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# Antimicrobial activity of cinnamon (*Cinnamomum verum*) essential oil and cinnamaldehyde against *Staphylococcus aureus*

**Atividade antimicrobiana da canela (*Cinnamomum verum*) óleo essencial e cinamaldeído contra *Staphylococcus aureus***

**Actividad antimicrobiana de la canela (*Cinnamomum verum*) aceite esencial y cinamaldehído contra *Staphylococcus aureus***

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**Abstract**
The aim of this study was to compare the antimicrobial activity of the essential oil (EO) of cinnamon (*Cinnamomum verum*) and its major compound cinnamaldehyde against *Staphylococcus aureus* (ATCC 14458) and bacteria isolated from mastitic milk. Cinnamon EO was chemically characterized by gas chromatography/mass spectrometry (GC-MS). The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of cinnamon EO and cinnamaldehyde were determined, as well as the effects of MIC and CBM on *S. aureus* cells, visualized by Scanning Electron Microscopy (SEM) and Microscopy Transmission Electronics (TEM). A synergistic evaluation of the EO of cinnamon and cinnamaldehyde with conventional antimicrobials was also performed. Results: The substance cis-cinnamaldehyde (58.7%) was identified as the major component of cinnamon EO. The MIC values determined against all tested strains ranged between 0.8 and 1.6 mg.mL$^{-1}$. SEM and TEM images (post-treatment with cinnamon EO and cinnamaldehyde) showed coarse-shaped *S. aureus* cells with residues adhered to the cell wall and irregular and irregular regions at the points of cell division. Conclusion: Both cinnamon EO and cinnamaldehyde showed undetermined antimicrobial activity combined with conventional antimicrobials, which brings the prospect of their ability to intensify the antimicrobial action of veterinary drugs used in the treatment of mastitis caused by multidrug-resistant bacteria.
**Keywords:** Antimicrobials; Mastitis; ATR- FTIR; GC-MS.

**Resumo**

O objetivo deste estudo foi comparar a atividade antimicrobiana do óleo essencial (OE) de canela (*Cinnamomum verum*) e seu composto majoritário cinamaldeído contra *Staphylococcus aureus* (ATCC 14458) e bactérias isoladas de leite mastítico. O OE de canela foi caracterizado quimicamente por cromatografia gasosa/espectrometria de massas (GC-MS). A Concentração Inibitória Mínima (CIM) e a Concentração Bactericida Mínima (CBM) de OE de canela e cinamaldeído foram determinadas, bem como os efeitos de CIM e CBM em células de *S. aureus*, visualizados por Microscopia Eletrônica de Varredura (MEV) e Microscopia Eletrônica de Transmissão ( TEM). Uma avaliação sinérgica do OE de canela e cinamaldeído com antimicrobianos convencionais também foi realizada. Resultados: A substância cis-cinamaldeído (58,7%) foi identificada como componente majoritário do OE de canela. Os valores de CIM determinados contra todas as cepas testadas variaram entre 0,8 e 1,6 mg.mL$^{-1}$. As imagens SEM e TEM (pós-tratamento com OE de canela e cinamaldeído) mostraram células de *S. aureus* com formato grosseiro com resíduos aderidos à parede celular e regiões irregulares e irregulares nos pontos de divisão celular. Conclusão: Tanto o OE de canela quanto o cinamaldeído apresentaram atividade antimicrobiana indeterminada combinada com antimicrobianos convencionais, o que traz a perspectiva de sua capacidade de intensificar a ação antimicrobiana de medicamentos veterinários utilizados no tratamento de mastite causada por bactérias multirresistentes.

**Palavras-chave:** Antimicrobiano; Mastite; FTIR- ATR; CG-MS.

**Resumen**

El objetivo de este estudio fue comparar la actividad antimicrobiana del aceite esencial (AE) de canela (*Cinnamomum verum*) y su compuesto mayoritario cinamaldehído contra *Staphylococcus aureus* (ATCC 14458) y bacterias aisladas de leche mastítica. El EO de canela se caracterizó químicamente mediante cromatografía de gases/espectrometría de masas (GC-MS). Se determinaron la Concentración Mínima Inhibitoria (MIC) y la Concentración Mínima Bactericida (MBC) de AE de canela y cinamaldehído, así como los efectos de MIC y CBM sobre células de *S. aureus*, visualizados por Microscopía Electrónica de Barrido (SEM) y Microscopía Electrónica de Transmisión (TEM). También se realizó una evaluación sinérgica del AE de canela y cinamaldehído con antimicrobianos convencionales. Resultados: La sustancia cis-cinamaldehído (58,7%) fue identificada como el componente mayoritario del AE de canela. Los valores de CIM determinados frente a todas las cepas probadas oscilaron entre 0,8 y 1,6 mg.mL$^{-1}$. Las imágenes SEM y TEM (post-tratamiento con AE de canela y cinamaldehído) mostraron células de *S. aureus* de forma gruesa con residuos adheridos a la pared celular y regiones irregulares e irregulares en los puntos de división celular. Conclusión: Tanto el AE de canela como el cinamaldehído mostraron una actividad antimicrobiana indeterminada en combinación con los antimicrobianos convencionales, lo que abre la perspectiva de su capacidad para intensificar la acción antimicrobiana de los medicamentos veterinarios utilizados en el tratamiento de la mastitis causada por bacterias multirresistentes.

**Palabras clave:** Antimicrobianos; Mastitis; FTIR- AT; GC-MS.

## 1. Introduction

Bovine mastitis is a disease frequently seen in dairy cattle (Langoni et al., 2017), which generates physical-chemical changes in the composition of milk because of the increase of somatic cells and the presence of pathogens. In addition, it can cause economic losses such as drop in milk production, compromised animal health, spending on medicines, and early disposal of animals (Maiochi et al., 2019). As it is an inflammation that occurs in the mammary glands of dairy cows because of the invasion and multiplication of pathogenic bacteria, the destruction of the milk-producing tissues can occur in clinical cases of mastitis (Mushtaq et al., 2017).

The prevalence of mastitis in cattle is related to the health management of animals and milking (Langoni et al., 2017). *Staphylococcus aureus* is one of the main etiologic agents of bovine mastitis (Pereira et al., 2011; Wang et al., 2015), followed by *Escherichia coli* and *Streptococcus uberis* (Langoni et al., 2017). The genus *Staphylococcus* spp. is among the pathogens that most cause problems in herds, with isolation rates ranging from 8.3% to 49.23% (Oliveira & Medeiros, 2015).

The inappropriate use of antimicrobials associated with the resistance mechanisms of the strains of *S. aureus* are the main factors that contribute to the increase in the rates of microbial resistance (Oliveira & Medeiros, 2015; Costa et al., 2013; Loureiro et al., 2016).

Bacterial resistance to antimicrobials can be classified initially as intrinsic or acquired. Intrinsic resistance is one that is part of the natural characteristics of individuals of a species. Acquired resistance occurs when some individuals of a species mutate or receive plasmids with resistance genes, thus acquiring this characteristic and differentiating them from parental cells.

A simple genetic change can lead to the appearance of a very resistant bacterial strain. Both types of resistance are a defense mechanism of bacteria, which is a serious problem in the treatment of infections and has substantially reduced the activity of important antibiotics (Barbosa & Torres, 2010).

The misuse of antimicrobials is a public health concern, as many microbial species are becoming resistant to antimicrobials and generating a series of consequences for society, such as: prolonged disease treatment, increased mortality rate, and inefficient preventive treatments (Brasil, 2017). The development of new antimicrobials means increasing the options for compounds that can be used against pathogenic bacteria resistant or not to conventional antimicrobials (Silva et al., 2010). Among the proposals for new antimicrobial bioactives, essential oils (EOs) are known for having activity against a wide variety of microorganisms. An important characteristic of EOs and their components is hydrophobicity (Ferro et al., 2016), as this property causes changes in cellular structures because of the permeability of hydrophobic particles of EOs by the bacterial cell membrane (Chouhan et al., 2017).

The aim of this study was to compare the antimicrobial activity of cinnamon EO and its major compound cinnamaldehyde against *Staphylococcus aureus* and bacteria isolated from mastitic milk.

## 2. Methodology

For this study, EO extracted from Ceylon - Sri Lanka cinnamon bark (*Cinnamomum verum*), obtained commercially from the company LASZLO (batch 1378) and cinnamaldehyde with purity ≥95% (Sigma Aldrich) were used. Seven bacterial strains isolated from milk from cows with clinical and subclinical mastitis were used in a previous study (data not shown), which showed growth of typical colonies (black and ± 1 mm) of *Staphylococcus* sp. in Baird-Parker agar, differentiated as positive coagulase (codes K04, K30, K31, and K34) and negative coagulase (codes K13, K16, and K26). A standard strain of *Staphylococcus aureus* INCQS 00005 (ATCC 14458) from the Collection was also used from the Reference Microorganisms in Health Surveillance - CMRVS, FIOCRUZ-INCQS, Rio de Janeiro, RJ, Brazil.

### 2.1 Chemical inference by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)

The spectra of cinnamon EO and cinnamaldehyde samples were obtained using the ATR-FTIR technique in the medium- infrared 4000-400 $cm^{-1}$ region in a Cary 630 (Agilent) equipment. The method described by Ruschel, et al., (2014) was used with adaptations. The sample was spread over the surface of the ATR crystal, and then the spectrum was obtained using the software Microlab Pc (Agilent). The spectra were interpreted according to Lopes and Fascio (2004).

### 2.2 Chemical characterization of cinnamon EO by gas chromatography - mass spectrometry (GC-MS)

Chemical characterization was performed in a gas chromatograph coupled to a mass spectrometer (GC-MS) (Shimadzu, model GCMS-plus-QP2010), in which a capillary column DB-5 measuring 30 meters x 0.25 mm x 0.25 mm was used. The method described by Beraldo et al. (2013) was used with adaptations. The injector and interface temperature were at 250ºC. The detector was operated in Electron Impact (EI) mode at 70 eV and helium was used as the carrier gas. The chromatographic conditions for cinnamon EO were an initial temperature of 40ºC (2min) at a rate of $3ºC.min^{-1}$, heating up to 250ºC and maintaining this temperature for 10 minutes, 1:10 split injection, and injected volume of 1 µL of sample. A mixture of linear alkanes ($C_{10}$ to $C_{39}$) was injected into the chromatograph under the same conditions used as the standard to calculate the retention index with linear temperature programming. Identification was performed by comparing the retention index with libraries (Adams, 2017; Nist, 2019).

**2.3 Antimicrobial activity**

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of cinnamon EO and cinnamaldehyde were determined using the microdilution method (CLSI, 2009) in microplates with adaptations. The concentrations of 25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.1, and 0.05 $mg.mL^{-1}$ were tested.

For the preparation of the highest concentration, cinnamon EO or cinnamaldehyde was diluted in equal parts of propylene glycol and subsequently in Mueller Hinton (MH) broth with 0.5% Tween 80, which resulted in a double concentration. From this concentration, serial dilution was performed on the microplate to obtain 50 µL of each concentration (columns) in the microplate wells.

The tested bacteria were activated in 3 mL of Tryptic Soy Broth (TSB) and incubated at 35ºC for 24hours. The standardization of cultures was performed on the 0.5 scale of MacFarland ($1.0 \times 10^8.UFC.mL^{-1}$) using a spectrophotometer (Kasuaki, IL-227) with a wavelength of 625 nm. Then, the inoculum was prepared with two serial dilutions, the first dilution being 1:10 in saline ($1.0 \times 10^7$ $UFC.mL^{-1}$) and the second dilution 1:10 in MH broth ($1.0 \times 10^6.UFC.mL^{-1}$).

In the microplate, 50 µL of the inoculum were added to each well, thus resulting in a cell concentration of $5.0 \times 10^5.UFC.mL^{-1}$ in addition to the concentrations of cinnamon EO or cinnamaldehyde to be tested. The microplates were incubated in a bacteriological incubator at 37ºC for 24hours. The MIC was determined with the addition of 50 µL of the 0.01% resazurin indicator in each well. After two hours of incubation at 37ºC, a visual reading of the stain was made, with bacterial inhibition being considered blue and bacterial growth being pink.

The verification of MBC was determined by streaking each well of the microplates in Petri dishes containing MH agar (MHA) before adding the resazurin indicator. The plates were incubated under aerobic conditions for 24hours at 37°C. The non-development of bacterial colony at the peak of each well indicated bactericidal activity.

Negative controls were prepared (only MH, higher and lower concentrations of cinnamon EO or cinnamaldehyde) and positive controls (only inoculants in MH broth). All tests were performed in triplicate. The MIC and MBC of each strain were considered the lowest concentration with the respective effect, in at least two replicates. From all MIC and MBC values, $MIC_{90\%}$ and $MIC_{50\%}$ were calculated, capable of inhibiting 90% and 50% of strains, respectively. $MBC_{90\%}$ and $MBC_{50\%}$ were also calculated, capable of killing 90% and 50% of strains, respectively.

**2.4 Synergism by disk diffusion of cinnamon EO and cinnamaldehyde with conventional antimicrobials**

Synergistic assessments of cinnamon EO and cinnamaldehyde were performed with conventional antimicrobial drugs: penicillin (PEN), tetracycline (TET), oxacillin (OXA), cefepime (CPM), ciprofloxacin (CIP), sulfazotrim (SUT), gentamicin (GEN), vancomycin (VAN), clindamycin (CLI), erythromycin (ERI), chloramphenicol (CLO), and rifampicin (RIF) against the standard culture of *S. aureus* (ATTC 14458). The method described by Albano et al. (2016) was used with adaptations. The Mueller Hinton Agar (MHA) culture medium was prepared with 0.5% Tween 80 and cinnamon EO or cinnamaldehyde, in the respective concentrations equivalent to 90% MIC, ½ 90% MIC, and ¼ 90% MIC.

The bacterial suspension, prepared with overnight culture in Brain Heart Infusion (BHI) broth, was standardized at a concentration equivalent to the 0.5 McFarland scale and then inoculated with the aid of sterile swabs in Petri dishes containing MHA. The antimicrobial drug disks were placed on the surface of the culture medium after drying the inoculum.

Control plates for bacterial growth were prepared without the addition of antimicrobial disks. The plates were incubated at 37ºC for 24hours and then the inhibition halo was measured (mm). Seven repetitions were performed for each treatment and the mean and standard deviation of the inhibition halos were calculated. The results of the control antibiogram were evaluated according to CLSI (2017).

**2.5 Electron Microscopy**

The techniques of Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) were performed according to the method described by Santos et al. (2008) with adaptations. The cells of *Staphylococcus aureus* bacteria (ATCC 14458) were treated separately with concentrations of cinnamon EO or cinnamaldehyde in the values established by $MIC_{90\%}$ and $MBC_{90\%}$, in addition to the control treatment (bacterial culture only).

The bacterial cultures were prepared in BHI broth and incubated at 37ºC for 24hours, standardized in the cell concentration equivalent to the 0.5 McFarland scale ($1.0x10^8$.UFC.$mL^{-1}$). In a conical tube, a 1:10 dilution was prepared with 0.5% Tween 80 MH culture medium and standardized bacterial culture ($1.0x10^7$.CFU.$mL^{-1}$) and cinnamon EO or cinnamaldehyde, in concentrations established for MIC and MBC. The tubes were incubated in a bacteriological oven at 37ºC for 24hours. After the incubation time, the samples were centrifuged and washed in phosphate buffer solution (pH 7.2). The supernatant and precipitates from the samples (pellet) were discarded and used for the next steps. During fixation, a 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer containing 1.0 mM CaCl2 was added over the pellet, stirred manually, and left to stand at room temperature for 2 hours. After fixation, the solution was discarded and the pellets were washed three times with sodium cacodylate buffer solution. In a glass coverslip with previously adhered poly-L-lysine, 30µL of pellet resuspended in sodium cacodylate buffer were added. After one hour, the samples were dehydrated in ethanol gradient solutions (30%, 50%, 70%, 80%, 90%, and 95% once and 100% three times) with 15-minute intervals for each concentration of ethanol. To finish the dehydration, the samples were submitted to the critical point of $CO_2$, metallized with gold, and observed in a scanning electron microscope Shimadzu SS-550.

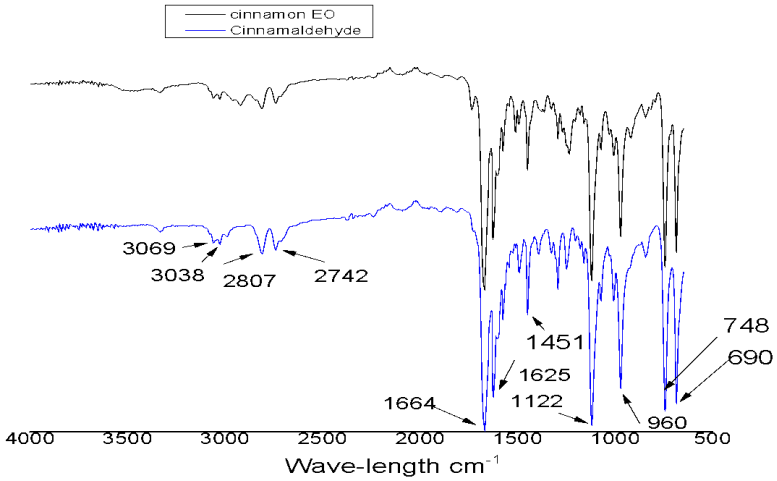
For TEM, biological samples prepared in the SEM procedure up to the fixation stage were used. After this step, the sample material was washed with 0.1 M sodium cacodylate buffer and post-fixed for one hour at room temperature in 1% osmium tetroxide plus 0.8% potassium ferrocyanide and calcium chloride 5 mM. After three washes in sodium cacodylate buffer solution, the pellets were dehydrated in acetone gradients (50%, 70%, and 90% once and 100% three times), at intervals of 5 minutes for each concentration. Then the pellets were soaked in dilutions of Epon® resin (EPON / acetone (v/v) (1:2), EPON/Acetone (1:1), EPON/Acetone (2:1), and pure EPON) with an 8h interval for each dilution. Polymerization (packaging) was carried out in silicone form with pure EPON and sample pellets at the ends of the form, and drying was carried out in an oven at 60ºC for 72h. The blocks obtained were a pyramid so that the sample was centered at the tip of the block. The pyramid blocks were placed on the ultramicrotome (Reichert Ultracut E) for the ultrathin sections of the sample. The cuts were collected in water, placed on a copper grid, and directed to the contrast phase with 5% uranyl acetate and lead citrate for 30 minutes each. After this period, the grids were washed with distilled water, dried with filter paper, and observed under the Zeiss CEM-900 transmission electron microscope.

## 3. Results

**3.1 Chemical inference of molecular functional groups and chemical characterization**

Infrared spectroscopy allows the checking of the purity of a product and the identification of an unknown substance. It is an easy, low-cost analysis and the analysis principle is based on molecular vibrations (Boughendjioua et al., 2017). Figure 1 illustrates the spectrum generated in FTIR for cinnamon EO and cinnamaldehyde.

**Figure 1 -** Spectrum of samples of cinnamon bark (*Cinnamomum verum*) essential oil and cinnamaldehyde obtained by infrared spectroscopy (FTIR).




Source: Authors based on survey data (2019).

The presence of bands 1664 and 1625 $cm^{-1}$ (C=O) and strong intensity, associated with the double band 2742 and 2807 $cm^{-1}$ (O=C-H, intensity w-m), is indicative of aldehyde function. Bands 1451 cm-1 (C-C, ArH) and 3069 cm-1 (ʊCsp2-H) with medium / low intensity confirm the presence of the aromatic structure. Band 1122 $cm^{-1}$ (C-O stretch) with strong intensity is indicative of alcohol or phenol, as well as bands 960 $cm^{-1}$ (C-H out-of-plane deformation) and 690 $cm^{-1}$ (C-H out-of-plane deformation) and the intensities of the olefin visual media. Band 748 $cm^{-1}$ (C-H out-of-plane deformation) can indicate benzene and derivatives, with a monosubstituted one. Table 1 presents, in an organized way, the main wave numbers, theoretical bands, functional group, type of vibration, and peak intensity.

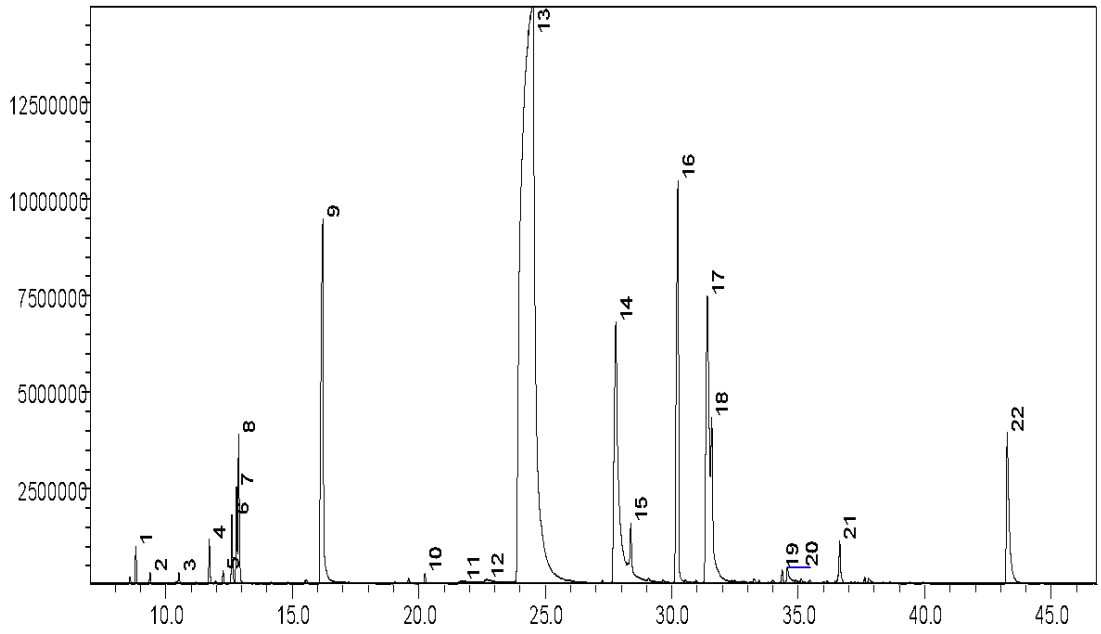
**Table 1** - Interpretation of the FTIR spectrum.

| Wave # | Band($cm^{-1}$) * | Functional Group | Type of Vibration | Peak intensity | Organic compost |
|---|---|---|---|---|---|
| 3069 | 3100-3000 | $Csp^2$-H | Stretch | w-m | Aromatic |
| 3038 | 3100-3000 | $Csp^2$-H | Stretch | w-m | Aromatic |
| 2807 | 2830-2700** | O=C-H | Stretch | S | Aldehyde |
| 2742 | 2830-2700** | O=C-H | Stretch | S | Aldehyde |
| 1664 | 1820-1630 | C=O | Stretch | S | Aldehyde |
| 1625 | 1820-1630 | C=O | Stretch | m | Alkeno |
| 1451 | ~1450 | C-C | Stretch | m | Aromatic |
| 1122 | 1300-1000 | C-O | Stretch | S | Alcohol |
| 960 | 1000-680 | C-H | Deformation outside the plane | m | Olefin |
| 748 | 770-730 | C-H | Deformation outside the plane | S | Benzene*** |
| 690 | 735-680 | C-H | Deformation outside the plane | m | Olefin |

* Theoretical Reference Lopes & Fascio (2004); ** Fermi resonance: doublet; *** Monosubstituted benzene;
Frequency Intensity: S = strong, m = medium, w = weak, W = wide, v = variable
Source: Authors based on survey data (2019).

The bands found in the FTIR spectrum of this study are in accordance with the literature, which confirms the presence of aldehydes, aromatic structures, and derivatives in cinnamon EO. The identification and detailed quantification of chemical compounds, present in the EO, can be obtained by gas chromatography analysis with mass spectroscopy (GC-MS). The chemical composition of cinnamon EO, analyzed by GC-MS, has the chromatogram in Figure 2.

**Figure 2** - Gas chromatography and mass spectrometry of cinnamon (*Cinnamomum verum*) essential oil, intensity (eV) versus time (mm).




Source: Authors based on survey data (2019).

In the list of chemical compounds shown in Table 2, the following chemical classes are present: monoterpene hydrocarbons ($C_{10}H_{16}$), oxygenated monoterpenes ($C_{10}H_{18}O$), sesquiterpene hydrocarbons ($C_{15}H_{24}$), oxygenated sesquiterpenes ($C_{15}H_{26}O$), and other oxygenated compounds.

**Table 2** - Chemical composition of cinnamon (*Cinnamomum verum*) essential oil.

| Peak | Compounds | Retention Index | Area (%) | Formula | Identification Method |
|---|---|---|---|---|---|
| 1 | α-Pinene | 929 | 0.31 | $C_{10}H_{16}$ | a, b, c |
| 2 | α-Fenchone | 943 | 0.10 | $C_{10}H_{16}$ | a, b, c |
| 3 | β-Pinene | 972 | 0.10 | $C_{10}H_{16}$ | a, b, c |
| 4 | α-Phellandrene | 1.002 | 0.40 | $C_{10}H_{16}$ | a, b, c |
| 5 | α-terpinene | 1.014 | 0.12 | $C_{10}H_{16}$ | a, b, c |
| 6 | p-Cimene | 1.022 | 0.67 | $C_{10}H_{14}$ | a, b, c |
| 7 | β-Phellandrene | 1.026 | 0.89 | $C_{10}H_{16}$ | a, b, c |
| 8 | Eucalyptol | 1.028 | 1.45 | $C_{10}H_{18}O$ | a, b, c |
| 9 | Linalool | 1.102 | 7.07 | $C_{10}H_{18}O$ | a, b, c |
| 10 | α-Terpineol | 1.190 | 0.11 | $C_{10}H_{18}O$ | a, b, c |
| 11 | *trans*- Cinnamaldehyde | 1.223 | 0.13 | $C_{9}H_{18}O$ | a, b, c |
| 12 | 2-methoxy-benzaldehyde | 1.245 | 0.13 | $C_{8}H_{18}O$ | a, b, c |
| 13 | *cis*-Cinnamaldehyde | 1.286 | 58.70 | $C_{9}H_{8}O_{2}$ | a, b, c |
| 14 | Eugenol | 1.362 | 8.53 | $C_{10}H_{12}O_{2}$ | a, b, c |
| 15 | α-Copaene | 1.376 | 1.00 | $C_{15}H_{24}$ | a, b, c |
| 16 | (E)- Caryophyllene | 1.420 | 6.79 | $C_{15}H_{24}$ | a, b, c |
| 17 | (E)- cinnamyl acetate | 1.449 | 6.43 | $C_{9}H_{10}O$ | a, b, c |
| 18 | α-Humulene | 1.453 | 3.05 | $C_{15}H_{24}$ | a, b, c |
| 19 | δ-cadinene | 1.523 | 0.15 | $C_{15}H_{24}$ | a, b, c |
| 20 | Eugenol Acetate | 1.528 | 0.22 | $C_{12}H_{14}O_{3}$ | a, b, c |
| 21 | Caryophyllene oxide | 1.582 | 0.55 | $C_{15}H_{26}O$ | a, b, c |
| 22 | benzyl benzoate | 1.766 | 3.10 | $C_{14}H_{12}O_{2}$ | a, b, c |
| Total | | | 100.00 | | |

[a] Compound listed according to the elution order of column DB-5; [b] Retention Index (IR) calculated using a homologous series of n-alkanes (C10-C39) in a capillary column (DB-5); [c] Identification based on the comparison of Nist (2019) and Adans (2017) data libraries. Area (%): is the percentage of the area occupied by the compound within the chromatogram.
Source: Authors based on survey data (2019).

A total of 22 components were identified. The cis-cinnamaldehyde (58.70%), eugenol (8.53%), and linalool (7.07%) were the major components. Regarding chemical classes, the EO presented 62.28% of oxygenated compounds, followed by 36.64% of oxygenated sesquiterpenes, 3.99% of hydrocarbon sesquiterpenes, 17.16% of oxygenated monoterpenes, and 2.59% of monoterpenes hydrocarbons.

Correlating the results of the GC-MS with that of the FTIR, it can be observed that the aldehyde bands identified in the FTIR correspond to the cis-cinnamaldehyde (58.70%) identified in the GC-MS and the bands with alcohol stretching, which correspond to the chemical compounds eugenol, linalool, and α-terpineol.

### 3.2 Antimicrobial activity

The determined values of MIC and MBC for cinnamon bark EO and cinnamaldehyde are shown in Table 3. The values of $MIC_{50\%}$ and $MIC_{90\%}$ calculated for cinnamon EO were 0.8 and 1.6 $mg.mL^{-1}$, respectively. The $MBC_{50\%}$ and $MBC_{90\%}$ values

were identical to the MIC value of cinnamon EO. For cinnamaldehyde, the $MIC_{50\%}$ and $MIC_{90\%}$ calculated were 0.8 and 1.6 $mg.mL^{-1}$, respectively, and $MBC_{50\%}$ and $MBC_{90\%}$ were 1.6 and 6.4 $mg.mL^{-1}$, respectively.

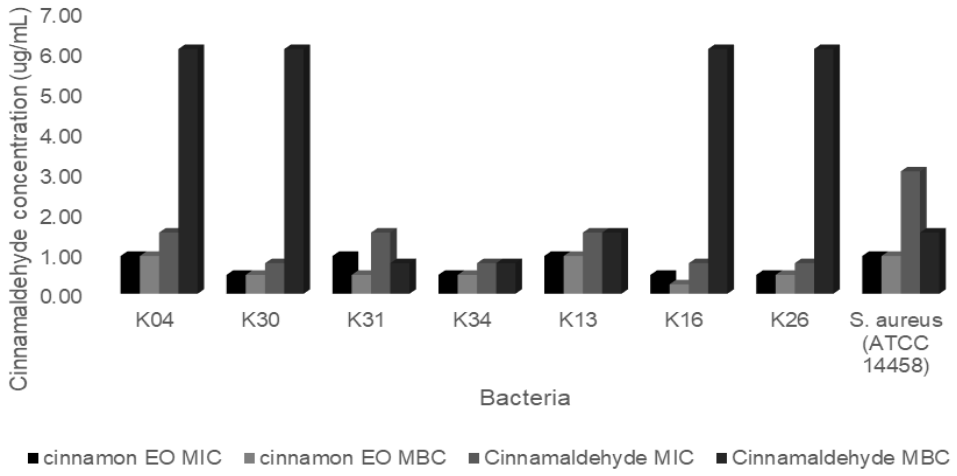
The cinnamon EO under study showed 59.7% cis-cinnamaldehyde as the major compound. Considering that the purity of the concentrated cinnamaldehyde used was ≥95%, the amount of cinnamaldehyde present in each determined MIC and MBC could be calculated. Multiplication was performed between the percentages of cinnamaldehyde present and the determined MIC or MBC. Figure 3 graphically shows the concentration of cinnamaldehyde ($mg.mL^{-1}$) present in MICs and MBCs determined for cinnamon EO and concentrated cinnamaldehyde against the tested bacteria.

**Table 3** - Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Cinnamomum verum* bark essential oil and cinnamaldehyde against strains of *Staphylococcus* spp.

| **Bacteria** | | **Cinnamon EO** | | **Cinnamaldehyde** | |
|---|---|---|---|---|---|
| | | **MIC ($mg.mL^{-1}$)** | **MBC ($mg.mL^{-1}$)** | **MIC ($mg.mL^{-1}$)** | **MBC ($mg.mL^{-1}$)** |
| CPS | K04 | 1.6 | 3.2 | 1.6 | 6.4 |
| | K30 | 0.8 | 0.8 | 0.8 | 6.4 |
| | K31 | 16 | 0.8 | 1.6 | 0.8 |
| | K34 | 0.8 | 0.8 | 1.6 | 0.8 |
| CNS | K13 | 1.6 | 1.6 | 1.6 | 1.6 |
| | K16 | 0.8 | 0.4 | 0.8 | 6.4 |
| | K26 | 0.8 | 0.8 | 0.8 | 6.4 |
| | *Staphylococcus aureus* (ATCC 14458) | 1.6 | 1.6 | 1.6 | 3.2 |

*EO: essential oil; CPS: coagulase positive staphylococci; CNS: coagulase negative staphylococci.
Source: Authors based on survey data (2019).

**Figure 3 -** Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the major compound (cinnamaldehyde mg.mL$^{-1}$) in the tests with *Cinnamomum verum* bark essential oil (EO) and cinnamaldehyde concentrated against strains of *Staphylococcus* spp.




Source: Authors based on survey data (2019).

The graphical representation shows that the amount of cinnamaldehyde required for antimicrobial activity was much greater than its concentration in the EO. This reveals that the synergy between components present in cinnamon EO is much more effective than concentrated cinnamaldehyde.

### 3.3 Synergism with conventional antimicrobials

Table 4 shows the mean values of inhibition halos for each conventional antimicrobial against the standard strain of *Staphylococcus aureus* (ATCC 14458). In percentage, this strain showed 50% sensitive, 25% resistant, and 8.3% intermediate to conventional antibiotics. The strain was resistant to the antibiotics penicillin, tetracycline, and chloramphenicol. Rifampicin had an intermediate effect.

By this agar diffusion technique, cinnamon EO was shown to be bactericidal in all tested concentrations (MIC, ½ $MIC_{90\%}$, and ¼ of $MIC_{90\%}$) for the positive control. Even with this result for cinnamon EO, interactions of cinnamon EO and conventional antibiotics were performed. In all repetitions, no inhibition halos were formed. For combinations of cinnamaldehyde and conventional antibiotics, at all proposed concentrations, bacterial growth was also not detected in all replicates, even in 150 mm plates.

**Table 4 -** Antibiotic disk diffusion disk for *Staphylococcus aureus* (ATCC 14458) with the inhibition halo measurement in millimeters (mm).

| Antibiotics | average (mm) | Standard deviation | Interpretation |
|---|---|---|---|
| Penicillin | 12 | ±0.53 | R |
| Tetracycline | 6 | ±0.00 | R |
| Oxacillin | 13 | ±0.79 | S |
| Cefepime | 19 | ±0.76 | S |
| Ciprofloxacin | 23 | ±1.81 | S |
| Sulfazotrim | 16 | ±0.90 | S |
| Gentamycin | 20 | ±1.00 | S |
| Vancomycin | 16 | ±0.79 | S |
| Clindamycin | 24 | ±1.07 | S |
| Erythromycin | 21 | ±1.00 | * |
| Chloramphenicol | 6 | ±0.00 | R |
| Rifampicin | 19 | ±0.79 | I |

R: resistant; S: sensitive; I: intermediate; *: without reference value
Source: Authors based on survey data (2019).

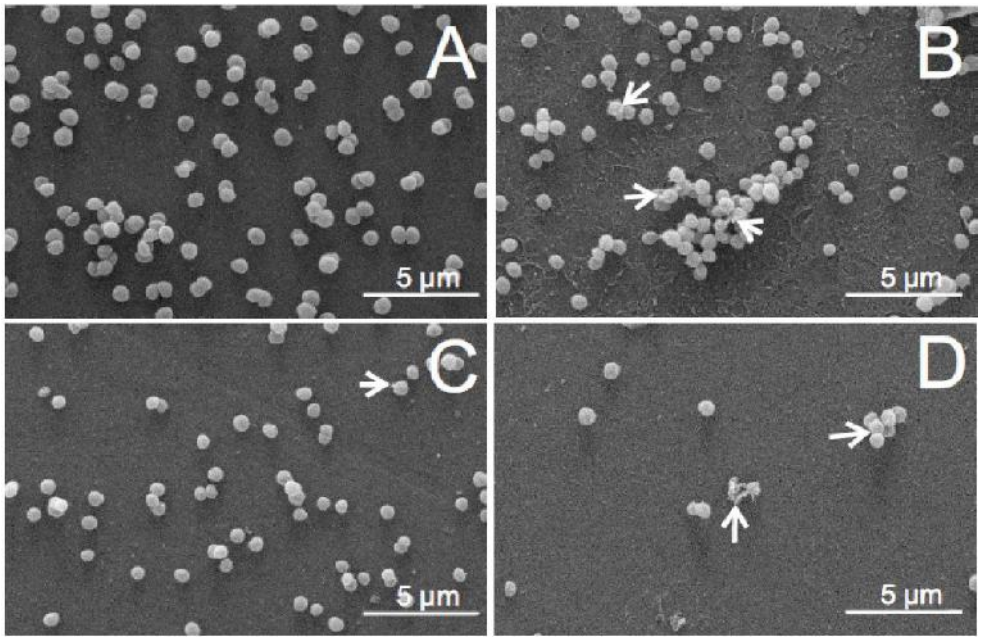
There was also no bacterial growth in the positive control plates (without conventional antimicrobial disks), in which the concentrations of $MIC_{90\%}$, ½ $MIC_{90\%}$, and ¼ $MIC_{90\%}$ of cinnamon EO were used; in addition, there was also no growth in the plates with concentrations of $MIC_{90\%}$, ½ $MIC_{90\%}$, and ¼ $MIC_{90\%}$ of cinnamaldehyde.

However, the same pattern of preparation of the culture medium and inoculation of the plates with the bacterial culture was followed, both for the treatments of cinnamon EO and cinnamaldehyde and for the controls (only the antimicrobial disks). It was possible to confirm that the inoculum was active, as there was growth in the positive control plate without antibiotics and cinnamaldehyde.

### 3.4 Electron microscopy

The cell morphology of *S. aureu*s bacteria (ATCC 14458) treated with cinnamon or cinnamaldehyde EO in MIC and MBC concentrations were analyzed by SEM (Figure 4) and TEM (Figure 5). Cell membranes are indispensable for many vital functions of cells, including maintaining the energy state of the cell, energy transducer processes coupled to the membrane, solute transport and metabolic regulation. In this way, by acting on the membrane, it is precisely these functions that EOs would harm.

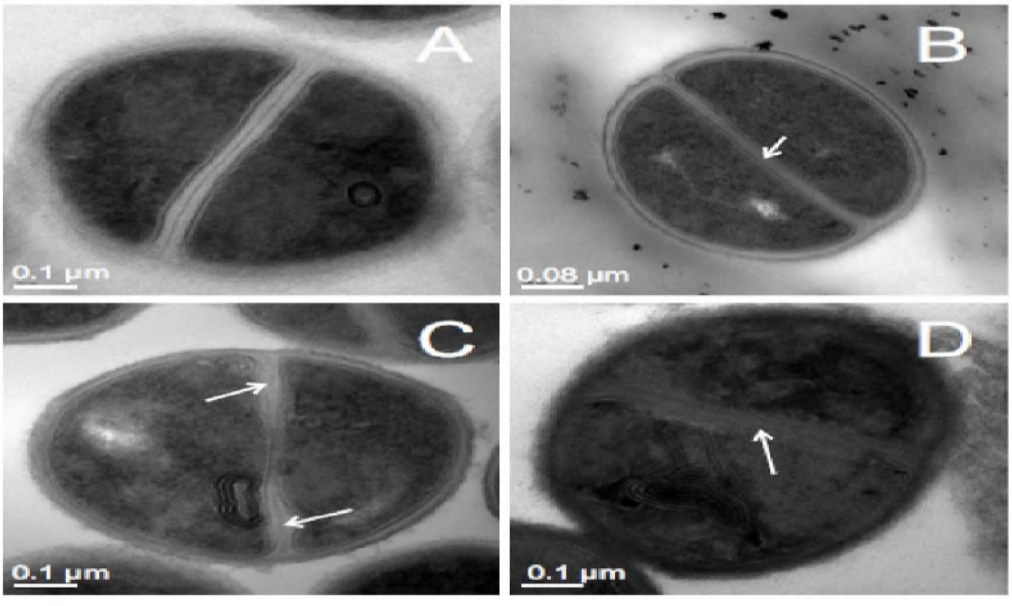
**Figure 4 -** Scanning electron microscopy with *Staphylococcus aureus* cells. A: Control treatment; B: Culture treated with 1.6 mg.mL$^{-1}$ of cinnamon essential oil; C: Culture treated with 1.6 mg.mL$^{-1}$ cinnamaldehyde; D: Culture treated with 6.4 mg.mL$^{-1}$ cinnamaldehyde. Arrows indicating cell leakage.




Source: Authors based on survey data (2019).

In Figure 4A, the S. aureus (control) cell is round, uniform, and has a smooth surface. In Figures 4B, 4C, and 4D the cells are rough and with residues adhered to the cell wall (arrows indicating), as if a slight cell leakage had occurred, however, this was more intense in the treatments in which there was bactericidal action (Figures 4B and 4D ). In Figure 4D, it was possible to register a cell with intense cell leakage.

**Figure 5 -** Transmission electron microscopy with *Staphylococcus aureus* cells. A: Control treatment; B: Culture treated with 1.6 mg.mL$^{-1}$ of cinnamon essential oil; C: Culture treated with 1.6 mg.mL$^{-1}$ cinnamaldehyde; D: Culture treated with 6.4 mg.mL$^{-1}$ cinnamaldehyde. Arrows indicate interruption of cell division.




Source: Authors based on survey data (2019).

In Figure 5A, the *S. aureus* (control) cell was at the time of cell division and the integrity of the cell wall could be observed, which was completely formed and uniform. In Figures 5B, 5C, and 5D, there are uneven and irregular zones in cell division sites, which allow inferring the cell wall as an action target for cinnamon EO and cinnamaldehyde with consequent interruption of cell division. The mechanism of action of EOs may involve several target points in the bacterial cell. As they present hydrophobic characteristics, EOs diffuse in the cell membrane from lipids, causing leakage of cell content. Certain conditions favor the action of EOs, such as low pH, low temperatures and low oxygen levels (Burt, 2004). According to Nazzaro et al. (2013), in a study on the effect of EOs in pathogenic bacteria, the mechanism of action of EOs has been shown to affect both the cell membrane and the cytoplasm.

## 4. Discussion

Boughendjioua et al. (2017) investigated the functional groups present in the EO of *Cinnamomum zeylanicum* bark using FTIR. The results were compared with the equipment's internal libraries (Euclidean, PSU / peak, MIX PSU, Peak Match, PEAK / psu, MIX PEAK). The authors were able to identify 10 volatile compounds (trans-cinnamaldehyde, cassia oil, n-formylmethamphetamine, isophorone (97%), N-butyl nitrite, 2,5-dimethoxycinmaldehyde, N, N-diethylformamide (99%), Citral in KBr, N, N-diethylformamide, isobutyl nitrite). The functional groups were identified from the following characteristic bands: 1500-1400 $cm^{-1}$ (CHx deformation), 1600-1500 $cm^{-1}$ (C=C stretch), and 1710-1700 $cm^{-1}$ (C=O stretch).

The functional groups present in the cinnamon bark EO grown in Tepi, southwestern Ethiopia, found by Adinew (2014) were: O-H stretch with broad band and average intensity between 3500-3200 $cm^{-1}$; H bond for alcohol and phenol with medium intensity with C-H stretch for alkane (3000-2850 $cm^{-1}$); at wavelength 1666 $cm^{-1}$ and medium intensity, they revealed the presence of a C=O bond for aldehyde; between 900-675 $cm^{-1}$ there was a strong band. They indicated the presence of aromatic C=C and weak-medium band between 1680-1600 $cm^{-1}$ and showed the presence of C=C stretching alkenes. Through GC-MS the authors identified 3-phenyl-2-propenal (87.01%), eugenol (9.31%), and O-methoxy cinnamic aldehyde (0.24%) as major compounds.

The results found on the chemical composition of cinnamon EO are in agreement with other scientific researches, such as Choi et al. (2016) who investigated the chemical composition of *Cinnamomum verum* bark EO and found 24 components, among them cinnamaldehyde (56.3%), cinnamyl acetate (7.1%), β-felandrene (6.3%), linalool (4.1%), p-cymene (4.1%), and eugenol (2.0%). Huang, et al., (2014) identified 34 components in Cinnamomum cassia bark EO, and trans-cinnamaldehyde (68.52%), copaene (4.66%), propane benzene (3.67%), γ-cadinene (3.41%), cis-Cinnamaldehyde (2.15%), α-cadinol (1.85%), and cinnamyl alcohol (1.24%) were considered the main compounds. Other components were found but in quantities less than 1.00%.

Firmino et al. (2018) evaluated the antimicrobial activity of cinnamon EOs (*Cinnamomum zeilamycun* (EOCz) and *Cinnamomum cassia* (EOCc)) and trans-cinnamaldehyde against biofilm-forming strains of *S. aureus*. EOCz, EOCc, and trans-cinnamaldehyde, exhibited bacteriostatic activity at MIC values of 0.50, 0.25, and 0.25 $mg.mL^{-1}$, respectively. The authors state that the similarity of the bacteriostatic activity of EOCc and trans-cinnamaldehyde can be explained by the amount of trans-cinnamaldehyde present in the composition of the EO. EOCc presented 90.22%, while EOCz presented 68.7% trans-cinnamaldehyde.

However, it is possible to observe a wide variation in the MIC and MBC values of the studies with cinnamon EO, since Huang et al. (2014) investigated the antimicrobial activity in Cinnamomum cassia bark EO against *S. aureus* (ATCC 25923) and found MIC and MBC values of 2.5 and 5.0 $mg.mL^{-1}$, respectively, with the major compounds being trans-cinnamaldehyde (68.52%), copaene (4.66%), and benzene propanal (3.67%). On the other hand, Teles et al. (2019) investigated the antimicrobial activity in *C. zeylanicum* leaf EO against *S. aureus* (ATCC 12600) and found an MIC value of 0.216 $mg.mL^{-1}$ and the major

compounds were cinnamic aldehyde (46.7%), α-copaene (16.35%), and trans-β-karyophylene (8.26%).

In this study, cinnamon EO presented cis-cinnamaldehyde (58.70%), eugenol (8.53%), and linalool (7.07%) as major compounds. Observing the two references mentioned above, the chemical composition of the EO can be seen interfering with the antimicrobial activity and the smallest MIC found does not depend exclusively on the major compound of the sample, but on the complex chemical composition of the EO. The cinnamaldehyde used in this study had an MIC of 90%, identical to cinnamon EO, but the bactericidal effect was achieved at a concentration of 6.4 $mg.mL^{-1}$, that is four times greater than the MBC value of cinnamon EO. Correlating these results, it can be observed that the major compound of cinnamon EO, cinnamaldehyde, has an inhibitory activity equivalent to EO, but it needs a higher concentration than cinnamon EO itself to exert bactericidal activity, which is not of economic and technological interest depending on the cost necessary for its isolation.

Padalia et al. (2017) investigated synergism between *C. verum* EO and antibiotics (ampicillin, gentamicin, chloramphenicol, penicillin-G, and tetracycline) using the disk diffusion method against *S. aureus* (ATCC 29737). The antibiotic disks were impregnated with 20µL of EO diluted in ethanol. Cinnamon EO showed synergistic antibacterial activity with the antibiotics ampicillin and penicillin-G with inhibition halos of 30 mm and 27 mm, respectively. The authors stated that the discovery of minimal and effective combinations of conventional antibiotics with natural products can minimize the side effects of antibiotics and prevent the emergence of bacterial resistance to antibiotics.

Comparing the protocols of the methods of antimicrobial activity employed in this study –microdilution and disk diffusion –, an unusual step between the methods which may have a possible interference in the chemical composition of the cinnamon EO and cinnamaldehyde is their solubilization in the culture medium. In the microdilution method, solubilization is performed with the culture medium at room temperature, as it is a broth. However, in the disk diffusion method used in the synergism study, cinnamon EO and cinnamaldehyde were added to the culture medium at a temperature of approximately 60ºC, as the culture medium was still in a liquid state, and then they poured into the plate Petri.

These observations are indications of a possible improvement in the antimicrobial activity of cinnamon EO and cinnamaldehyde as a result of heating, with intensification of their bactericidal action with other antimicrobial drugs. It can also be a peculiarity of the analysis method, in which it promotes the permanence of the active compounds in the form of vapor inside the Petri dish, in direct contact with the bacterial cells, right after their sowing, promoting a better interface with the bacterial surface. The investigation of the antimicrobial activity of natural products in vitro presents obstacles due to the peculiarities that each EO may present (Teles, 2019), in this study, they made several attempts to adapt the method, in order to control the volatilization and insolubility characteristics that cinnamon EO and cinnamaldehyde present.

Although no data were obtained from the halos of inhibition, for comparison between treatments (conventional drugs plus cinnamon or cinnamaldehyde EO) and controls (conventional drugs only), the method of analysis used in this study provides an opportunity to discuss the application proposal of cinnamon EO or cinnamaldehyde, as antimicrobials through biofilms, which could be positioned on superficial lesions. Consequently, these active ingredients would volatilize and remain in direct contact with the surface contaminated by the infectious agent.

However, the mechanism of action of EOs in bacterial cells depends on structural differences in cell walls. The cell wall of Gram-negative bacteria is more complex, has a thin layer of peptideoglycan and an outer membrane composed of a double layer of phospholipids, the outer layer being linked to the inner layer by lipopolysaccharides (LPS). The outer membrane can act as a barrier for some types of antimicrobial drugs. The cell wall of Gram-positive bacteria, on the other hand, has an extensive layer of peptideoglycan, the presence of theoretical acids and proteins, which facilitate the entry of hydrophobic molecules through the cell wall into the cytoplasm. In turn, the phenolic compounds present in EOs, can benefit from this cellular structure to obtain greater antimicrobial activity in cells (Padalia et al., 2017).

In Gram-positive bacteria, the mechanism of action of cinnamaldehyde, consequently of cinnamon EO, is the

interference in the formation of the Z disk (cell division point), by interacting with the FstZ protein (Filamentation Temperature Sensitive Protein Z). This action has the consequence of inhibiting cell division (Balouiri et al., 2016). Consequently, the cells do not divide and / or cell lysis occurs at the time the two daughter cells divide due to the complete formation of the cell wall on the Z disk.

Huang et al. (2014) investigated the possible changes that can occur in bacterial cells after the treatment of *C. cassia* bark EO in MIC values (2.5 $mg.mL^{-1}$). They observed by SEM in the control treatment that *S. aureus* cells were round, regular, bulky, and with a smooth surface, whereas the cells treated with cinnamon EO were irregular and with a rough surface and holes. By TEM, the authors observed that the bacteria in the control treatment had defined cytoplasmic walls and membranes with uniform distribution of genetic material within the cytoplasm. After treatment with cinnamon EO, the cell wall and cytoplasmic membrane became irregular and with interruptions, in addition to a disorder of the genetic material.

## 5. Conclusion

Cinnamon EO had bactericidal activity at the same inhibitory concentration, while its major compound required a concentration four times higher to cause cell death of *Staphylococcus aureus*. The cell wall was the target of action for both cinnamon EO and cinnamaldehyde with consequent inhibition of cell division. Both cinnamon EO and cinnamaldehyde showed undetermined antimicrobial activity combined with conventional antimicrobials, which brings the prospect of their ability to intensify the antimicrobial action of veterinary drugs used in the treatment of mastitis caused by multidrug-resistant bacteria.

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