Article

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# Extract of *Mangifera indica* L. leaves may reduce biofilms of *Staphylococcus* spp. in stainless steel and teatcup rubbers

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**Abstract**

Human ingestion of antimicrobial residues in dairy products is a problem of public health. In this study we evaluated antibacterial effectiveness of aqueous and ethanolic leaf extracts of *Mangifera indica* L. as a natural alternative for reduction of *Staphylococcus* spp. biofilms. Eight *Staphylococcus* spp. strains from cows with mastitis and the *Staphylococcus aureus* strain (ATCC 25923) were evaluated. High performance liquid chromatography indicated the presence of phenolic compounds in the aqueous extract. Agilent Technologies gas chromatography revealed the presence of phenolic compounds, carbohydrates, and gallic acid in ethanolic extract. The tannin contents were $2.27 \pm 0.01$ and $2.67 \pm 0.01\%$ for ethanolic extract and aqueous extract, respectively. Extracts were evaluated in agar diffusion test and the size of inhibition zones were compared. The ethanolic extract was the most effective, showing minimum inhibitory concentrations and minimum bactericidal concentrations between 1.8–7.5 and 15.1–45.3 mg/ml, respectively. After tannin removal, the extracts were less effective, indicating these secondary metabolites as one of the main antibacterial compounds. The ethanolic extract at 45.3 mg/ml reduced mature biofilms in teat rubber (average: 3.91 log reduction) and stainless steel (average: 3.87 log reduction) after 5 min of contact. The extracts of *M. indica* leaves represent natural alternatives against *Staphylococcus* spp. strains and the ethanolic extract shows potential as a natural sanitizer.

**Keywords**

Bovine mastitis, mango, tannin, bacterial adhesion, alternative sanitizer

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## INTRODUCTION

Mastitis is a frequent cause of economic losses in dairy cows, compromising animal health, milk composition and public health. The incorrect and indiscriminate antimicrobial use in animals has selected multiresistant bacteria (Moritz and Moritz, 2016), as evidenced for *Staphylococcus* spp. strains isolated from cows (Liu et al., 2017; Unakal and Kaliwal, 2010; Wang et al., 2013). The species *Staphylococcus aureus* is the most important mastitis pathogen, presenting multiresistant strains in different continents (Saidi et al. 2013; Sampimon et al. 2011).

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The disinfectants and antibiotics ineffectiveness have been related to bacterial biofilm formation in food industry (Mafu et al., 2011; Simões et al., 2010), conducting pathogens to consumers and causing outbreaks of foodborne illnesses (Chmielewski and Frank, 2003). Bacterial cells in biofilms can be up to 1000 times more resistant to antibiotics and sanitizing agents (Nader et al., 2014). Due to the difficulties to reduce cells adhered to surfaces, new strategies for the biofilm control have been studied (Donlan, 2002), such as plants containing flavonoids that may break outer membrane and inhibit bacteria enzymatic activity (Nascimento et al., 2000). Recent studies have showed potential of some plant metabolites to reduce biofilm formation (Bazargani and Rohloff, 2016; Borges et al., 2014; Rocha et al., 2014).

Plants have aroused interest of both scientists and of the pharmaceutical industry (Mostafa et al., 2018). Anacardiaceae family is commonly used for medicinal purposes and presents phenols and tannins which have showed antimicrobial properties (Cajado et al., 2016; Kabongo-Kayoka et al., 2016). *Mangifera indica* L. var. ''Ubá,'' is a member of this family, common in most continents, with world production of approximately 40 million tons per year (Mitra, 2016). Its seeds have antimicrobial action, when formulated in ointments and creams for topical use in humans, showing inhibition zones against bacteria (El-Gied et al., 2015).

In the present study, we investigated the effects of leaf extracts of this plant against *Staphylococcus* spp. and their activity against mature biofilms on stainless steel and teatcup rubber surfaces.

## MATERIAL AND METHODS

### Bacterial strain

Eight isolates of *Staphylococcus* spp. from bovine mastitis in Northern of Minas Gerais, Brazil and the *S. aureus* strain (ATCC 25923) were evaluated. These bacteria were isolated and cultured on mannitol salt agar and evaluated based on colony characteristics, Gram staining, catalase reaction, and coagulase test.

For molecular identification of the isolates, the extraction and amplification of DNA was promoted as described by Chapaval et al. (2008). A pair of primers was annealed in conserved regions of the 16S and 23S, 16-1A genes (5′ GAATCGCTAGTAATCG 3′) and 23-1B (5′ GGGTTCCCCCATTCGGA 3′). The 16S rRNA gene was sequenced using the MegaBACE$^{TM}$ 1000 automated sequencer and the primers used were 27F (5′ AGAGTTTGATCCTGGCT CAG 3′) and 1492R (5′ GGTTACCTTGTTACGAC TT 3′) in the Myleus Biotechnology Laboratory (Reysenbach et al., 2000). Sequencing was verified with SeqScanner *software* and results compared *online* in BLAST (NCBI database—https://blast.ncbi.nlm.nih.gov/Blast.cgi). Bacteria were identified with a minimum level of 99% similarity.

Additionally, the presence of the femA gene was screened by the PCR technique. The primers FemA1-5′ AAAAAAGCACATAACAAGCG 3′ and FemA2-5′ GATAAAGAAGAACCAGCAG 3′ were used and PCR conditions were according to Teixeira et al. (2014). Expected amplicons of 132 base pairs were visualized on 1.5% agarose gel. The standard *S. aureus* strain (ATCC 25923) was used as the positive control.

### Antimicrobial susceptibility

Microorganisms were characterized by the resistance profile to main antimicrobial used in mastitis control, through diffusion test in agar, according to CLSI (2016), conducted in duplicate. Each bacterium inoculum was prepared by direct suspension of grown colonies on plates containing mannitol salt agar in sterile saline solution. Bacterial suspensions were adjusted to McFarland turbidity standard No. 0.5, corresponding to approximately $10^8$ colony-forming unit (CFU)/ml. Two hundred microliters of inoculum was spread on Mueller–Hinton agar plates, with sterile swabs. The antimicrobial disks, erythromycin 15 μg, vancomycin 30 μg, gentamicin 10 μg, penicillin 10 μg, ampicillin 10 μg, ciprofloxacin 5 μg, were placed on surface plates, as described in NCCLS (2005) and incubated in a BOD oven at 35 °C for 24 h. Diameters of inhibition zones (mm) were measured by a digital pachometer. Bacteria were classified as resistant or sensitive according to CLSI (2016). The assay was performed in triplicate.

### Preparation of extracts and quantification of tannin

Leaves were collected at the Institute of Agricultural Sciences of the UFMG in Montes Claros at 16 °51′ latitude and 44°55′ longitude. The region climate is tropical humid with dry summer (As) according to classification of Köppen (Alvares et al., 2014), is marked by a long dry season from May to September and a rainy period in January and February.

During February and March of 2016, leaves were collected and the damaged ones were discarded. The selected leaves were washed in running water, dehydrated in a forced circulation stove of air at 40 ± 5 °C for 72 h, crushed in an industrial blender, and were stored in the dark paper bags at 25 ± 2 °C.

To prepare the aqueous extract (AE), dehydrated and crushed leaves (10 g) were added on 50 ml sterile water and incubated on water bath at 40°C for 60 min.

To prepare the ethanolic extract (EE), dehydrated and crushed leaves (100 g) were stored in amber glass containers by adding ethanol PA (1000 ml) and stored in a dark place at room temperature for seven days. Extracts were filtered with funnel, cotton wool, and gauze and were dehydrated in a forced air circulation oven at 40 °C until reaching constant weight. After dehydration, these extracts were scraped and stored in dark place at 4 °C (Morais-Costa et al., 2015, 2016). Both extracts were completely soluble in distilled water, requiring no solvents.

Total condensed tannin content was quantified after acid-catalyzed solvolysis with 37% n-BuOH/HCl (95:5). Absorbance was read on spectrophotometer at 540 nm and the values were expressed as cyanidin chloride. Results corresponded to the average of three determinations, followed by the standard deviations (Morais–Costa et al., 2015, 2016). For verification of the possible antimicrobial action of tannins, we removed these secondary metabolites, according to the methodology described by Nyman et al. (1998). These extracts were dissolved, 1 g in 20 ml of water at 90 °C. After cooling to room temperature, four drops of NaCl (10%) were added and 1 ml of this solution was mixed with 4 ml of gelatin solution (1%) and centrifuged for 6 min.

## Characterization of extracts

The AE was evaluated in high performance liquid chromatography (HPLC) system, Waters Alliance 2695. The Waters Empower program was used to control the chromatographic system and data collection. Analyses were performed on LiChrospher 100 RP-18 column (250 × ID 4 mm, 5 µm) combined with a 100 RP-LiChrospher 18 column (4 × 4 mm id, 5 µm) at 40 °C. Water (A) and acetonitrile (B) were used as eluents, both containing 0.1% (v/v) $H_3PO_4$, with a flow rate of 1.0 ml/min such as follows: 0 min, 95% A and 5% B; 60 min, 5% A and 95% B followed by 10 min isocratic elution.

The solvents used were HPLC grade and were degassed by ultrasound prior to use. The chromatogram was obtained at 210 nm, and the UV spectra were recorded on line at 190–400 nm. Dried extracts were dissolved in methanol (HPLC grade), ultrapure water, or hydroethanolic solutions, according to solubility, at concentrations of 10 mg/ml. After centrifugation at 8400 × *g*, the sample solutions (10 µl) were automatically injected into the chromatograph.

The EE was characterized in Agilent Technologies gas chromatograph (GC 7890A) equipped with electron impact ionization detector (CG-EM) and DB-5MS capillary column (Agilent Technologies, 30 m length × 0.25 mm internal diameter × 0.25 µm film thickness). Pure helium was used as the entrainment gas at the rate of 1 ml/min. Using a self-injector (CTC combiPaL), 1 µl of the sample was injected into the chromatograph at a 1:10 split ratio. The split/splitless injector was maintained at 290 °C. The chromatographic column initially at 80 °C, isotherm for 5 min, was heated at a rate of 4 °C/min to 260 °C for 10 min. After separation of compounds, the temperature was raised to 300 °C and remaining for 2 min (post run). The interface temperature was maintained at 280 °C and the ionization was performed by 70 V. The scanning range of m/z was from 30 to 600 Da.

## Antagonism of extracts in agar diffusion tests

The extracts were filtered on a millipore membrane (0.2 µm pore diameter) and aliquots were then submitted to dry matter (DM) determination in an oven at 105 °C, to calculate the concentrations to be tested (Cunnif, 1995). After DM determination, the extracts were evaluated in the concentrations 25.87–103.5 and 30.47–121.9 mg/ml of the EE and AE, respectively.

In vitro activity of plant extracts on each bacterial isolate was determined according to NCCLS (2003). Bacteria inoculations were prepared by direct suspension of colonies grown on plates containing mannitol salt in sterile saline solution. We inoculated 200 µl of the bacterial suspensions (McFarland turbidity standard No. 0.5; approximately $1.3 \times 10^8$ CFU/ml) and spread on Mueller–Hinton agar plates, with sterile swabs. Filter paper disks (5 mm) containing the solution of plant extracts (previously prepared and homogenized in vortexer for 1 min) were placed on seeded plates and incubated at 35 °C for 24 h. Paper filter disks with sterile saline solution (without extract) and discs containing the extracts incubated without bacteria were used as controls. The diameters of inhibition zones (mm) were measured as described by CLSI (2016).

This assay was performed in triplicate and the results were evaluated using analysis of variance with the means compared by the Scott–Knott test and regression analysis, considering the significance of 5%, in the statistical program SAEG 9.1.

## Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC were evaluated by broth microdilution method according to CLSI (2011). In 96-well polystyrene microplates, eight concentrations of the extracts were prepared and evaluated against strain ATCC 25923 and to isolates SH135, SH182, SA178, SA348A, SA353C, which presented greater resistance in previous step.

The initial concentration (IC) of the extracts was defined as 150 mg/ml and the corresponding DM was quantified in 103.5 mg/ml (EE) and 121.9 mg/ml (AE). After filtration of the extracts through Millipore® membrane (0.22 μm pore diameter) and again quantification of DM, the IC corresponded to 60.5 mg/ml (EE) and 71.0 mg/ml (AE); from these, we calculated the final concentrations: 0.9, 1.8, 3.7, 7.5, 15.1, 30.2, 45.3 mg/ml EE and 1.1, 2.2, 4.4, 8.8, 17.7, 35.5, 53.2 mg/ml AE.

The experiment was conducted in four replicates. In each well was added 80 μl of brain heart infusion (BHI) broth, 20 μl of the plant extract, and finally 100 μl of standardized microorganism in saline solution at 0.5 McFarland scale ($1.5 \times 10^8$ CFU/ml). In the positive control, 100 μl of saline, 80 μl of BHI, and 100 μl of the bacterial inoculum were used. In the negative control, we used 80 μl of BHI and 100 μl of saline solution. The microplates were sealed and incubated at 37 °C for 18 h. After incubation, the solution of triphenyl tetrazolium at 0.1% was used to verify bacterial growth. This reagent indicates cellular multiplication through the development of a reddish color, thus enabling MIC determination (Klancnik et al., 2010).

For MBC determination, 100 μl aliquots from the microdilution wells of the MIC assay and the control without extracts were inoculated on Mueller–Hinton agar and incubated at 37 °C for 24 h. The absence of bacterial growth on the agar plates was checked to determine the MBCs. All these procedures were carried out in triplicate.

The assay was realized again using the extracts after removal of tannins in four replicates. The positive control of the test using extract without condensed tannin was elaborated with the highest concentration in each initial extract (150 mg/ml).

### Inhibition of bacterial biofilms in steel coupons and teatcup rubbers

Of each bacterium species were selected the most resistant isolate and the standard strain for biofilm formation. Stainless steel coupons (AISI 304 #4) used in the food industry and teatcup rubber (4212) used in mechanical milking of cows with dimensions of 2.0 cm × 2.0 cm × 0.1 cm were previously sanitized and sterilized according to Rossoni and Gaylarde (2000).

For the experimental system of biofilm formation, we inoculated 1 ml (5 log CFU/ml) of isolates SH182, SA178, and ATCC 25923 strain in 99 ml of skimmed UHT milk and four coupons, of each surface type, inside a sterile glass jar. This experimental system was maintained at 28 °C/24 h under constant agitation on an orbital shaker table at 60 r/min to simulate the agitation of the milk in the expansion tank.

After 24 h of conduction of the experimental system, the coupons were rinsed with distilled water and transferred to new sample of sterilized skim milk without inoculation. This new system was maintained under agitation at 60 r/min for a further 24 h, totaling 48 h of bacterial adhesion. The period was scaled based on the current legislation of the quality of raw milk, which establishes the maximum time between milking and receiving milk at the establishment where it will be processed.

To evaluate the biotransfer potential of the cells adhered to the coupons for the noninoculated skim milk, aliquots of 1000 μl were withdrawn and subjected to successive serial decimal dilutions, followed by plating in mannitol salt agar at 35 ± 2 °C for 24 h (Careli et al., 2009). The results were expressed in CFU/ml.

For quantification of the adhered bacterial cells, the coupons were removed, rinsed for 1 min in 10 ml of 0.85% sodium chloride solution to remove planktonic cells, transferred to 10 ml of 0.85% sodium chloride solution, and submitted to ultrasonic bath for 2 min to detach sessile cells. Decimal dilutions of each sample were performed with plating in mannitol salt agar at 35 ± 2 °C for 24 h and the results were expressed in CFU/cm$^2$.

### Sanitizing activity of extracts on bacterial biofilms

The most effective extract was selected in the disk diffusion test because it presented CBM for all bacterial strains. To test the sensitivity of cells adhered to the *M. indica* leaves EE, coupons were subjected to rinsing with 0.85% sterile sodium chloride solution. Then, these were transferred to the sanitizing solution containing EE at CBM detected in the previous tests.

Sterilized distilled water was used as the control. The contact times of the coupons adhered to the extract and control were 5 and 10 min. For quantification of the bacterial cells after contact with the EE solutions, we carried out procedures for the removal of adhered cells, described by Careli et al. (2009), plating on mannitol salt agar at 35 °C for 48 h and the results were expressed in CFU/cm$^2$. The results were compared by means of a nonparametric analysis, in a 2 × 2 × 3 factorial scheme (surfaces, times, bacterial isolates). The Duncan's test was used at 5% significance level to compare the means.

## RESULTS AND DISCUSSION

### Bacterial strain

DNA sequencing analysis identified isolate 178 as *S. aureus* (SA178), as *Staphylococcus haemolyticus* (SH135, SH182). Strains 376C, 372A, 348A, 364B,

and 353C showed a specific 132-base pair amplicon corresponding to the *femA* genetic marker and were identified as *S. aureus*.

## Antimicrobial susceptibility

SA178 strain was resistant to erythromycin, vancomycin, and penicillin. ATCC 25923 was resistant to penicillin and vancomycin and SA353C strain was resistant to penicillin (Table 1). B-lactam antibiotics are often used to treat mastitis and their indiscriminate use has increased the selection of resistance genes due to the expression of the β-lactamase enzyme (Keserű et al. [2011]), which may justify the results detected.

**Table 1.** Antimicrobial susceptibility profile for strains of *S. aureus* (SA), *S. haemolyticus* (SH) isolated from milk of cows with mastitis and *S. aureus* ATCC 25923.

| Bacteria | AMP | CIP | ERI | GEN | PEN | VAN |
|---|---|---|---|---|---|---|
| ATCC 25923 | S | S | S | S | R | R |
| SH135 | S | S | S | S | S | S |
| SH182 | S | S | S | S | S | S |
| SA178 | S | S | R | S | R | R |
| SA376 C | S | S | S | S | S | S |
| SA372 A | S | S | S | S | S | S |
| SA348 A | S | S | S | S | S | S |
| SA364 B | S | S | S | S | S | S |
| SA353 C | S | S | S | S | R | S |

AMP: ampicillin 10 μg; CIP: ciprofloxacin 5 μg; ERI: erythromycin 15 μg; GEN: 10 μg gentamycin; PEN: penicillin 10 μg; VAN: vancomycin 30 μg. R: resistant; S: sensitive according to NCCLS (2005).

## Characterization of extracts

The presence of tannin, flavone, and flavonoid peaks was observed (Figure 1) in different retention times in AE, with a maximum absorbance intensity of 294.7 nm. Gallic acid, carbohydrates, and fatty acids were observed in the EE (Figure 2). The content of condensed tannins in the DM was $2.67 \pm 0.01$ and $2.27 \pm 0.01\%$ to the AE and EE, respectively.

The gallic acid (3,4,5-trihydroxybenzoic acid), detected in the chromatogram of the *M. indica* EE (Annex A), has been reported as a phenolic structure showing antimicrobial activities (Llivisaca et al., 2018).

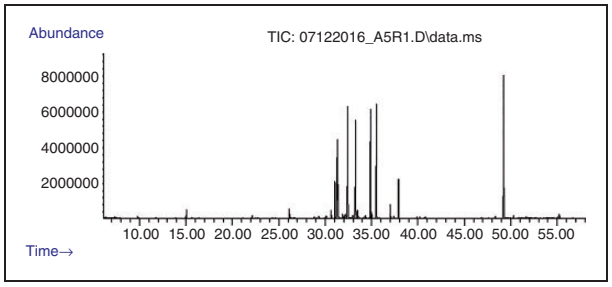


**Figure 2.** Chromatographic profile obtained by gas chromatograph from Agilent Technologies (GC 7890A) from ethanolic extract of the *M. indica* L leaves. The peaks indicate the phenolic compounds and presence of carbohydrates.

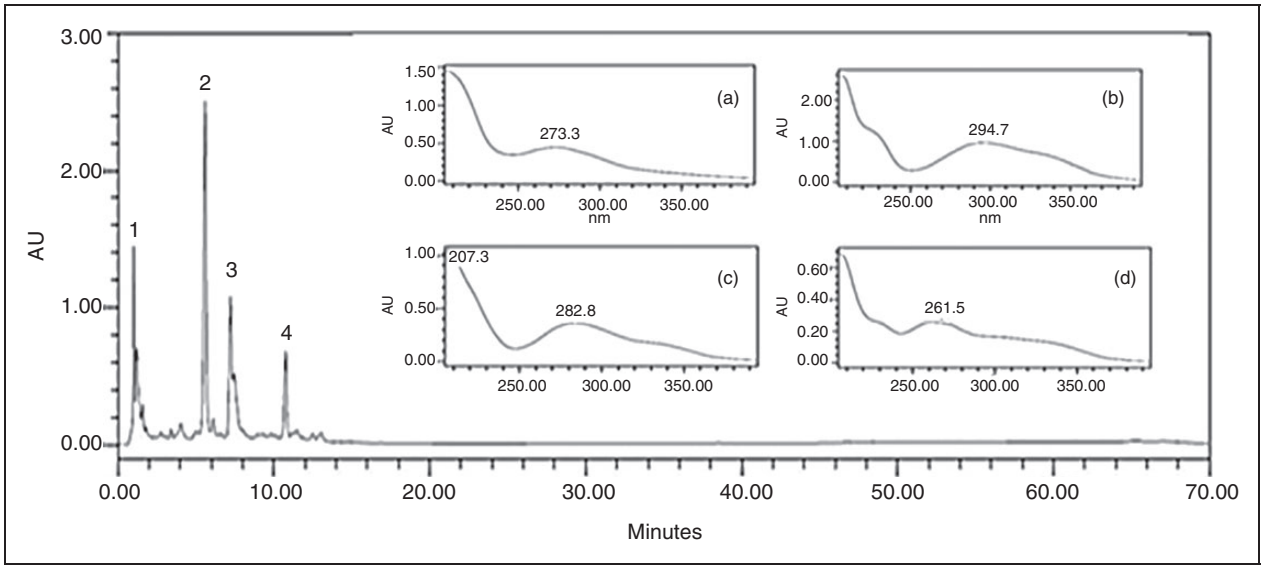


**Figure 1.** Chromatographic profile obtained by HPLC in reverse phase of the AE from *M. indica* L. leaves in UV: Peaks related 1, 2, 3, and 4, indicating tannins.

The carbohydrates inositol, thalose, galactopyranose, D-fructose, and α-D-glucopyranoside were also identified and the latter with a higher peak retention (Figure 2). According to Nobmann et al. (2009), α-D-glucopyranoside showed effects against *Listeria* spp. and these authors suggest that the alpha and beta configuration of carbohydrate fractions was involved with the mechanism of action.

These results corroborate the phytochemical study of seed, stem bark, and leaves of *Mangifera pajang*, performed by Ahmad et al. (2015), that identified terpenoids and flavonoid derivatives.

## Antagonism in diffusion test in agar

The diameters of inhibition zones were directly related to the EE and AE concentrations and influenced by *Staphylococcus* spp. strains (Tables 2 and 3, $p < 0.05$).The EE showed higher antimicrobial efficacy compared to AE (Graph 1, supplementary material). The isolate SA353C was the most resistant to the evaluated concentrations of EE and SA372A, the most sensitive (Table 2, $p<0.05$). However, using AE, strain ATCC 25923 was the most resistant and SA364B was the most sensitive (Table 3, $p < 0.05$).

Average inhibition zones of EE and AE were 8.62 and 6.66 mm, respectively (Tables 2 and 3) and statistically different ($p < 0.05$). Cajado et al. (2016) analyzing hydroalcoholic extract of *M. indica* fruits at 37.5–150 mg/ml observed antimicrobial effect against *S. aureus* with the inhibition zones varying between 9.0 and 9.5 mm. Other study evaluated antimicrobial activity of peel flour and mango kernel EE at 1 mg/ml. Antimicrobial activity against *Escherichia coli, Salmonella* spp., *Pseudomonas aeruginosa*, and *S. aureus* were observed by inhibition zones varying between 14.1 and 19.1 mm (Arbos et al., 2013).

**Table 2.** Inhibition zones average in millimeters for *Staphylococcus* spp. isolated from cows with mastitis and *S. aureus* ATCC 25923 treated with disks containing concentrations (mg/ml) of the ethanolic extract of *M. indica* leaves.

| Bacteria | 0.00 mg/ml | 25.87 mg/ml | 51.75 mg/ml | 77.62 mg/ml | 103.5 mg/ml |
|---|---|---|---|---|---|
| ATCC 25923 | 0.00Da | 6.05Cd | 9.00Bb | 10.43Ab | 11.35Ab |
| SH135 | 0.00Ea | 2.75De | 6.72Ce | 9.08Be | 10.80Ae |
| SH182 | 0.00Ea | 6.25Dh | 8.45Ch | 9.27Bh | 10.32Ah |
| SA178 | 0.00Ea | 4.22Dg | 7.53Cg | 9.43Bg | 10.38Ag |
| SA376C | 0.00Ea | 6.18Dd | 7.50Cd | 8.82Bd | 11.10Ad |
| SA372A | 0.00Ea | 8.18Da | 9.70Ca | 10.33Ba | 11.65Aa |
| SA348A | 0.00Ea | 8.65Dc | 10.23Cc | 10.70Bc | 11.28Ac |
| SA364B | 0.00Ea | 6.07Df | 6.82Cf | 8.67Bf | 10.50Af |
| SA353C | 0.00Ea | 6.20Di | 7.38Ci | 8.40Bi | 9.98Ai |

Capital letters in the rows and lowercase in the columns indicate difference by the Scott–Knott test at 5% significance. Variation coefficient = 17.2%.

**Table 3.** Inhibition zones average in millimeters for *Staphylococcus* spp. isolated from cows with mastitis and *S. aureus* ATCC 25923 treated with disks containing d concentrations of the aqueous extract from *M. indica* leaves.

| Bacteria | 0.00 mg/ml | 30.47 mg/ml | 60.95 mg/ml | 91.42 mg/ml | 121.9 mg/ml |
|---|---|---|---|---|---|
| ATCC 25923 | 0.00Ca | 1.55Bh | 1.58Bi | 6.35Ai | 6.75Ai |
| SH135 | 0.00Da | 0.00De | 2.98Ce | 7.50Be | 9.23Ae |
| SH182 | 0.00Ea | 6.50Db | 7.95Cb | 8.65Bb | 10.40Ab |
| SA178 | 0.00Ea | 5.60Df | 6.68Cf | 7.18Bf | 8.40Af |
| SA376 C | 0.00Ea | 5.28Dd | 5.88Cd | 7.00Bd | 9.50Ad |
| SA372 A | 0.00Ea | 6.12Dc | 7.38Cc | 8.62Bc | 9.88Ac |
| SA348 A | 0.00Da | 0.00Dh | 6.03Ch | 6.43Bh | 7.20Ah |
| SA364 B | 0.00Ea | 7.12Da | 9.50Ca | 9.90Ba | 10.82Aa |
| SA353 C | 0.00Ea | 5.62Dg | 6.05Cg | 6.92Bg | 7.50Ag |

Capital letters in the rows and lowercase in the columns indicate significant difference by the Scott–Knott test at 5% significance. Variation coefficient = 19.95%.

### MIC and MBC determination

The extracts presented antibacterial activity against all the strains evaluated, with MIC values of 1.8–7.5 mg/ml and CBM of 15.1–45.3 mg/ml (EE). The EE MIC required to inhibit 90% bacteria growth ($MIC_{90}$) was 30 mg/ml. The AE MIC varied of 8.8–71 mg/ml and CBM of 71 mg/ml for SA348A isolate. CBM values were not obtained for the other bacteria, demonstrating AE lower efficiency if compared to the EE (Table 4). Lowest AE CIM was observed for ATCC 25923, indicating this bacterium as the most sensitive.

In tannin absence, AE did not present MIC for any of the evaluated strains while EE was less effective against SH182, SH135, and SA178, and showed absence of MIC to the other bacteria (Table 5). These results may indicate this metabolite as the main antibacterial agent in these extracts. Antimicrobial activity of plant origin extracts has been attributed to the phenolic compounds (Narayani et al., 2011). Tannins present antimicrobial activity already well established in the literature (Monteiro et al., 2005). Mirghani et al. (2009), in a study with mango almonds, associated the antimicrobial action to the hydrolyzed tannin content, for it interacts with proteins, inhibiting enzymatic activity.

### Adhesion and biotransfer potential of the evaluated strains

Initial inoculum of each bacterial strain in ultrahigh temperature milk presented 5 log CFU/ml at room temperature. All bacterial strains grew up to 4 log cycles after 48 h of contact, obtaining an average concentration of 9 log CFU/ml after time of bacterial adhesion. For biotransfer potential, there was no difference between cell counts in steel and rubber and there were no differences between bacterial strains ($p > 0.05$). The high microbial load is worrisome; when there is a count of *S. aureus* equal to or greater than $10^5$ CFU/g of food, it becomes potentially capable of causing food poisoning (Ciupescu et al., 2018).

There was no difference ($p > 0.05$) between bacteria adhered counts to the rubber and steel surfaces. The strains do not differ from each other in relation to their potential to form biofilms ($p > 0.05$). The isolates SA178 and SH182 obtained average counts of $7.27 \pm 1.09$ and $7.13 \pm 0.95$ log CFU/cm$^2$, respectively, while ATCC 25923 showed $7.26 \pm 0.99$ log CFU/cm$^2$.

In order to consider which adhered cells constitute a biofilm, Andrade et al. (1998) suggest that a minimum number of adhered cells per cm$^2$ of surface is required. Ronner and Wong (1993) consider biofilm when the adhered cell count is $10^3$ CFU/cm$^2$. Based on these researches, we formed biofilms by *Staphylococcus* spp. on both surfaces.

All bacteria were able to form biofilms on stainless steel surfaces and teat rubber, with values greater than 7 log CFU/cm$^2$. This is a worrying result, since the formation of these biofilms can hinder the procedures for cleaning and disinfection of equipment made of stainless steel or teatcup rubber. The concentration of biotransferred cells can lead to the production of milk-borne thermotolerant toxins, causing food poisoning in humans (Ciupescu et al., 2018).

**Table 4.** Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of aqueous and ethanolic extracts from *M. indica* leaves against *Staphylococcus* spp. isolates from cows with mastitis and *S. aureus* ATCC 25923.

| | Concentrations (mg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | Ethanolic extract | | | Aqueous extract | | |
| Bacteria | MIC | $MIC_{90}$ | MBC | MIC | $MIC_{90}$ | MBC |
| ATCC 25923 | 1.8 | 7.5 | 15.1 | 8.8 | – | – |
| SH135 | 7.5 | 7.5 | 15.1 | 17.7 | – | – |
| SH182 | 3.7 | 15.1 | 30.2 | 8.8 | – | – |
| SA178 | 3.7 | 30.2 | 45.3 | 8.8 | 71.0 | – |
| SA348A | 7.5 | 15.1 | 30.2 | 8.8 | 53.2 | 71.0 |
| SA353C | 7.5 | 15.1 | 30.2 | 71.0 | – | – |

$MIC_{90}$: minimum inhibitory concentration required for inhibition of 90% growth of bacteria; MBC: minimum bactericidal concentration; SA: *Staphylococcus aureus*; SH: *Staphylococcus haemolyticus*; (–): inhibition absence.

**Table 5.** Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the aqueous and ethanolic extracts (without tannin) of *M. indica* leaves against *Staphylococcus* spp. isolates from cattle and *S. aureus* ATCC 25923.

| | Concentrations (mg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | Ethanolic extract | | | Aqueous extract | | |
| Bacteria | MIC | $MIC_{90}$ | MBC | MIC | $MIC_{90}$ | MBC |
| ATCC 25923 | – | – | – | – | – | – |
| SH135 | 45.3 | 45.3 | 60.5 | – | – | – |
| SH182 | 60.5 | 60.5 | 60.5 | – | – | – |
| SA178 | 15.1 | 30.2 | 45.3 | – | – | – |
| SA348A | – | – | – | – | – | – |
| SA353C | – | – | – | – | – | – |

$MIC_{90}$: minimum inhibitory concentration required to inhibit 90% growth of *Staphylococcus* spp.; MBC: minimum bactericidal concentration; (–): inhibition absence.

**Table 6.** Cell counts of *Staphylococcus* spp. adhered on stainless steel or teat rubber surfaces, expressed as (log CFU/cm$^2$) and efficacy of reduction (%) after 5 and 10 min of contact with control solution or ethanolic extract of *M. indica* leaves at 45.3 mg/ml.

| | 5 min | | | 10 min | | |
|---|---|---|---|---|---|---|
| | Control | Extract | Efficacy[1] | Control | Extract | Efficacy[1] |
| Stainless steel | | | | | | |
| SA178 | 5.77Aa | 0.00Cb | 100.0Aa | 5.44Aa | 0.00Cb | 100.0Aa |
| SH182 | 6.21Aa | 2.01Bb | 65.70Bb | 6.15Aa | 2.26Bb | 61.6Bb |
| ATCC 25923 | 5.65Aa | 3.93Ab | 33.14Cb | 5.88Aa | 4.12Ab | 29.9Cb |
| Teat rubber | | | | | | |
| SA178 | 5.89Aa | 1.82Bb | 69.0Ab | 6.35Aa | 2.77Bb | 56.3Aa |
| SH182 | 6.19Aa | 2.85Bb | 53.9Aa | 6.34Aa | 1.57Bb | 74.5Ab |
| ATCC 25923 | 6.51Aa | 2.19Bb | 64.6Aa | 6.64Aa | 2.54Bb | 59.0Ab |

Separate upper case letters indicate differences between *Staphylococcus* spp. strains and lower case letters between lines indicate differences between the treatments evaluated by the Duncan test at 5% significance.
Lowercase letters in the efficacy column indicate difference between steel and rubber, by the Duncan test at 5% significance.
Variation coefficient (VC) of log CFU/cm$^2$ in steel = 16.45%; log CFU/cm$^2$ in the rubber = 19.08% and CV of the efficacies = 34.33%.
[1]Efficacy of reduction (%) = 100 [1− (log treatment/control log)].

### Sanitizing activity of the extracts on the biofilms of Staphylococcus spp

A lower number of cells adhered to the stainless steel and rubber coupons were observed after contact with EE when compared to the control solution ($p < 0.0001$, Table 6). There was susceptibility difference between strains adhered in stainless steel after the treatments with the EE ($p > 0.05$). It was verified a greater susceptibility of the SA178 isolate adhered to the stainless steel with the EE, presenting 100% effectiveness for biofilm reduction in stainless steel.

In other study, Borges et al. (2014) reported that there was prevention of the formation of *S. aureus* biofilms with 1 mg/ml of phenolic acids and isothiocyanates. However, there was no total reduction of adhered bacterial cells.

We detected differences of biofilms reduction between bacterial strains. The EE promoted better efficacy in biofilms reduction in stainless steel compared to teat rubbers for the isolate SA178 (Table 6). However, for ATCC 25923 strain, the higher efficacy was detected to biofilms in teat rubbers ($p < 0.05$).

There was no difference ($p > 0.05$) between times. Therefore, 5 min of contact with the EE was considered sufficient for reduction of *Staphylococcus* spp. cells adhered. The efficacies of biofilms reduction in this contact period were SA178: 69–100%; SH182 53.9–66.70%, and ATCC 25923: 33.1–64.6%. These results emphasize the potential of plant extracts to the control these bacteria. It is possible that EE antibiofilm activity of *M. indica* is related to the presence of phenolic compounds of the plant, mangiferin, and the content of condensed tannin. Trentin et al. (2013) reported that tannins from medicinal plant may prevent the formation of biofilms. The diterpene isolated from the *Croton antisyphiliticus* root chloroform extract was not able to completely reduce biofilms, but efficient inhibitory activity was found, capable of promoting significant antibiofilm activity by eliminating 56% *S. aureus* at 250 μg/ml (Nader et al., 2014).

In this research, we used the leaves of *M. indica* that presents great bioavailability in tropical and sub-tropical regions and produced simple extracts of easy preparation. We observed the inhibition of *S. aureus* strains and reduction of mature biofilms and, using a plant extract, is the first study recording reduction of adhered biofilms in teacup rubber, which represent an initial and relevant point of contamination in the processing of dairy products.

## CONCLUSIONS

The extracts of *M. indica* leaves show antibacterial effects that could be attributed especially to the tannins, because after removal of these compounds, the extracts reduced its effectiveness. The EE is more potent than AE, showing lower MICs and reducing mature biofilms on steel or teacup rubber after 5 min. The EE shows antibacterial activities against *Staphylococcus* spp. from cattle with mastitis and is effective to reduction of mature biofilms, indicating potential as a natural sanitizer. The EE compounds may be considered as an alternative to conventional antimicrobials in the prevention and control of bovine mastitis, after toxicity and in vivo tests.

## DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## ETHICAL APPROVAL

The bacterial isolates were collected in previous experiments submitted and approved by the Ethics Committee on Animal Experiments of the UFMG (protocol no. 39/2009 and 145/2013), regulated by the National Council for Control of Animal Experimentation of Brazil.

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## SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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