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ARTICLE

Protective Properties in *Hymenaea martiana* Hayne against Multi-drug-resistant *Staphylococcus aureus*

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ABSTRACT

Antibiotic resistance represents a widespread problem in milk production. The identification of compounds for a topically applied ointment used in mastitis therapy remains elusive. Compounds from the genus *Hymenaea* can be administered in cases of multi-drug-resistant *Staphylococcus aureus* infection for ruminant species, but the protective properties are not well known. Wi this research the aim is verify the protective effects of *H. martiana* against *S. aureus* infection in bovine mammary epithelial cell line (MAC-T) and to obtain an antioxidant profile evaluation *in vitro*. The MAC-T cells were challenged with *S. aureus* after being exposed to the extract of the *H. martiana* in the protective assay. For the verification of the viability of the MAC-T cells, the MTT assay was performed, and was used dilutions of the plant extract, starting at 2.5%. The extract of *H. martiana* was evaluated for antioxidant aspect in different dilutions by FRAP, ORAC and DPPH. A variety of flavonoids (quercetin, luteolin, etc.) have been identified as the main components by using mass spectrometry, reinforcing our *in vitro* findings that flavonoids, especially quercetin, have a medicinal profile capable of killing mastitis-causing bacteria. An excellent antioxidant pattern was observed in the 2.5% solution; however, membrane integrity in MAC-T cells was compromised. Those findings suggest low dilutions of *H. martiana* extract has a desirable protective effect from *S. aureus* pathogenesis. Our *in vitro* studies can be gleaned upon for further in vivo studies.

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1. Introduction

Bacteria in the genus *Staphylococcus* are widely known to cause inflammation in ruminant species vital for milk production. As a result of an increasing resurgence in multi-drug- resistant pathogens, there is a significant demand to find antibacterial chemicals that can protect against bacteria such as *Staphylococcus spp*. and prevent mastitis. Mastitis is a disease caused by microorganisms presenting a resistance profile to current treatment. Plants of the genus *Hymenaea* have been previously discovered for medicinal usage due to a broad spectrum of desirable effects [1,2] that are considered capable of being included in new drug formulations. Recent research suggests the bark from the genus *Hymenaea* shows potential non-antibiotic treatment for goats with mastitis [3], and negatively affect the growth of bacteria from the genus *Staphylococcus* [1].

Hymenaea plants have already demonstrated great versatility in medicinal use, indicated from a study conducted in the State of Rio de Janeiro, Brazil [4]. Specifically, these properties are composed of bio-components such as anthracene derivatives, flavonoids, and naphthoquinones [3], phenols, flavonoids, steroids, and terpenoids [2]. These components contribute to the highly attractive antibacterial properties to multi-drug-resistant pathogens such as *S. aureus* [3].

Due to an increasing number of antibiotic-resistant bacteria that affect milk production and export, this research was focused on ruminant species responsible for production, namely cows and goats. One of the key aspects that give the protective effects from this extract is the redox potential. The antioxidant activity of phenolic compounds exhibits mainly in redox properties, which play an essential role in the absorption and neutralization of free radicals known to inhibit various types of oxidizing enzymes [2,5].

H. martiana is one such plant with antioxidant and antibacterial properties that are known however, which phenolic compounds that are contributing to the protection due to the redox potential are not fully known [3,6]. Based on this premise, the effect of *H. martiana* leaf extract on the MAC-T cells and the extract's potential in defending these cells from bacteria such as *S. aureus* has not been previously investigated. With this research it is hypothesized that an extract based on H. martiana has antioxidant compounds that protect against the pathogenesis of bacteria resistant to multiple drugs, such as S. aureus. The present study aimed to: (i) evaluate the antioxidant profile; (ii) the effect on MAC-T cells in the presence or absence of *S. aureus*; (iii) and the polyphenols and flavonoids content of *H. martiana* leaf extract.

2. Materials and Methods

2.1 Plant Material and Extraction

The leaves of *H. martiana* Hayne have the specimen number 21868, which is deposited in the Herbarium (Vale do São Francisco- HVASF) located in the northeast region of Brazil. The extraction and production of crude ethanolic extract (CEE) from the leaf was performed, according to Peixoto *et al.* [3], with some modifications since its material was the bark of the plant.

2.2 Phytochemical Determination by UHPLC-PDA-qTOF-MS / MS

The experiments were performed using an ACQUITY UPLC H-Class liquid chromatograph[1] coupled to a Quadrupole-Tof mass spectrometer (Xevo G2-XS QTof)[1] with ionization by electrospray (ESI). Chromatographic separations were performed using an ACQUITY UPLCTM BEH C18 (2.1 x 50mm, 1.7µm)[1] column at 40°C. The binary mobile phase consisted of water 0.1% formic acid (mobile phase A) and acetonitrile 0.1% formic acid (mobile phase B). The flow rate was 0.4 mL/min, and the injection volume was 5.0µL. The elution gradient used was: 0.0 to 8.0 min 10% - 50% B; 8.0 to 9.0 m - 50% -95% B and in 9.1m and 10%B, monitoring was done at 340 nm. The mass spectrometer was operated in negative ionization mode (ESI-) in the sensitivity mode. Detection was implemented in MS^E centroid mode at a mass band of 50-1200Da. All analyses were performed using a lockspray to ensure the accuracy and reproducibility of mass values. Leucine enkephalin (5 ng mL^{-1}) was used as a standard/reference for calibration. Data acquisition and analysis were performed using Waters MassLynx 4.1 software.

2.3 Total Polyphenol Content in Leaves (µg mL^{-1})

The total polyphenol concentration of the extracts was analyzed according to the Folin-Ciocalteu method [5]. The total polyphenol content was determined by interpolating the absorbance of the samples on the analytical curve constructed with a gallic acid standard. For the preparation of the analytical curve, gallic acid was used in the concentrations of 25 to 500 µg mL^{-1} diluted in absolute ethyl alcohol. The concentrations of phenols were expressed in µg mL^{-1} of gallic acid equivalents.

2.4 Total Flavonoid Content (µg mL^{-1})

The dosage of flavonoids was performed according to Yao *et al.* [7], using rutin as standard, in absolute ethyl alcohol and aluminum chloride. The spectrophotometer was read at 510nm; the blank used contains all reagents except the

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sample. The results were expressed as μg mL^{-1} (Table 1).

2.5 Free Radical Scavenger Activity DPPH

For the evaluation of the antioxidant capacity in the sequestration of the 2,2-diphenyl-1-picryl-hydrolyzed free radical (DPPH •), the methodology described by Blois [8] with modifications was used. A graph of %AA x Concentration (μg mL^{-1}) was constructed with the values obtained. For the calculation of the Inhibitory Concentration (IC_{50}), the equation of the line was used, replacing the value of y by 50 to obtain the sample concentration capable of reducing 50% of the DPPH • (Table 2).

2.6 The Absorption Capacity of Oxygen Radical (ORAC)

In the leaf extract assay (concentration 2.5%) of *H. martiana*, 20μL of the sample was mixed to 120μL of a fluorescein solution diluted in phosphate buffer (pH 7.4) in black microplates and incubated at a constant temperature of 37°C/15min. All reagents were prepared in 75mM phosphate buffer, pH 7.4. Subsequently, 60 μL of the solution of 2,2-azobis 2-amidinopropane dihydrochloride (AAPH) was added, initiating the reaction.

The fluorescence intensity (485nm excitation/520 nm emission) was checked every 10 minutes for 80 minutes in a quartz cuvette. The phosphate buffer was also used to clear the equipment. To control the reaction, 20μL of methanol was added to the fluorescence solution.

The ORAC values are expressed in μmoles equivalents of Trolox, using a standard Trolox curve. The area of the fluorescence loss of a sample is calculated by subtracting the area corresponding to that of the control. Fluorescence determination was performed using a spectrophotometer. All analyses shall be carried out in triplicate, and the values expressed as μmoles equivalents of Trolox/g sample, on a dry basis.

2.7 Antioxidant Power by Iron Reduction (FRAP)

The FRAP reagent was prepared from the light with 300 mmol/L acetate buffer (pH 3.6), 10 mmol TPTZ in a solution of 40 mmol/L HCl, and 20mmol/L $FeCl_3$. Samples and standard solutions are mixed with deionized water and the FRAP reagent after being placed in a water bath for 30 min. at 37°C. Upon cooling to room temperature, the absorbance of the samples and a standard solution were read at 595nm. The standard Trolox curve was prepared using concentrations of 10 to 800μmol TE/L. The results are expressed in μmol TE/L.

2.8 Cell Culture

The bovine mammary alveolar cells (MAC-T) were assigned by Dr. Maria Aparecida Scatamburlo Moreira, Federal University of Viçosa (UFV), in Viçosa, Minas Gerais, Brazil. MAC-T cells were cultured in 96-well flat-bottom culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100μg mL^{-1}) and streptomycin (100μg mL^{-1}). Cells were incubated at 37 °C with 5% CO_2 for 24 hours (Forma ™ Series II 3110 Water Jet Co Incubators)[2]. Cell growth was visualized and monitored in an inverted microscope IX70)[3] until reaching confluency ($1x10^5$ cells).

2.9 *Staphylococcus* Aureus Isolates used in the Research

The *S. aureus* used in the research belongs to the bacterial "library" of the Laboratory of Bacterial Diseases (LDBAC) of the preventive sector of the UFV. Six isolates of *S. aureus* were used, three strains isolated from cases of acute mastitis, and three isolated cases of remitting mastitis. These bacteria come from the milk of goats with mastitis. TSB (tryptone soy broth) was used to reactivate them at 37 °C for 24 hours. Each group has one strain with less than two resistance/virulence genes, one strain with four resistance/virulence genes, and another strain with more than ten genes. In this way, they were classified as low resistance (2 genes), medium resistance (4-8 genes), and strong resistance (> 10 genes) in regards to virulence. At the time of use, three to five colony forming units (CFU) were inoculated in TSB broth (tryptone soy broth) at 37 °C until reaching the optical density (OD) 0.1 to 595 nm measured in a spectrophotometer apparatus (Biomate 3). This concentration corresponds to approximately 10^7 CFU mL^{-1}, pre-calibrated by standard plate count.

2.10 Concentrations of the Compounds

Nine dilutions of the *H. martiana* Hayne leaf extract were tested starting from the dilution of 25 mg/mL to 97.65 mg/mL, which were diluted in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 μg mL^{-1}) and streptomycin (100 μg mL^{-1}). Antibiotics were withdrawn when the effect of the extract on the bacteria was tested.

2.11 MTT Assay and Cytotoxic Effect

The MTT tetrazolium salt (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide) has its ring cleaved by mitochondrial dehydrogenases forming dark blue formazan crystals. Briefly, upon reaching confluency, MAC-T cells were exposed to the compounds in nine dilu-

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tions starting from 25,000 mg/mL (as reported) of extract for 24 hours. Then 10 µL of MTT (stock solution of 5 mg mL^{-1} in PBS pH 7.6)[5] was added in 100 µL of the medium, and the plate was incubated for four hours. The supernatant was discarded, and 200 µL of DMSO was added per well to dissolve the crystals formed. The plate was shaken slowly, after which the developed color was read in a plate reader spectrophotometer at 550 nm. Cells that were not exposed to the compounds served as controls. The experiment was performed in technical triplicates from each well.

2.12 Protective Effect on MAC-T

The isolates were incubated in TSB broth (tryptone soy broth) until reaching the concentration of 10^5 CFU mL^{-1}, and the cells reached $1x10^5$ confluency in Dulbecco's modified Eagle's medium with 10% FBS antibiotics. The extract of the leaves of *H. martiana* was diluted in Dulbecco's modified Eagle's medium without antibiotics with 10% FBS until reaching ninth dilution, but only the dilutions with 1562.5 mg/mL and 781.25 mg/mL, are bactericidal dilutions of *S. aureus* isolates [3] and that in previous tests have observed that do not cause severe damage to MAC-T cell structures.

2.13 Quantification and Statistical Analysis

Results are presented as mean ± SEM. For the data related to phytochemical determination, a descriptive analysis of the data and observed alterations were performed. For cell viability tests, the Kruskal-Wallis test was used to test whether samples originate from the control with n=3. Statistical analysis and plotting for cell viability studies were performed in GraphPad Prism 8. On the protective effect and antioxidant activity by DPPH•, after the normality test, the data were submitted to analysis of variance, and the means were compared by the Tukey test, with a significance considered as $p < 0.05$ compared to control.

3. Results

3.1 Compound Identification and Antioxidant Activity

The major compounds of the leaves in *H. martiana* were identified from high-resolution mass spectrometry (UHPLC-qTOF-MS/MS) as flavonoids (Figure 1). In our study, the main flavonoids identified were derived from quercetin and luteolin (Table 1). An increase in total polyphenols (2,604.16 µg/mL) and total flavonoids (3,661.33 µg/mL) was evidenced at a concentration of 25,000 mg/mL (Table 1).

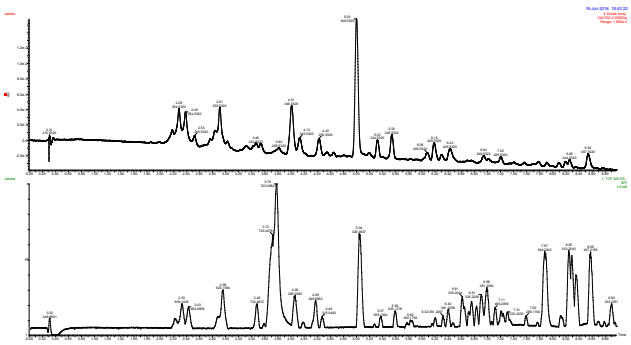

Figure 1. UPLC-DAD (340 nm) of the SPE fraction of *Hymenaea* martiana leaves (A) and Chromatogram of the total ions obtained by the MSE method (UPLC-qTOF/MSE) in negative mode

Table 1. Flavonoids identified by UPLC-DAD-ESI (-) - QTOF-MS/MS in SPE fraction of *Hymenaea* martiana leaves

Peak	Retention time	λ_{max}	$[M-H]^-$	MS^2	Possible identification
1	2.23	253. 357	609.1422	463.0853; 197.8061	Quercetin-hexo-side-rhamnoside
2	2.33	263.357	609.1425	463.0859; 197.8061	Quercetin-hexo-side-rhamnoside
3	2.43	254. 357	463.1584	300.0251; 197.8055	Quercetin-hexoside
4	2.96	252. 356	623.1584	477.1018; 197.8080	Quercetin-O-methyl-hexoside
5	4.06	250. 345	285.0383	265.1434; 197.8062	Luteolin
6	4.18	245. 357	315.0484	300.0258; 197.8060	Quercetin-O-methyl
7	4.48	246. 357	315.0492	300.0264; 197.8066	Quercetin-O-methyl
8	5.04	245. 346	329.0637	299.0177; 197.8059	Quercetin-di-O-methyl

One method is FRAP (antioxidant activity via reduction of iron), and the other is ORAC (antioxidant capacity via absorption of oxygenated radicals). To explore the redox potential, the FRAP (383.5 mmol TE/g) and ORAC (1,206.9 mmol TE/g) were screened for antioxidant potential, in which the concentration is 2.5% of leaves of *H. martiana*.

Table 2. Antioxidant activity by the radical sequestration method DPPH (IC_{50}) and total compounds of the concentrations of the hydroalcoholic extract of *H. martina*

Concentration of *H. martina*	Antioxidant activity	Total compounds	
(mg mL^{-1})	IC_{50}	(µg mL^{-1})	
	DPPH	Total polyphenols	Total flavonoids
25000	27.71± 0.319**	2604.16±10.874**	3661.33±0.000**
12500	560.63±0.125	1564.25±0.000	1978.83±0.000
6250	1039.86±0.574	890.88±0.138	990.09±0.861

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3125	2579.61±2.867	550.72±0.111	823.33±0.127
1562.5	4321.32±12.828b	320.27±0.055	534.00±0.083
781.25	2116.25±6.413	170.38±0.027	315.19±0.121
390.6	3225.36±4.783	104.47±0.073	176.27±0.027
195.3	11747.35±92.399	54.16±0.220	106.63±0.028
97.65	27388.03±1061.490	36.08±0.096	59.47±0.027
CV (%)	8.67	0.90	0.04

Legend: Values presented as the mean and standard error. ** significant at the 1% probability level ($p < 0.01$)

3.2 Interference of *H. martiana* in the MAC-T Development

The MAC-T cells were exposed to the dilutions of the extract for 24 hours and were then followed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The mean values of D.O. (optic density) obtained are illustrated in Figure 2. A comparison from our values obtained by the MTT test shows no statistical difference ($p < 0.001$) in formazan formation by the mitochondrial dehydrogenases at the final dilutions of 781.25 up to 97.65 mg/mL; thus, no toxic profile was shown in MAC-T cells.

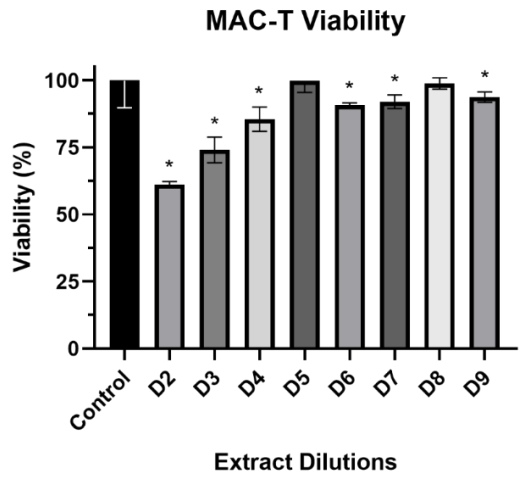

Figure 2. Results of the cell viability in MAC-T cells. Legend: D2 = 12,500 mg/mL, lower error bar is shorter than height of bar; D3= 6,250 mg/mL; D4 = 3,125 mg/mL; D5 = 1,562.5 mg/mL, upper error bar is shorter than height of bar; D6 = 781.25 mg/mL, lower error bar is shorter than height of bar; D7 = 390.6 mg/mL; D8 = 195.3 mg/mL; D9 = 97.65 mg/mL. * Indicates P < 0.05 in relation to control. The first dilution (D1) was excluded due to its impregnating profile, which is difficult to read

The 5th dilution of our plant extract at 1,562.5 mg/mL was found significantly different ($p = 0.0412$), indicating a cytotoxic effect on MAC-T cells. When was analyzed the optical aspect of the cells, there was no difference when compared to the 6th dilution (781.25mg/mL).

3.3 Viability of MAC-T Cells infected with *S. aureus*

To evaluate the protective effect in *H. martiana*, was visualized looked at the protection of MAC-T cells against *S. aureus* mutants. From those results, was founded the survivability of MAC-T cells was dependent on the genetic profile of *S. aureus*. Was investigated that *S. aureus* in acute and recurrence mastitis cases with two virulence/resistance profiles (Figure 3).

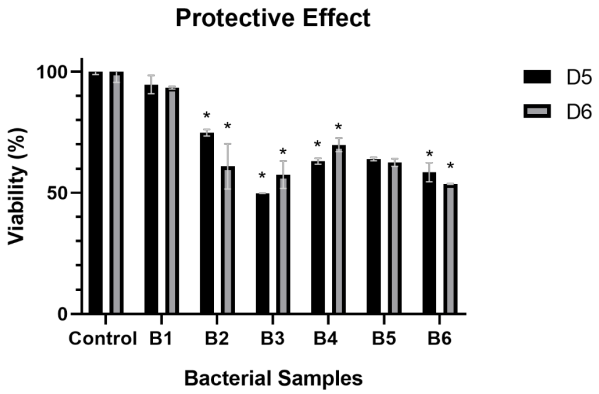

Figure 3. Protective effect on MAC-T cells from acute mastitis (B1-B3) and chronic mastitis (B4-B6). D5 = 1,562.5 mg/mL, D6 = 781.25 mg/mL

With the resistance profile from the isolates, was observed that there was a more significant protective effect of the extract when *S. aureus* had a lower resistance profile. In the dilution of 1562.5 mg/mL, there was a significant protective effect of the extract in acute mastitis, as well as at the 781.25 mg/mL dilution. The impact on both the dilutions was significantly reduced (Figure 4) when a high antimicrobial resistance profile (B3 and B6) was detected.

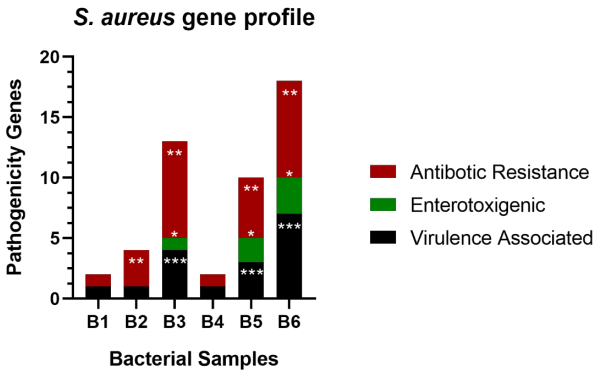

Figure 4. *S. aureus* gene profile related to pathogenesis. Symbols indicate common genes: * norA and TET 38; ** TETK and TETM; *** FNBB, ETA, and LUC

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Table 3. Protective effect of dilutions of *H. mariana* leaf extract on MAC-T cells with *S. aureus* isolated from several cases of caprine mastitis

Protective effect (Absorbance)								
	Bacteria samples							
Diluitions	**Control**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	***P***
5[a] (1.562.5 mg/mL) $\overline{X}_5 \pm S_5$	0.655 ± 0.0120[a]	0.620 ± 0.0431[a]	0.490 ± 0.0155[b]	0.326 ± 0.0007[c]	0.413 ± 0.0141[bc]	0.418 ± 0.0077[bc]	0.383 ± 0.0445[c]	<0.0001
6[a] **(**781.25 mg/mL) $\overline{X}_6 \pm S_6$	0.642 ± 0.0495[a]	0.559 ± 0.0064[ab]	0.391 ± 0.1040[bc]	0.369 ± 0.0630[bc]	0.448 ± 0.0311[abc]	0.401 ± 0.0176[bc]	0.345 ± 0.0021[c]	0.0051

Legend: Averages followed by different lowercase letters indicate significant differences in the lines.

C = control; = mean absorbance of the 5th dilution; S5 = standard deviation of the 5th dilution; = mean absorbance of the 6th dilution; S6 = standard deviation of the 6th dilution; C (MAC-T control); B1 (2 genes/acute mastitis); B2 (4-8 genes/acute mastitis); B3 (> 10 genes/acute mastitis); B4 (2 gene/recurrence mastitis); B5 (4-8 genes/recurrence mastitis); B6 (> 10 genes/recurrence mastitis). (HLA, NUC, FNBA, FNBB, ETA, ETB, LUC, TST, sea, seb, sec, se, se, seh, sej, sei, MEC, ANT, BLAz, APH3, ERMA, ERMB, ERMC , TETK, TETM, MRSA, norA, norB, norC, LmRS, TET 38).

4. Discussion

Antibiotic-resistant pathogens are a significant threat to milk production in developing countries that rely on milk production for export [3]. Among several alternatives to antibiotic administration to ruminants for mammary gland protection are the use of plant-based ointments [3]. Some of those products contain plant extracts that are rich in flavonoids. In this research the hipothesis is that compounds such as quercetin and luteolin contribute to the protective properties in *H. martiana* at concentrations protecting against pathogenesis while remaining harmless to mammary gland cells. Polyphenols represent one of the most abundant compounds present in plants and the flavonoids led our group and others to study the effects recently and demonstrating a therapeutic potential [9]. These compounds reflect a large benefit for protection against mastitis." at a small risk for mammary gland inflammation in ruminant species.

Due to the vast number of free radicals and forms of action in living organisms, different complementary methods were used to evaluate the antioxidant activity of the extract. Silva *et al.* [2] reported that *H. martiana* bark extract has extraordinary antioxidant potential and can protect the health of milk-producing animals. Thus, the redox states were sought due to bacterial infection, which leads to an increase in reactive oxygen species (ROS) and the use of antioxidants for protection. The leaves contain many compounds based on flavonoids, specifically quercetin, which can contribute to the health of ruminants affected by mastitis.

The MAC-T cells were exposed to the dilutions of the extract for 24 hours and were then followed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The mean values of D.O. (optic density) obtained are illustrated in Figure 2. From the data in this research, was demonstrated more protective potential in *H. martiana in vitro*.

Our *in vitro* findings help contribute to the antimicrobial and antioxidant profile often reported for plants in *Hymenaea*. In this regard, it is necessary to move to further understand the therapeutic profile in our extract dilutions in response to *S. aureus* infection, leading to the study carried out on the effect that the extract could have on the possibility of cell infection after exposure to the product.

To evaluate the protective effect in *H. martiana*, was investigated the protection of MAC-T cells against several *S. aureus* mutants. In this experiment, was founded our 5th and 6th dilutions of the plant extract would give protection in our multi-drug resistant strains (Table 3). Thus, was used these dilutions to look at the effect in preventing the invasion of *S. aureus* into MAC-T cells (Figure 2). The accession process and internalization are essential in establishing mastitis in a study of *Streptococcus uberis* [10]. From this results, the survivability of MAC-T cells was dependent on the genetic profile of S. aureus. With the investigation of S. aureus in acute and recurrence mastitis cases with two virulence/resistance profiles (Figure 4).

Survivability stands out in the cause of the initial stages of infection [10,11]. Barros *et al.* [11] reported that adhesion is the first step in the initial stage of the disease and the first step in the formation of microbial biofilm. The biofilm structure is categorized into stages as primary and reversible accession secondary adhesion and reversible and biofilm formation [12]. Was noticed a similar trend in our MAC-T cells with *S. aureus*. One supposition is the extract has a protective effect in the MAC-T cells, creating a hostile environment on invading microbes.

In animal studies, initial infection is followed by an anti-inflammatory process by the host's immune system, which slows the progression of mastitis. Li *et al.* [13] report quercetin exhibits anti-inflammatory potential and is a

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potential therapeutic agent for mastitis. In our study, Was founded quercetin and luteolin metabolites, which may be essential for the protection of the MAC-T cells against initial stages of infection. The mechanism of action flavonoids utilize starts with the B-ring in the C2-position, a C2=C3 double bond in the C-ring and an OH-group at C5- and C7- positions in the A-ring [i.e. the flavones, apigenin, and luteolin], are associated with good anti-inflammatory effects [14].

Studies both *in vitro* and *in vivo* have attempted to understand how plant extracts affect the mammary gland, but it is challenging to control pathophysiology aspects *in vivo* due to the complexity of autocrine signaling. A recent discovery from Barros *et al.* [11] observed the cellular impact on their synthetic product. Bacteria with a significant mutagenic profile have many different molecular mechanisms to survive in hostile environments, reflecting the severity during infection stages.

Dilution of the extract (leaf) at a concentration of 781.25 mg/mL has a bactericidal potential in *S. aureus* strains in some *in vitro* and *in vivo* studies, in which has not been previously reported. Thus, the extract's ability to relate to the cell without damaging or destroying may lead to further research suggesting *H. martiana* extract's concentration at 781.25 mg/mL may be used for the treatment of mastitis without causing damage to the cells of the mammary gland. Due to MAC-T cells did not show intoxication profile at this concentration, our information can be gleaned upon for future *in vivo* studies with *H. martiana* leaf extracts.

5. Conclusion

Antibiotic resistance has developed into a significant problem in milk production. In the search for the development of a compound that is a topically applied ointment for mastitis therapy, was founded that *H. martiana* is one such non-antibiotic that can be refined and used for the treatment of mastitis in ruminants. *H. martiana* did not cause damage in the alveolar cells of MAC-T cells in lower concentrations. *H. martiana* has medicinal protection against *S. aureus*, and our data may reflect future studies with synergisms and other techniques to leverage the power of drugs and combat diseases such as in mastitis and multi-drug resistant bacteria.

Manufactures:

(1) Waters Corporation. Milford, USA.

(2) Thermo Scientific ™.Waltham, USA.

(3) Olympus®, Tokyo, Japan.

(4) Thermo Spectronic, Madison, USA.

(5) Sigma-Aldrich, St. Louis, USA.

(6) BioTek. Winooski, USA

Declaration of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and the writing of the manuscript.

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