Supplementary File

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**Methodology**

**Plant material and extraction**

The leaves of *H. martiana* Hayne were harvested from the voucher specimen number 21868, which is deposited in the Herbarium Vale do São Francisco (HVASF), located in the city of Petrolina, Pernambuco, Brazil. The extraction and production of crude ethanolic extract (CEE) from the leaf was performed according to Peixoto *et al.* (2015), with some modifications since its material was the bark of the plant. The leaves spent seven days in an air circulating oven at 45°C and were then macerated in absolute ethyl alcohol, and the extract concentrated in a rotary evaporator.

**Phytochemical determination by UHPLC-PDA-qTOF-MS / MS**

The experiments were performed using an ACQUITY UPLC H-Class liquid chromatograph1 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 MSE 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.

**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 calculation of the loss of fluorescence, or area under the curve (ASC), are carried out using the following formula: ASC = 1 + f1 / f0 + ........ fi / fo + ........ + f80 / f0

where: f0 is represented by fluorescence obtained at time 0 and fi fluorescence obtained at times between 0 and 80 minutes.

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 (Prior *et al.* 2003; Davalos *et al.* 2004).

**Antioxidant power by iron reduction (FRAP)**

The ferric reduction capacity of the extract at 2.5% concentration is determined by the FRAP method (Benzie *et al.* 1996) with some modifications. 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 FeCl3. 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.

**Total polyphenol content in leaves (μg mL-1)**

The total polyphenol concentration of the extracts was analyzed according to the Folin-Ciocalteu method (Stagos *et al.* 2012). The procedure was performed in test tubes with a capacity of 3.0 mL. A volume of 25μL of the test solutions was mixed with 125 μL of the Folin-Ciocalteu reagent and 1250μL of distilled water. The homogenized tubes were resting for 3 min, and then 350μL of 25% (w/v) sodium carbonate solution and 750 μL of distilled water were added. The test tubes were shaken and held in the dark at room temperature for 1 hour. After one hour, the absorbance was measured at 765nm using a spectrophotometer.

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.

**Total flavonoid content (μg mL-1)**

The dosage of flavonoids was performed according to Yao *et al.* (2013), using rutin as standard, in absolute ethyl alcohol and aluminum chloride. For the assay, 10mg of rutin was measured then transferred to a 10mL volumetric flask and then dissolved and quenched with absolute ethyl alcohol. The remaining solution has the concentration equal to 1000 μg mL-1 and thus made the standard curve reading.

To quantify the total flavonoid content in the leaves of *H. martiana*, a 100μL aliquot of the sample was added to the test tube along with 400 μL of 70% alcohol and 50μL of 5% NaNO2. After 6 minutes, 50μL of the 10% aluminum chloride (AlCl3) solution, 300μL of NaOH (1 M), and 100μL of distilled water were added. The spectrophotometer was measured at 510nm; the blank used contains all reagents except the sample. The results are shown as μg mL-1 (Table 2).

**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 (1958) with modifications was performed. A mixture containing 1mL of acetate buffer (100 mM/ H 5.5), 1.25mL of absolute ethanol, 250μL of 500mM DPPH ethanolic solution, and 50μL of extracts diluted in ethanol were vortexed and rested for 30 minutes in a low light environment. DPPH • exhibits maximum absorbance at 518 nm, which decreases in the presence of H+ donor molecules, indicated by the change from purple to yellow. The absorbances were determined in a UV-visible spectrophotometer at 518 nm. All determinations were performed in triplicate for statistical analysis, and the sequestering activity of the DPPH • radical was expressed as a percentage being calculated according to the following equation: %AA = (Acontrol – Asample/Acontrol) x 100 where: control is the absorbance of the DPPH and Sample is the absorbance of the sample after 30 minutes.

A graph of %AA x Concentration (μg mL-1) was constructed with the values obtained. For the calculation of the Inhibitory Concentration (IC50), 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).

**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% CO2 supplementation 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 confluence (1x105 cells) (Silva *et al.* 2014).

***S. aureus* isolates used in the research**

The *S. aureus* used in the study belongs to the bacterial “library” of the Laboratory of Bacterial Diseases (LDBAC) of the preventive sector of the UFV. 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. Six isolates of *S. aureus* were used, three strains isolated from cases of acute mastitis, and three isolated cases of remitting mastitis. 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 spectrophotometer apparatus (Biomate 3)4. This concentration corresponds to approximately 107 CFU mL-1, pre-calibrated by standard plate count.

**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.

**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. This reaction only occurs in metabolically active cells, therefore living cells (Mosmann, 1983). The percentage of viable cells was measured as described by Al-Sheddi *et al.* (2015). Briefly, upon reaching confluency, MAC-T cells were exposed to the compounds in nine dilutions 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.

**Protective effect on MAC-T**

The isolates were incubated in TSB broth (tryptone soy broth) until reaching the concentration of 105 CFU mL-1, and the cells reached 1x105 confluency in Dulbecco's modified Eagle's medium without 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 (Peixoto *et al.* 2015; Vieira *et al.* 2018) and that in previous tests have observed (Vieira *et al.* 2018) that do not cause severe damage to MAC cell structures -T.

**Figures and Table legends**

Supplementary Figure 1. 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.

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

Supplementary Table 2. Antioxidant activity by the radical sequestration method DPPH (IC50) and total compounds of the concentrations of the hydroalcoholic extract of *H. martina*.

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

Supplementary 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

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).

**Figures**

Supplementary Figure 1



**Tables**

Supplementary Table 1

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Peak | Retention time | λmax | [M-H]- | MS2 | Possible identification |
| 1 | 2.23 | 253. 357 | 609.1422 | 463.0853; 197.8061 | Quercetin-hexoside-rhamnoside |
| 2 | 2.33 | 263.357 | 609.1425 | 463.0859; 197.8061 | Quercetin-hexoside-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-methylhexoside |
| 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 |

Supplementary Table 2

|  |  |  |  |
| --- | --- | --- | --- |
| Concentration of *H. martina* | Antioxidant activity | Total compounds | |
| (mg mL-1) | IC50 | (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 |
| 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 |

Supplementary Table 3

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Protective effect (Absorbance)** | | | | | | | | |
|  | **Bacteria samples** | | | | | | | |
| **Diluitions** | **Control** | **B1** | **B2** | **B3** | **B4** | **B5** | **B6** | ***P*** |
| **5a** (1.562.5 mg/mL) | 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 |
| **6a (**781.25 mg/mL) | 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 |

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