllene oxide (1%) and spathulenol (0,9%). These terpenes are characteristically described for this specie (Oliva *et al.*, 2010).

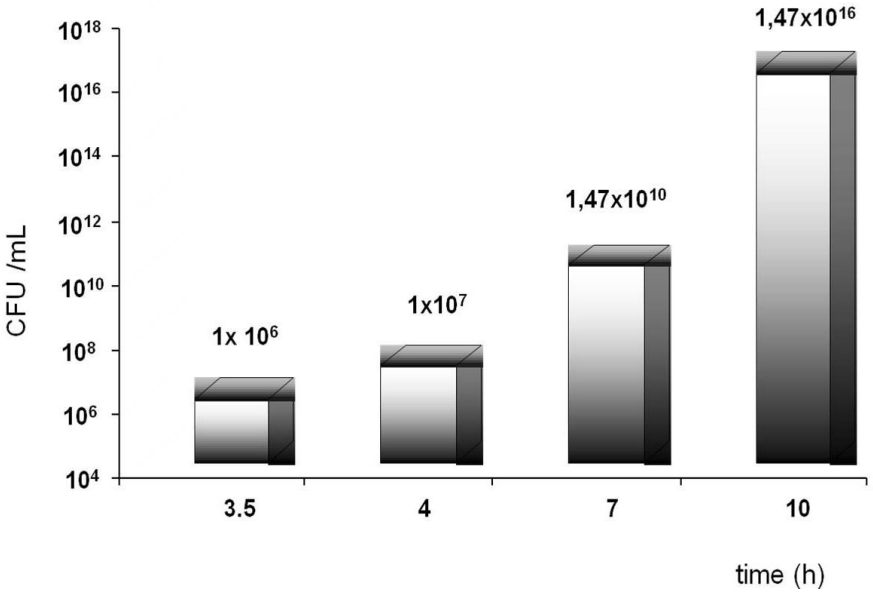
Inoculumm effect on MIC and MBC

The Minimun Inhibitory Concentration (MIC) and the Minimun Bactericidal Concentration (MBC) of the essential oil were evaluated using three different cellular concentrations: 6.3 x 10^4 CFU/mL, 5.7 x 10^5 CFU/mL and 9 x 10^6 CFU/mL. The MIC results obtained for an inoculumm of 6.3 x 10^4 CFU/mL was 2.3 µg/mL, for 5.7 x 10^5 CFU/mL the MIC was 9.2 µg/mL and for an inoculum of 9 x 10^6 CFU/mL the MIC was 200 µg/mL. MIC value for penicillin was 9.4 UI/mL. All cellular concentrations showed a MBC of 5900 µg/mL.

Bacterial killing assays

The time that is necessary to kill *S. aureus* cells (killing time) at the MBC (5900 µg/mL) was determined at different cells concentrations. Results are shown in Figure 1. The time necessary to kill a bacterial population of 8.9 x 10^6 CFU/mL was 3.30 h. For a cellular concentration of 1 x 10^7 CFU/mL, the killing time was 4 h; for 1.4 x 10^{10} CFU /mL the killing time was 7 h and for 1.4 x 10^{16} CFU /mL was 10 h. These data showed that for a unique essential oil concentration the killing time was dependant on cell concentration. In all cases the viability control (cells without treatment) presented a macroscopically visible growth.

Figure 1
Killing times (h) for Staohylococcus aureus after treatment with Aloysia triphylla essential oil (5.9 mg/mL)



In the experiences described above was exposed how the microbial population size had influence on the killing time. In this section is described the effect that different essential oil concentrations had on the killing time using two cellular concentrations: 10^6 and 10^9 CFU/mL. The results showed that when very high essential oil concentrations were used, 56.2 mg/mL, killing time was 4 and 5 min for both inoculums, respectively. When smaller essential oil concentrations were used (5.9 mg/mL) the killing time increased for both celullar concentrations (Table 1).

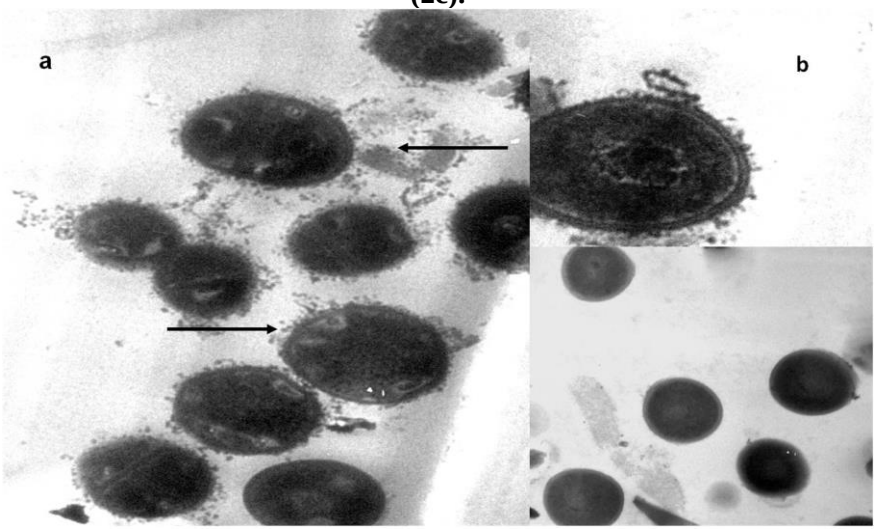
Table 1
Killing time of S. aureus at different Aloysia triphylla essential oil concentrations

EO (mg/mL)	Killing time (min)	
	106 CFU/mL	109 CFU/mL
56.2	4	5
28.1	20	60
5.9	210	260
2.9	235	300

Transmission Electronic Microscopy (TEM)

In TEM assays the images clearly showed the damage that *A. triphylla* essential oil had on the cellular structure. Microstructural observations showed that *S. aureus* cells were affected when they were treated with 5.9 mg/mL (MBC) of the oil. It was observed that cytoplasm of some cells seem to be less dense and altered than non treated cells. The cytoplasm contained multilamellar mesosome-like structures and seems to have lost its distribution, observing the intracellular materials aggregated near the cell wall. In addition, the contents of some treated cells appeared depleted and amorphous material was seen surrounding the cells, indicating that cell membrane structures were severely affected and damaged by the antimicrobial agent (Figure 2a). It was also observed that bacterial membrane was disrupted and a complete loss of membrane integrity was evident showing deformation of the surface (Figure 2b). Furthermore, it could be observed that the intracellular content was leaked through the membrane (Figures 2a & 2b). When the experience was made in the absence of the essential oil (untreated cells), *S. aureus* presented the typical rounded morphology and integrity of the cell membrane (Figure 2c).

Figure 2
Electronic microscopy of S. aureus cells treated with essential oil (5.9 mg/mL) (16700x). Figure show multimellar, mesosomelike structures, cellular contents depleted and amosphous (2a). The membrane is disrupted and it is observed surface deformation (2b). S aureus cells without essential oil treatment (control) (2c).



DISCUSSION

The essential oils are produced in specific glandular structures of aromatic plants called trichomes. The quantity and composition of the essential oil of *A. triphylla* vary according to the part of the plant from which it was extracted, the age of the plant (young/old) and the harvesting season of the plant. The components commonly found in the essential oil of *A. triphylla* are: neral, geranial limonene, geranyl acetate, betacaryophyllene, *ar*-curcumene, and spathulenol. Other compounds that could be found in specific chemotypes are carvone, cedrol, 1,8-cineol, thujone isomers and citronellal (Gil *et al.,* 2007). The chemical composition determines the biological properties of the essential oils. The variability in this composition influences the antimicrobial activity of the oils. Previous studies made with the essential oils of this plant collected from different provinces of Argentina: Salta, Mendoza, Córdoba and San Luis, showed that the highest relation of the main components of the oil, citral/limonene, presented the best antimicrobial activity against Gram positive and Gram negative bacteria and yeasts. In that work, the oil obtained from plants collected in Cordoba (La Paz) presented the highest relation and was the most active (Oliva *et al.,* 2010). Consequently, this essential oil was chosen to perform the present study.

The antimicrobial activity of essential oil on Gram positive and Gram negative bacteria has been largely documented. In this way, the effective action of oily compounds derived from aromatic plants on *S. aureus*, was largely demonstrated in studies with *Melaleuca alternifolia* (tea tree) oil, *Rosmarinus officinalis*, *Caryophyllus aromaticus* L, *Z. officinalis*, *C. citratus*, *Mentha piperita*, *Cinnamomum zeilanicum* Blume, *A. triphylla*, *Bacharis flabellata, Psila spartioides* and *Achyrocline satureioides* (Burt. 2004; Demo *et al.,* 2005; Yasunakaa *et al.,* 2005; Gil *et al.,* 2007; Silva & Fernandes Jr. 2010). *S. aureus* is a major human pathogen that causes a wide spectrum of infections, ranging from superficial wound infections to life-threatening septicaemia and toxic-shock syndrome (Bore *et al.,* 2007). Methicillin-sensible *S. aureus* and methicillin-resistant *S. aureus* (MRSA) have created major problems for burn units and intensive care units. Alternative therapies are being sought for treatment of MRSA and one area of interest is the use of essential oils and their constituents (Edwards-Jones *et al.,* 2004). It is also a major food poisoning bacterium posing a great risk to consumer health, mainly through its production of heat-stable enterotoxins (Bore *et al.,* 2007).

Antimicrobial activity varies according to the methodology employed. In previous studies, the antimicrobial activity and MIC of *A. triphylla* essential oils using the disk diffusion method was reported (Demo *et al.,* 2005; Oliva *et al.,* 2010). The usefulness of this method is limited to the generation of preliminary, qualitative data only, as the hydrophobic nature of most essential oils prevents the uniform diffusion of these substances through the agar medium (Hammer *et al.,* 1999). The broth dilution methods are also commonly used to determine antimicrobial activity and results obtained with these methods are more sensitive. The differences obtained with both methodologies may be attributed to the microbial growth, the solubility of the oil components, the use and quantity of an emulsifier and the time of exposure of microorganisms to plant oil (Hammer *et al.,* 1999). In this work, MIC was determined with a coloured (rezasurine) broth microdilution method that resulted in a total time of contact of the microorganism with the essential oil of five and a half hours, approximately. This exposition time was enough to observe the inhibition effect of the oil on the microbial growth. Carson *et al*. (1995) demonstrated that the time of exposition of the microorganism to the oil was dependent of the microorganism species (Carson *et al.,* 1995).

There is not an agreement on the acceptable inhibition level for plant extracts when they are compared with standards levels. It was proposed a classification of plant materials based on MIC results (strong inhibitors: MIC up to 0.5 mg/mL; moderate inhibitors: MIC between 0.6 and 1.5 mg/mL; weak inhibitors: MIC above 1.6 mg/mL (Texeira Duarte *et al.,* 2007). Taking into consideration these values, the *A. triphylla* essential oil can be considered a strong inhibitor of *S. aureus*, since MIC values were $\leq$ 0.2mg/mL.

This work has demonstrated that the MIC of *A. triphylla* essential oil varied with the inoculums, requiring more oil concentration to inhibit more cells. In other words, MIC increased as the concentration of cells did. In contrast, the MBC was the same for all cell concentrations used, meaning that there was a bactericidal effect that was not dependant on the inoculums concentration, but on the time of exposition of the bacteria to the oil.

The time-kill assay was a useful weapon to confirm that the essential oil had bactericidal activity and helped to know when the cell death was produced. Killing time results were in agreement with other investigations made with *Cumminum cyminum* L. and *Rosmarinus officinalis* which were able to kill *S. aureus* (10^7 UFC/mL) at 180 and 240 min, respectively (Gachkar *et al.,* 2007). Similar results were obtained in this work with 10^6 and 10^9 CFU/mL, where the killing time was 210 and 260 min, respectively. It could be observed from the results that the time required for a complete bactericidal effect of the oil (MBC: 5.9 mg/mL) increased when cellular concentrations increased. When high concentrations of the oil were used (> 5.9 mg/mL) the killing time was shorter for both cellular concentrations used (10^6 CFU/mL: 4 min and 10^9 CFU/mL: 5 min). This means that the time necessary to kill *S. aureus* with *A. triphylla* essential oil was dependant on the cellular concentrations used and the essential oil concentration.

A. triphylla essential oil did not provoke whole cell lysis of *S. aureus* but compromise the structural integrity of the plasmic membrane and induce a loss of the cytoplasmic contents. It showed multilamellar, mesosome-like structures and the contents of some treated cells appeared depleted and amorphous. These morphological alterations could be observed in TEM studies. Similar results of the effect of oily compounds on the cellular structure of *S. aureus* were obtained by other authors. Carson *et al.* (2002) who studied the effect of terpinen-4-ol observed damaged in the cytoplasmic membrane suggesting that nucleic acids were lost through it (Carson *et al.,* 2002). Aggregation of intracellular material near the cell wall caused density cytoplasmic changes in *S. aureus* cells put under the action of *I. graveolens* and *S. corsica* essential oils. As a consequence, unusual thickenings were observed in the cell wall more pronounced near the membrane invaginations to the cell wall, which loses its uniformity and tends to become rough (Guinouseau *et al.,* 2010). The electron micrographs of *S. typhimurium* and *S. aureus* cells treated with the essential oil of *Zataria multiflora* and the essential oil combined with nisin, showed important morphological damages and disruption in membranes, increasing the permeabilization of cells to dyes appearing the contents of the cells depleted and amorphous (Moosavy *et al.*, 2008). Other studies of scanning electron microscopy made on Gram negative bacteria treated with mustard essential oil showed disruption in membranes and damaged morphology, lost of cellular components, decrease of intracellular ATP concentration, increase of extracellular ATP and a significant diminution of the internal pH (Turgis *et al.,* 2009). Researches made with *Salmonella typhi* treated with eugenol, showed deformation in their surface and disruption of the cellular membrane with a complete loss of membrane integrity (Pandima *et al.,* 2010).

The results of the experiments exposed in this work demonstrate that *A. triphylla* essential oil exerted its effect mainly in the cytoplasmic membrane, causing severe damages that finally caused the death of *S. aureus* cells. This killing ability may be associated to the large chemical composition of the essential oil, provoking that multiple mechanisms in the cell became altered. Research works of the mechanisms of action of essential oils agrees that the primary site of action of essential oils is the cytoplasmic membrane. This effect may be directly related to the hydrophobicity of the constituents of essential oils, allowing them to partition into the bacterial lipid bilayer, disturbing its structure and increasing its permeability to protons, ions and other cell constituents (Sikkema *et al.,* 2005; Carson *et al.,* 2006; Guinouseau *et al.,* 2010). What is more, terpenic compounds have the ability to cross the lipid bilayer and interact with the intracellular material causing severe damages to cells with several invasive targets which could lead to to the inhibition of bacterial pathogens such as *S. aureus* (Cristani *et al.*, 2007; Guinouseau *et al.*, 2010).

The pathogenic ability of *S. aureus* is important in clinics and in the food industry. It could become easily resistant to antibiotics, like methicillin resistant *S. aureus* (MRSA), making it difficult to treat. The essential oil of *A. triphylla* is an effective antimicrobial natural product and it could be considered a potential antibacterial compound that could be used against pathogenic species such as *S. aureus*. As other oily compounds, its primary target is the cytoplamic membrane structure, causing structural and functional alterations that provoke cellular death. In the future *A. triphylla* essential oil could be used for terapheutical formulations in replacement of antibiotic to treat diseases caused by resistant microorganisms such as methicillin resistant *S. aureus* (MRSA) and in the food industry to avoid the development of this microorganism.

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