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Biological properties and phytochemical characterization from *Miconia chamissois* Naudin aqueous extract

[Propiedades biológicas y caracterización fitoquímica del extracto acuoso de *Miconia chamissois* Naudin]

Lorena Ferreira Gomes[1], Diegue Henrique Nascimento Martins[1], Sandra Marcia Mazutti da Silva[2], Yuri Yabu de Barros[1], Paula Monteiro de Souza[1], Marcela Medeiros de Freitas[1], Cristopher William Fagg[2], Luiz Alberto Simeoni[1], Pérola Oliveira Magalhães[1], Damaris Silveira[1] & Yris Maria Fonseca-Bazzo[1]

[1]Department of Pharmacy, Health Sciences School, University of Brasília, Brasília, Distrito Federal, Brazil
[2]Ceilândia Faculty, University of Brasília, Brasília, Distrito Federal, Brazil

Abstract: The objective of this study was to evaluate biological and phytochemical properties of the aqueous extract from the leaves of *Miconia chamissois* Naudin (AEMC). Phytochemical properties were assessed by analyzing the chromatographic profile and the polyphenol content of AEMC. Biological properties evaluation was conducted based on cytotoxicity assay and by evaluating the antioxidant, antimicrobial, and enzymatic inhibition activities. Results indicated the presence of phytochemicals in AEMC such as flavonoids and polyphenols, including rutin, isoquercitrin and vitexin derivatives. AEMC showed antioxidant activity, which may be attributed to the high polyphenolic content. Moreover, AEMC demonstrated in vitro enzyme inhibition activity against tyrosinase and alpha-amylase, as well as showed low cytotoxicity. On the other hand, AEMC exhibited weak antimicrobial activity against *S. aureus* and *C. albicans*. Thus, AEMC is a promising alternative in search of potential drugs for the treatment of diseases induced by oxidative stress and inflammation, conditions due to hyperpigmentation processes, such as melisma, as well as for diabetes.

Keywords: Antioxidant; Antimicrobial; Polyphenols; Tyrosinase; Alpha-amylase; *Miconia chamissois*

Resumen: El objetivo de este estudio fue detectar las propiedades biológicas y fitoquímicos del extracto acuoso de las hojas de *Miconia chamissois* Naudin (AEMC). Las propiedades fitoquímicas se evaluaron analizando el perfil cromatográfico y el contenido de polifenoles de AEMC. La evaluación de las propiedades biológicas se realizó en base al ensayo de citotoxicidad y evaluando las actividades de inhibición antioxidante, antimicrobiana y enzimática. Los resultados indicaron la presencia de fitoquímicos en AEMC, como flavonoides y polifenoles, que incluyen derivados de rutina, isoquercitrina y vitexina. AEMC mostró una actividad antioxidante considerable, que puede atribuirse al alto contenido polifenólico. Además, AEMC exhibió actividad de inhibición enzimática in vitro contra tirosinasa y alfa-amilasa, así como mostró baja citotoxicidad. Por otro lado, AEMC demostró actividad antimicrobiana débil contra *S. aureus* y *C. albicans*. Por lo tanto, AEMC es una alternativa prometedora en busca de posibles drogas para el tratamiento de enfermedades inducidas por el estrés oxidativo y la inflamación, afecciones debidas a procesos de hiperpigmentación, como el melasma, así como para la diabetes.

Palabras clave: Antioxidante; Antimicrobiano; Polifenoles; Tirosinasa; Alfa-amilasa; *Miconia chamissois*

INTRODUCTION

Melastomataceae is one of the largest families of flowering plants widespread globally and comprises 4,079 accepted species names and 167 genera, being found in tropical and subtropical regions (Abdullah & Yong, 2007; The Plant List, 2020). In Brazil, this family is found in all regions, with 1,506 known species distributed in 73 genera (Baumgratz *et al.*, 2015). The most important genera of Melastomataceae families are *Miconia* Ruiz & Pav., *Leandra* Raddi & Pav. Lee, and *Tibouchina* Aubl, which are the most studied and reported, particularly pertaining to economic, botanical, and therapeutic aspects (Goldenberg *et al.*, 2012).

The genus *Miconia* has 289 species described for Brazil, distributed across four phytogeographic domains -Amazonia, Caatinga, Cerrado (Brazilian Savannah), and Mata Atlântica. In addition, 123 species from this genus are considered endemic to Brazil (Baumgratz *et al.*, 2015).

Miconia chamissois Naudin is a native species found in Caatinga, Brazilian Savannah, and Mata Atlântica biomes (Goldenberg *et al.*, 2012). The following six botanical synonyms are attributed to this species: *Acinodendron chamissoi* (Naudin) Kuntze, *Melastoma calyptratum* Schrank & Mart. ex Triana, *Miconia calyptrata* Mart. ex Triana, *Miconia langlassei* Standl., *Miconia panamensis* Gleason, and *Oxymeris calyptrata* Cham. ex Naudin (Tropicos, 2019). Species of *Miconia* genus are popularly named as "pixirica", "jacatirão", or "sabiazeira" (Goldenberg, 2004). *Miconia chamissois* Naudin has been described popularly as "Maria Preta" (Araujo *et al.*, 2017).

In traditional medicine, some species of the *Miconia* genus have been used for cold and fever [*M. cinnmonifolia* (DC.) Naudin], as antirheumatic and eupeptic [*M. albicans* (Sw.) Tr.] (Cruz & Kaplan, 2012) agents, as well as for throat disorders [*M. rubiginosa* (Bompl.) DC.] (Rodrigues & Carvalho, 2001). The use of *M. chamissois* as food by inhabitants of Mato Grosso do Sul had also been described (Bortolotto *et al.*, 2018).

In general, most studies on the Melastomataceae family have focused on botanical, taxonomic, or environmental quality characterization (recovery of degraded areas or quality indicators) (Zampieri *et al.*, 2013). Thus, there are few studies published on the biological activity and the phytochemical constituents of *M. chamissois* Naudin. One of the studies that screened for phytochemical content of the aqueous extract of *M. chamissois* leaves (AEMC) found that it was composed of

anthraquinones, triterpenoids, saponins, and tannins. This extract was phytotoxic to the germination of maize and cucumber, and the authors related the effect to the increase in triterpenoids and tannins content during the dry season (Pinto & Kolb, 2016). Alves (2016) described groups of compounds from *M. chamissois* extract, such as steroids, triterpenoids, flavonoids, saponins, tannins, alkaloids, and coumarins (Alves, 2016), and showed that *M. chamissois* extract demonstrated *in vitro* inhibition of MMP-2 and MMP-9 activities. Another study demonstrated, *in vitro*, the antineoplastic potential of *M. chamissois* extract in glioma cell lines (Morais, 2019). Silva *et al*. (2020) investigated the cytotoxicity of the chloroform partition from *M. chamissois* Naudin and its sub-fraction in glioblastoma cell lines and one normal cell line of astrocytes, and the isolated compound matteucinol presented selective cytotoxic for the tumour cells. Recently, Gimenez *et al*. (2020) showed seasonal variations in the chemical composition of *M. chamissois* from Brazilian Savannah. Among the characterized compounds - miconioside B, matteucinol 7-O-β-apiofuranosyl(1→6)-β-glucopyra-noside, ursolic acid and oleanolic acid - the content of the last three compounds was decreased in the rainy season.

Brazil possesses the largest biodiversity in the world, which is a potential source for bioeconomy (Valli & Bolzani, 2019). However, only 8% of the 60,000 species of superior plants had their active compounds studied, while medicinal properties were investigated for only 1,100 species (Brazil, 2016). Thus, taking into consideration the limited number of studies about *M. chamissois,* the necessity to expand studies on Brazilian flora, the medicinal potential of *M. chamissois* Naudin, as well as its use in Brazilian traditional medicine, the aim of this work was characterize the biological and phytochemical properties of aqueous extract from the leaves of *M. chamissois* Naudin (AEMC).

MATERIAL AND METHODS
Plant material and extraction process
Miconia chamissois Naudin leaves (Melastomataceae) were collected in Brasilia, Brazil, at the Lagoa de Cedro (14.53.54S 47.58.38W) by botanist Christopher William Fagg in March 2015. An exsiccate was deposited in the Herbarium of the University of Brasília (UB) under the voucher CWF 2358.

The leaves were dried at room temperature. The dried leaves were powdered in a knife mill., and submitted to extraction by infusion in a ratio of 1:5 (Brasil, 2010). The aqueous extract was then lyophilized (VirTis SP Scientific Advantage Plus XL-70 Benchtop Freeze Dryer) and stored at -20°C.

This project was registered at SisGen (National System for the Management of Genetic Heritage and Associated Traditional Knowledge), under the number A215A9A.

Phytochemical screening: Total polyphenol content
Polyphenol content was determined using the method proposed by Kumazawa *et al*. (2004), with few modifications. Briefly, 0.5 mL of Folin-Ciocalteu reagent was added to 0.5 mL of sodium carbonate (10%) and 0.5 mL of sample or standard. After reaction at room temperature for 1 h, the absorbance was determined at 760 nm using a Shimadzu® UV-1800 (Software UVProve 2.33) spectrophotometer. Gallic acid was used as the standard, and a standard curve was generated using concentrations in the range of 1.0 - 9.0 μg/mL. The concentration of the sample tested was 5 μg/mL. The data obtained from the standard curve were used for the estimation of the polyphenol content in the sample, using linear regression. The results were expressed as μg of gallic acid equivalents per mg of extract (μg GAE/mg). The analyses were performed in triplicate.

Total flavonoid content
Flavonoid content was determined according to Kumazawa *et al*. (2004), with modifications. Quercetin was used as a standard, and a standard curve was generated using concentrations in the range of 0.8 - 25.0 μg/mL. The concentration of the sample was 12.5 μg/mL. In the assay, 0.5 mL of 2% aluminum chloride was added to 0.5 mL of sample/standard. After allowing a reaction time of 1 h at room temperature, the absorbance was determined using a Shimadzu® UV-1800 (Software UVProve 2.33) spectrophotometer at 420 nm. The data obtained from the standard curve were used for the calculation of the flavonoid content in the sample, using linear regression. The results are expressed as μg of quercetin equivalents per mg of extract (μg QE/mg). The analyses were performed in triplicate.

Thin-layer chromatography (TLC) analysis
The stationary phase used was silica gel on aluminium folium (Sobernt Technologies®, 200 μm, 20×20). The eluent was ethyl acetate, formic acid, acetic acid, and deionized water (100:11:11:26). Natural product/polyethylene glycol (NP/PEG) reagent (2% diphenylboryloxyethylamine methanolic

solution - solution A and 5% polyethylene glycol ethanolic solution 4000 - solution B) was used as the detection reagent (Wagner & Bladt, 1996). The sample was eluted at a concentration of 10 mg/mL, in methanol.

After elution, the chromatograms were visualized under a UV lamp at 365 nm, and the retention factor (Rf) was determined for each spot. Rf was used in the identified of the compounds in the sample by comparing with the authentic standards, caffeic acid, rutin, isovitexin, vitexin, and isoquercitrin, from Sigma-Aldrich® and hiperoside from Merck®.

High-performance liquid chromatography (HPLC) analysis

The HPLC analysis was carried on a Hitachi LaChrom Elite® HPLC System (L-2130 pump, L-2200 auto-sample, L-2300 column oven, and L-2455 DAD detector). A C18 column was used (5 µm, 250 × 4.6 mm; LiChroCART®150-4.6 Purospher® RP-18e) at 25°C; the flow rate was set at 0.6 mL/min, the injection volume was 10 µL, and detection was performed at 354 nm. Phosphoric acid 1% (A) (Sigma-Aldrich®) and acetonitrile (B) (Tedia®) were used as the mobile phase. Gradient elution was performed using the following combination of solvents: 0 min. 90% (A) and 10% (B), 40 min. 70% (A) and 30% (B), 50 min. 50% (A) and 50% (B).The data were analyzed using EZChrom Elite software, version 3.3.2 SP1. All solvents used were HPLC grade (Sigma-Aldrich® and Tedia®). The water used was obtained using a Millipore Mili-Q System (Leite *et al.*, 2014).

The compounds were identified by the retention time and UV spectra by comparing with authentic standards, such as hiperoside (Merck®), isoquercitrin (Sigma-Aldrich®), isovitexin (Sigma-Aldrich®), quercetin (Sigma-Aldrich®), quercetin 3-O-α-L-arabinopyranoside (Sigma-Aldrich®), quercitrin (Sigma-Aldrich®), rutin (Sigma-Aldrich®), vitexin (Sigma-Aldrich®), and vitexin 2-O-rhamnoside (Sigma-Aldrich®). A mixed point method with samples and standards was used to confirm the presence of the compound. For this method, isovitexin, vitexin, and vitexin 2-O-rhamnoside (Sigma-Aldrich®) at 100 µg/mL were used as standards. To determine the vitexin equivalents (VE) in the extract, a standard curve was generated using vitexin concentrations in the range of 8 to 100 µg/mL. The sample was analyzed at a concentration of 2.5 mg/mL in methanol. The data obtained from the standard curve was used for the estimation of the vitexin equivalents in the sample, using linear regression. The analyses were performed in triplicate.

Biological screening: Antioxidant activity
Antioxidant activity was evaluated by two methods: DPPH radical reduction and phosphomolybdenum method.

Antioxidant activity by DPPH radical

reduction was assessed by a method previously proposed by Blois (1958). Ascorbic acid (AA) (1.2 – 5.4 μg/mL) and butylated hydroxytoluene (BHT) (1.9–38.5 μg/mL) were used as positive controls. The

sample concentration range was 1.5–5.0 μg/mL. The inhibition potential was calculated using the absorbance of the sample and control using the following equation:

$$\% \text{ inhibition} = 100 - [(\text{Abssample} - \text{Abswhite}) / \text{Abscontrol}) \times 100)]$$

Where, Abssample is the absorbance of the sample, Abswhite is the absorbance of the reaction medium and Abscontrol is the absorbance untreated control

The concentration corresponding to 50% inhibition (IC_{50}) was calculated from a linear regression of the positive control data (Locatelli *et al.*, 2009).

Antioxidant activity by phosphomolybdenum method was evaluated according to Prieto *et al.* (1999). Ascorbic acid and BHT were used as positive controls, and standard curves were generated in the concentration ranges of 4.5 - 45.5 μg/mL and 13.0 - 74.0 for ascorbic acid and BHT, respectively. The sample concentration was 22.7 μg/mL. The assay was performed by adding 1.0 mL of the reagent solution to 0.1 mL of the sample or the standard. The reagent solution consisted of 28 mM phosphate solution, 4 mM molybdate, and 0.6 M sulfuric acid. After 90 min of reaction time in a water bath at 95°C, the absorbance was measured at 695 nm using a Shimadzu® UV-1800 (Software UVProve 2.33) spectrophotometer. The data obtained from the standard curve were used for the estimation of the equivalents of ascorbic acid or BHT content in the sample, using linear regression.

Antimicrobial activity
Determination of antimicrobial activity was carried out according to the method proposed by The National Committee for Clinical Laboratory Standards - NCCLS (NCCLS, 2002; NCCLS, 2003a; NCCLS, 2003b). The microorganisms used to evaluate the antimicrobial activity were *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Candida albicans* (ATCC 40277). *S. aureus* and *E. coli* were cultivated in Mueller-Hinton liquid medium at pH 7.2 - 7.4. in an oven at 35°C for 24 h. The fungus, *C. albicans* was cultivated in Sabouraud liquid medium at pH 5.6 in an oven at 35°C for 24 h.

Sensitivity testing of the antimicrobial agents was evaluated by using a microdilution method and identification of the presence of bacteria was assessed with resazurin. The evaluated sample concentrations

ranged from 5000 to 39 μg/mL. Bacteria were inoculated at 5.0×10^4 CFU/mL and fungus was inoculated at 2.5×10^3 CFU/mL. The positive controls were oxacillin and ampicillin at a concentration range of 1250 μg/mL to 0.01 μg/mL, and fluconazole at a concentration range of 800 μg/mL to 6.3 μg/mL.

In a 96-well plate, 100 μL of either the sample or the standard were added to 100 μL of inoculum. An untreated control was produced with 200 μL of inoculum. For the positive control, 100 μL of the antibiotic and 100 μL of inoculum were used, and for sterility control, the sterile medium (200 μL) was used. The plate was incubated at 35°C for 24 h. At the end of the incubation, 30.0 μL of resazurin at 0.1 mg/mL were added to all wells, and the plate was incubated for an additional 3 h (Alves *et al.*, 2008).

The results were expressed as the minimum inhibitory concentration (MIC). The wells on which the MIC was determined were used to seed a new petri dish to confirm the death of the microorganism. The contents of the wells of the next higher and lower concentrations of MIC were also seeded on to either the Sabouraud dextrose agar medium or Mueller-Hinton agar medium for fungus and bacteria, respectively.

For the disk diffusion method, the sample concentrations ranged from 1000 to 62.5 μg/mL. Bacteria were inoculated at 1.0×10^6 CFU/mL and the fungus at 1.0×10^5 CFU/mL. The standards used were 10 μg ampicillin and 32 μg fluconazole.

The disks were produced by adding 20.0 μL of sample to paper disks, which were kept for 24 h for sample absorption. In petri dishes containing the agar medium (Mueller-Hinton pH 7.2 - 7.4 for bacteria and Sabouraud pH 5.6 for fungus), 100 μL of the inoculum were added and over it, the disks with the respective sample/standard concentrations. Sterility control testing was performed with sterile water. The results were expressed as the halo diameter, which was measured with a pachymeter.

Enzymatic inhibition: Tyrosinase inhibition

Tyrosinase inhibitory capacity was evaluated according to Khatib *et al.* (2005) with modifications. Kojic acid was used as a positive control at concentrations ranging from 1000.0 to 1.0 µg/mL (concentration in the reaction medium). The sample was evaluated in the same concentration range as that of the positive control. The assay was performed in triplicate in a 96-well plate, adding 60 µL of buffer to each well (50 mM sodium phosphate buffer pH 6.5) and 10 µL of sample. Finally, 30 µL of 25 U/mL of mushroom tyrosinase were added, and the reaction mixture was incubated for 5 min at room temperature. Enzyme control was achieved by adding 70 µL buffer and 30 µL enzyme. At the end of the incubation period, 100 µL of L-tyrosine substrate (2 mM) were added to all wells, and after allowing 20 min of reaction time at room temperature, absorbances were measured at 475 nm, in a Multimode detector (DTX 800, Beckman Coulter®). All reagents were obtained from Sigma-Aldrich®. The percentage of inhibition was calculated using the equation:

$$\% \text{ inhibition} = ((C-A) \times 100) / (C)$$

Where C is the absorbance of the enzyme control, and A is the Sample/standard absorbance

The IC_{50} values of the sample and the positive control were determined using nonlinear regression analyses of the % inhibition and the log concentration values of the sample or positive control, respectively. All tests were performed in triplicate.

Alpha-amylase inhibition

Alpha-amylase inhibition activity was determined using the method proposed by Bernfeld (1955) with modifications. Acarbose was used as a positive control at concentrations ranging from 100 to 1.9 µg/mL. Samples were evaluated at concentrations of 1000 µg/mL. The following were added into microtubes: 930.0 µL buffer (0.02 M sodium phosphate buffer pH 6.9 with 0.0067 M sodium chloride), 50.0 µL alfa-amylase solution (porcine pancreatic α-amylase solution 40 IU/mL), and 20.0 µL of sample/standard. Before proceeding with the assay, these pre-incubates remained at room temperature for 30 min. For assaying the activity in the tube, the following were added: 250.0 µL buffer, 500.0 µL of 1% starch solution and 250.0 µL of the pre-incubate. Afterwards, the mixture was incubated in a water bath at 40°C for 20 min. After this, 500 µL of DNS (3,5-dinitrosalicylic acid) were added to all tubes that were submitted to additional 5 min incubation in a water bath at 100°C. After cooling, 4.5 mL of distilled water were added to all tubes. The absorbance was measured at 540 nm using a spectrophotometer (Shimadzu, UV - 1800). The inhibition percentage was calculated using the same equation describe above in tyrosinase inhibition.

Cytotoxicity

Murine macrophage cells (Raw 264.7), human keratinocytes (HaCat), and mouse fibroblasts (L929) were used for the cytotoxicity assays. Raw 264.7 (ATCC TIB-71) cells were obtained from the Adolf Lutz Institute Cell Bank, and L929 and HaCat cells were obtained from the American Type Culture Collection - ATCC. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with phenol red (GIBCO®), at pH 7, enriched with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics. Cells were grown in 75 cm^2 specific filter bottles at 37°C and 5% CO_2. The cytotoxicity test was performed in 96-well plates by the method proposed by Mosmann (1983) and Hansen *et al*. (1989). The sample was evaluated at the following concentration ranges: 500 to 15.6 µg/mL, 1250 to 31.3 µg/mL, and 1500 to 300 µg/mL, for HaCat, L929 and Raw 264.7 cell lines, respectively. The cell densities of L929 and HaCat cell lines were 1.5 x 10^4 cells/well and that for Raw 264.7 cell line was 2.0 x 10^4 cells/well. After 24 h of plating the cells, DMEM medium was removed and 200.0 µL sample concentrations diluted in DMEM were added. The plate was then again transferred to a 37°C and 5% CO_2 incubator. After 24 h, DMEM medium was removed and 50.0 µL MTT (Sigma-Aldrich®) at 1.0 mg/mL was added. The plate was again incubated at 37°C and 5% CO_2 for 4 h. Following this, 150 µL of acidified isopropanol (26 mL of isopropanol and 104 µL of HCl) were added to all the wells, and mixed to solubilize the formazan crystals formed. After complete solubilization, plate reading was performed using a Multimode detector (DTX 800, Beckman

Coulter®) at 570 nm. IC_{50} values and the percent cell viability were determined by averaging at least three independent assays, where each assay was performed in triplicate.

Statistical analysis
Results are expressed as mean ± standard deviation. Statistical analysis was performed using *t*-tests, using GraphPad Prism 5.0®. The confidence level was 95%.

RESULTS
Phytochemical screening: Total polyphenol and flavonoid content
Linear regression of the quercetin standard curve (y =

0.036x - 0.008 and r = 0.9998) and the gallic acid standard curve (y = 0.0943 x - 0.0145 and *r* = 0.996), were used to determine the flavonoid and polyphenol content present in the sample, respectively. The flavonoid content of AEMC was 252.8 μg of QE/mg extract, and the polyphenol content was 532.1 μg GAE/mg extract.

TLC analysis
In the TLC analysis for detection of flavonoids and phenolic compounds, six main spots were observed from AEMC (A), with Rf values of 0.9, 0.8, 0.7, 0.6, 0.5, and 0.3. The Rf values corresponding to the standards were: 0.6 for caffeic acid (B), 0.6 for hyperoside (C), 0.5 for rutin (D), 0.6 for isovitexin (E), 0.7 for vitexin (F), and 0.7 for isoquercitrin (G) (Figure No. 1). A similarity was observed between the Rf values for rutin and isoquercitrin. Therefore, a new TLC analysis was performed using the mixed point technique (i.e., sample plus standard), in order to confirm the presence of rutin and isoquercitrin flavonoids in the extract.
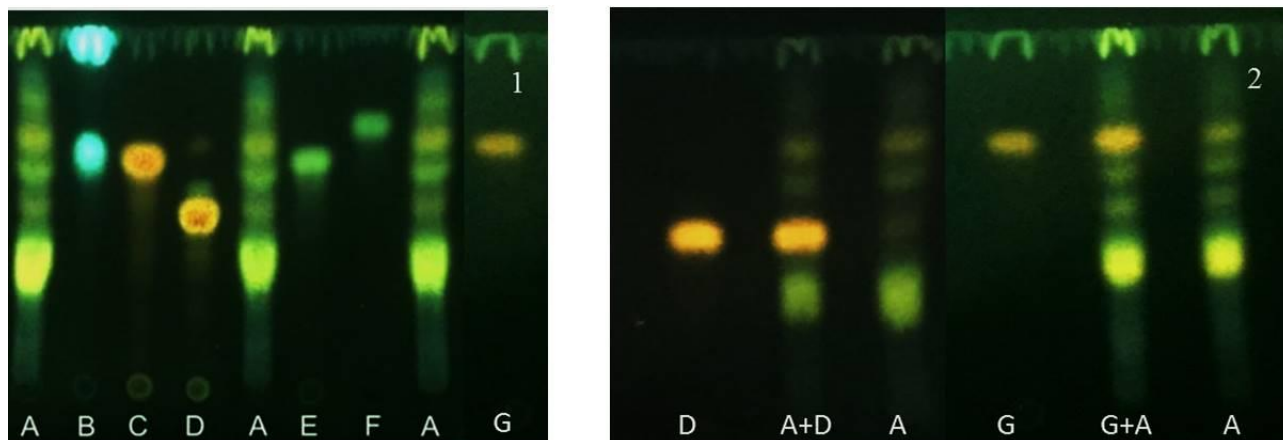

Figure No. 1
TLC analysis for detection of the following flavonoid and phenolic compounds. 1: aqueous extract from the leaves of *M. chamissois* Naudin (AEMC) (A), caffeic acid (B), hyperoside (C), rutin (D), isovitexin (E), vitexin (F), and, isoquercitrin (G), 2: mixed point. Eluent: ethyl acetate, formic acid, acetic acid and deionized water (100:11:11:26) and detection reagent: Natural product - Polyethylene glycol (NP / PEG) reagent, visualized by a UV lamp at 365 nm

HPLC analysis
The chromatographic profile of AEMC is shown in Figure No. 2. Nine main chromatographic peaks were observed. Table No. 1 shows the retention time, area, lambda maximum, and lambda minimum. In the identification process of AEMC compounds, the

similarity was observed in the UV spectra for one sample peak (retention time of 24.8 min – peak 6) when compared with the authentic standard; when compared with vitexin, isovitexine, and vitexin 2-O-rhamnoside standards, a similarity of 0.9968, 0.9963, and 0.9949, respectively, was observed. The mixed

point technique was used for AEMC and vitexin, isovitexin, and vitexin 2-O-rhamnoside standards, confirming different retention times between them.

The UV spectra similarity suggested the peak corresponding to the retention time of 24.8 min may be another derivative of vitexin.
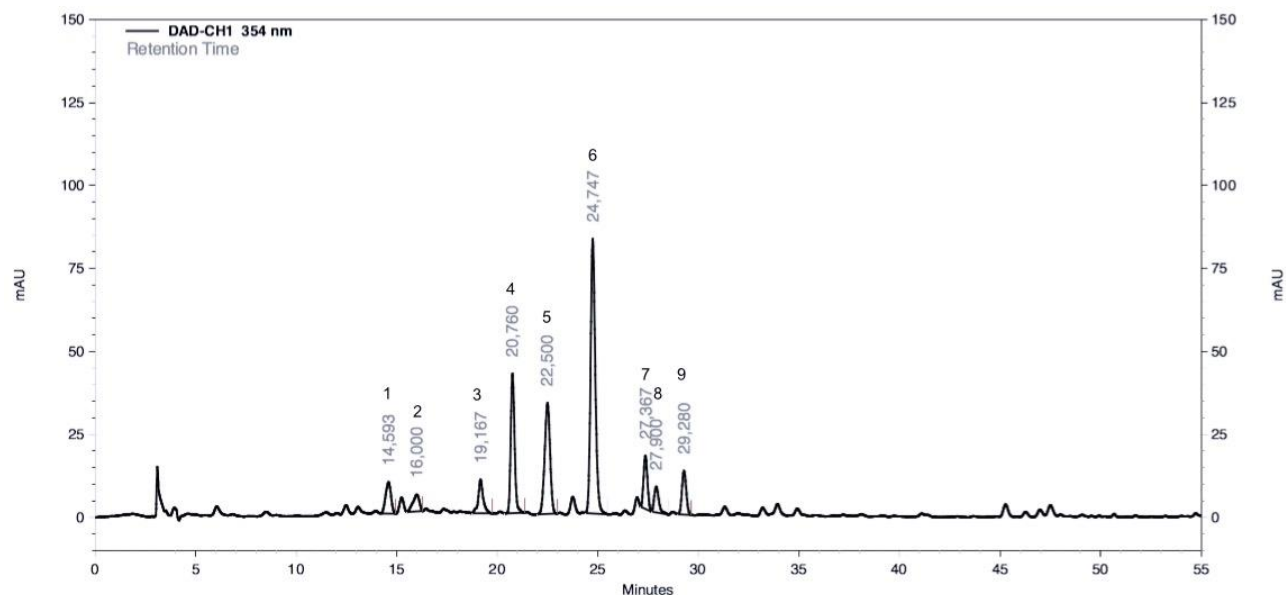

Figure No. 2
Chromatographic profile of aqueous extract from the leaves of *M. chamissois* Naudin (AEMC). Detection at 354 nm, C18 column, flow rate of 0.6 mL/min, eluent: phosphoric acid 1% (A) and acetonitrile (B) in gradient system

In order to quantify the AEMC compound corresponding to peak 6, linear regression of a standard curve generated using vitexin as the standard ($y = 104860x – 109129$, $r = 0.9981$) was used to determine a vitexin equivalent (VE) of 20.9 ± 0.1 µg VE/mg of AEMC.

Table No. 1
Peak area, retention time and lambda values of peaks detected from aqueous extract from the leaves of *M. chamissois* Naudin (AEMC) by HPLC

Peak	Peak area	Retention time (min)	Lambda maximum	Lambda minimum
1	708893	14.6	271	255
2	450709	16.0	268; 391	399; 388
3	688459	19.2	253; 360	399; 331
4	2262209	20.8	270; 349	309; 247
5	2506169	22.5	268; 349	309; 246
6	5380125	24.8	270; 338	247; 301
7	858342	27.4	268	249
8	489119	27.9	254; 366	399; 331
9	785116	29.3	258; 356	321; 241

Detection performed at 354 nm, C18 column, flow rate of 0.6 mL/min, eluent: phosphoric acid 1% (A) and acetonitrile (B) using a gradient system

Biological screening: Antioxidant activity

In the DPPH method, AEMC showed an IC_{50} of 2.9 ± 0.1 μg/mL. Positive controls, ascorbic acid and BHT, demonstrated IC_{50} values of 2.8 ± 0.1 μg/mL and 13.6 ± 0.2 μg/mL, respectively. There was no statistically significant difference in the IC_{50} for AEMC and ascorbic acid (Figure No. 3).
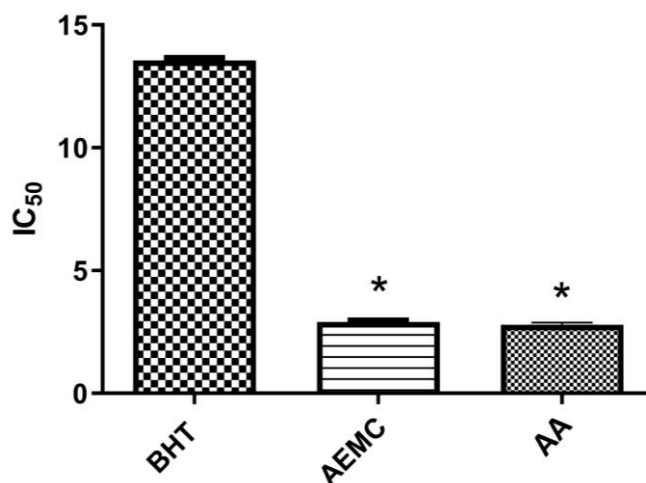

Figure No. 3
Antioxidant activity by the DPPH method IC_{50}: concentration corresponding to 50% inhibition, BHT: butylated hydroxytoluene, AA: ascorbic acid, and AEMC: Aqueous extract of *M. chamissois*. *$p<0.05$ *vs.* BHT by *t*-test. The data represent the mean ± SD (standard deviation) of three experiments, each performed in triplicate

AEMC also demonstrated antioxidant activity by the phosphomolybdenum assay, with equivalents of ascorbic acid content of 0.8 ± 0.01 μg/mg (y=0.0182x - 0.0524, r = 0.9967) and BHT content of 0.9 ± 0.05 μg/mg (y=0.0133x - 0.0137, r = 0.9985).

Antimicrobial activity

The results of the antimicrobial evaluation are summarized in Table No. 2. In the microdilution test, AEMC did not show antimicrobial activity against *E. coli*. However, AEMC showed activity against *C. albicans* and *S. aureus* with a MIC of 78.1 μg/mL and 312.5 μg/mL, respectively. Oxacillin and ampicillin (positive controls) showed activity against the microorganisms at all concentrations studied. Fluconazole did not induce death at a concentration of 800 μg/mL, indicating that *C. albicans* is resistant to the standard.

The wells at concentrations corresponding to the MIC, as indicated by the resazurin test, along with the wells at concentrations above the MIC as well as below the MIC, were seeded in either Sabouraud dextrose agar medium or Mueller-Hinton agar medium. After one day of incubation with *S. aureus*, the MIC-seeded plates showed growth of the bacterium, although in a small number of colonies, indicating that the MIC was bacteriostatic. The concentration level higher than the MIC (625 μg/mL) did not show any growth of the microorganism, whereas the concentration level lower than the MIC showed normal bacterial growth. Similarly, for *C. albicans* the MIC was seeded, but there was no growth of the fungus at the MIC or the concentration level higher than the MIC.

In the disk diffusion method, AEMC did not show any significant antimicrobial activity against *E. coli* or *C. albicans.* However, for *S. aureus* a small zone of inhibition (11.8 ± 0.3 mm) was observed compared to ampicillin. The positive controls, with ampicillin (10 μg) and fluconazole (800 μg/mL) showed inhibition zones of 26.3 ± 0.02 mm and 25.0 ± 0.4 mm, respectively. Enzymatic inhibition: Tyrosinase inhibition

The results of the tyrosinase inhibition assay are shown in Figure No. 4. AEMC inhibited the tyrosinase enzyme with an IC_{50} of 183.2 ± 6.2 μg/mL. Kojic acid, the positive control, showed an IC_{50} of 3.4 ± 0.4 μg/mL.

Table No. 2
Antimicrobial activity of aqueous extract from the leaves of *M. chamissois* Naudin (AEMC)

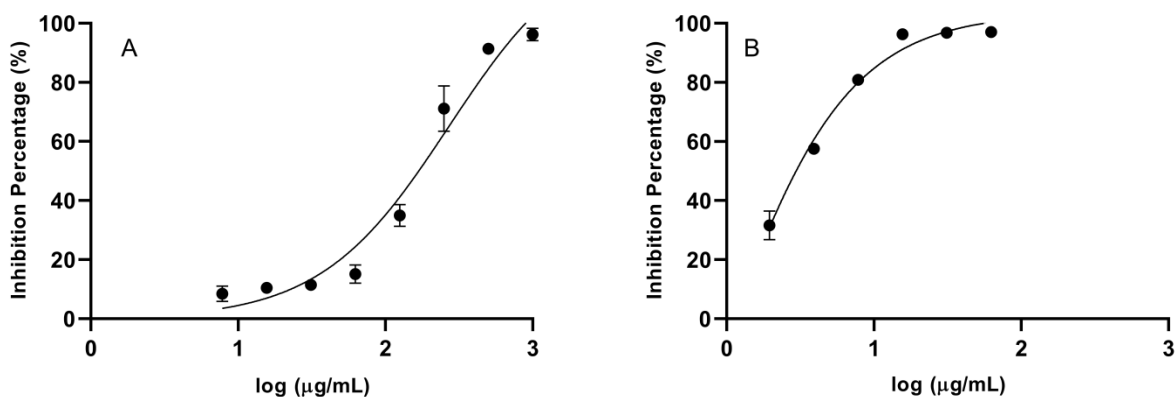| Microorganism	Zone of inhibition (mm)	Microdilution (resazurin) µg/mL
Staphylococcus aureus	11.8 ± 0.3	312.5
Escherichia coli	-	-
Candida albicans	-	78.1

Figure No. 4

Tyrosinase inhibition assay A: Percent inhibition for aqueous extract from the leaves of *M. chamissois* Naudin (AEMC). B: Percent inhibition for Kojic acid. The data represent the mean ± SD (standard deviation) of three experiments, each performed in triplicate

Alpha-amylase inhibition

The results of the α-amylase enzyme inhibition assay are shown in Figure No. 5. The IC_{50} value for AEMC was 15.1 ± 1.1 µg/mL, while the IC_{50} for acarbose was 9.2 ± 1.3 µg/mL. The inhibition activity of the AEMC sample was significantly lower than acarbose ($p<0.05$).
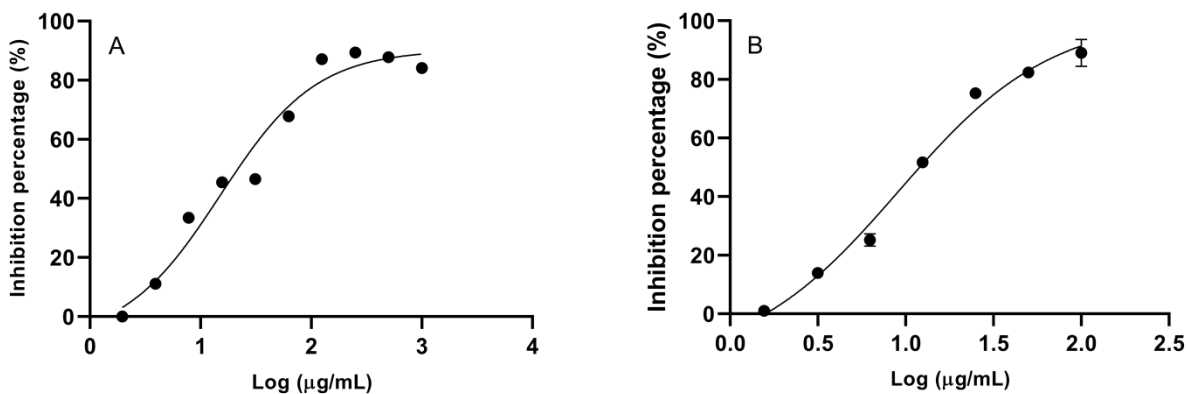

Figure No. 5
Alpha-amylase inhibition assay. A: Percent inhibition for aqueous extract from the leaves of *M. chamissois* Naudin (AEMC). B: Percent inhibition for acarbose. The data represent the mean ± SD (standard deviation) of three experiments, each performed in triplicate

Cytotoxicity

AEMC exhibited varied cytotoxicity responses in the cell lines evaluated. The extract showed IC_{50} values of 182.7 µg/mL, 414.6 µg/mL and 977.0 µg/mL for human keratinocyte (HaCat), mouse fibroblast (L929) and mouse macrophage (Raw 264.7), respectively (Figure No. 6).
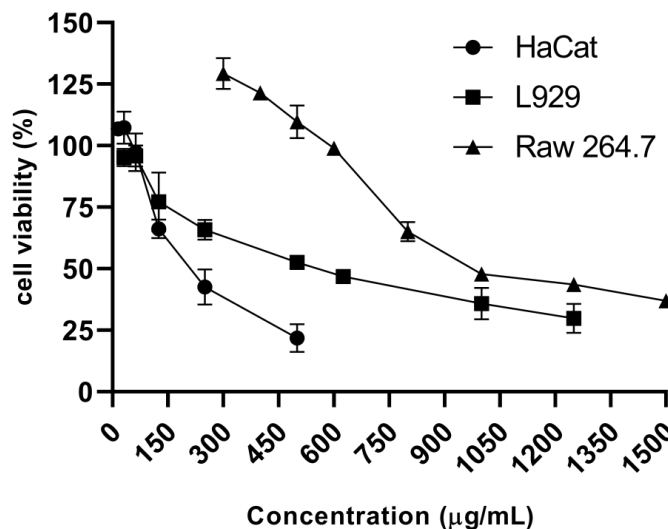

Figure No. 6
Cytotoxicity assay of aqueous extract from the leaves of *M. chamissois* Naudin (AEMC) in HaCat, L929, and Raw 264.7 cell lines. The data represent the mean ± SD (standard deviation) of three experiments, each performed in triplicate

DISCUSSION

Phenolic compounds such as flavonoids have been reported to promote antioxidant activity by reducing free radicals and by chelating metals, mechanisms also involved in inflammatory processes (Coutinho *et al.*, 2009; Tarawneh *et al.*, 2014). Compounds of vegetal origin with antioxidant properties have been highlighted by their potential to treat degenerative diseases that involve oxidative processes, such as heart disease, cancer, and changes in DNA (Santos *et al.*, 2010; Silva *et al.*, 2012).

Oxidative processes are mechanisms that occur in the normal functioning of the body, being reactive species of oxygen and nitrogen generated during cellular metabolism. However, the presence of an excess of these compounds has been related to the development of diseases such as atherosclerosis, changes in DNA, changes in proteins, changes in enzymes, and their effect on inflammatory processes (Barreiros *et al.*, 2006). Polyphenols have been reported to possess the ability to reduce free radicals, for example, by sequestering OH groups (Santos *et al.*, 2010). Therefore, there has been an increasing interest in the study of plants that have phenolic compounds and antioxidant activity.

Vitexin, a mono-C-glycoflavone is one of the most common phenolic compounds present in plants, and has been reported to have important antioxidant and anti-inflammatory activity (Praveena *et al.*, 2014). C-glycosylated compounds are also described as having antimicrobial activity (Michael *et al.*, 1998). Although the presence of the compound in the sample has not been confirmed by HPLC analyses, the presence of its derivative, which is the major compound in the sample, is thought to be responsible for its biological activity.

The results obtained from *M. chamissois* extract showed that this species had a higher polyphenol content than other species from the same genus. In the sample, the flavonoid content was 252.8 µg QE/mg extract and the polyphenol content was 532.2 µg GAE/mg extract. This was higher than that found in the methanolic extract of *Miconia minutiflora* (flavonoid content was 58.5 µg QE/mg extract, and polyphenol content was 151.4 µg GAE/mg extract). The polyphenol content in AEMC was also higher than that found in the methanolic extract of *Miconia albicans* (polyphenol content 70.0 mg GAE/g dry extract, i.e. 70µg GAE/mg extract) (Pieroni *et al.*, 2011).

The antioxidant activity of AEMC estimated by the DPPH method demonstrated an IC_{50} of 2.9 ±

0.1 µg/mL that was equivalent to that observed for ascorbic acid (IC_{50} of 2.8 ± 0.1 µg/mL), and was lower than that observed for BHT (IC_{50} of 13.6 ± 0.9 µg/mL), indicating more significant activity for AEMC in comparison to BHT. The AEMC sample also had higher antioxidant activity than the methanolic extract of the species *M. minutiflora*, which exhibited an IC_{50} of 3.6 µg/mL. However, the sample was less active than the aqueous extract of *M. latecrenata,* which demonstrated an IC_{50} of 1.1 µg/mL ± 0.7 (Forte, 2012; Gontijo, 2012).

In the phosphomolybdenum method, AEMC showed considerable antioxidant activity, equivalents of ascorbic acid content 18.4 ± 0.1 µg/mL and equivalents of BHT content 22.2 ± 1.1 µg/mL.

The antioxidant test results can be explained based on the content of phenolic compounds present in the sample, that was similar to the findings of Macedo *et al.* (2014), who evaluated the flavonoid content in ethanolic extracts of *Davilla rugosa,* obtained during different seasons at different times of the year, and determined the antioxidant activity in the extracts using the DPPH method. It was thus concluded that the highest antioxidant activity was observed in extracts with the highest phenolic content, corroborating the relationship between antioxidant activity and the concentration of polyphenols present (Macedo *et al.*, 2014).

Antimicrobial activity was observed only against *S. aureus* using the disk diffusion test. Resazurin is a chromogenic compound used as an indicator of microbial growth, whereby it acquires a pink colour in the presence of live microorganisms as a result of it being reduced by products of cellular metabolism (Palomino *et al.*, 2002; O'Brien *et al.*, 2000). In resazurin test, it was observed antimicrobial activity at a concentration of 312.5 µg/mL against *S. aureus* and 78.1 µg/mL against *C. albicans,* respectively; however, for *S. aureus,* the MIC represented bacteriostatic activity.

Considering the current increase in resistance to antibiotics, the search for compounds from plant extracts possessing antimicrobial properties has gained pace. Some authors consider plant extracts as having the advantage of inducing less adverse effects compared to synthetic antimicrobials (Celotto *et al.*, 2003; Silva *et al.*, 2012).

Despite these results, other species of the genus *Miconia,* such as *M. latecrenata*, have been reported to have antimicrobial action against *S aureus* and *E. coli* by the disk diffusion test in agar (Gontijo, 2012). The species *M. argyrophylla* has also been shown to have antimicrobial activity against *Candida*

sp. by the dilution method (for the aqueous extract, methanolic extract, and extract in dichloromethane) (Rodrigues *et al.*, 2014). Ahmad *et al.* (1998) showed that alcohol appears to extract antimicrobial compounds better than water. Thus, studies evaluating the antimicrobial activity using an ethanolic extract of *M. chamissois* would be of interest.

Tyrosinase inhibitors have the potential for being used in the treatment of skin disorders such as hyperpigmentation. Plant-derived natural compounds, such as polyphenols, have been reported to have this ability. In a study described by Yao *et al.* (2012), the inhibitory activity of the vitexin compound isolated from *Vigna radiatae* L. exhibited an IC_{50} of 6.0 mg/mL, which was higher than that of AEMC (Erdogan Orhan *et al.*, 2015). Other studies have shown tyrosinase inhibitor activity from rutin (Ren *et al.*, 2018; Fan *et al.*, 2019), another compound found in *M. chamissois*. Docking studies have shown that rutin interacts with the side chains of key amino acid residues and with the copper atom found at the active site of tyrosinase (Uysal *et al.*, 2018). The docking results suggest that rutin mostly interacts with histidine residues located at the active site (Si *et al.*, 2012). Therefore, the inhibition activity observed in our study can be attributed to the synergistic action of these compounds or to the presence of other polyphenols, which may also contribute to the reported other activities.

In the Melastomataceae family, tyrosinase inhibitory activity was observed only in *Macairea viscosa,* which exhibited 17% inhibition at 100 µg/mL concentration (Basile *et al.*, 1999), and in *Tibouchina semidecandra*, whose activity was attributed to the presence of quercetin, which when isolated from the extract, showed a 95% inhibition at a concentration of 2700 µg/mL (Bourgaud *et al.*, 2001).

Comparing the activity of AEMC with other plant extracts, a study by Adhikari *et al.* (2008) showed that the ethanolic extracts of *Azadirachta indica* leaves, *Curcuma longa* rhizome, and *Dolichos lablab* leaves showed 38.3%, 38%, and 37% tyrosinase inhibition at concentrations of 500, 50, and 500 µg/mL, respectively (Adhikari *et al.*, 2008). In the study by Baurin *et al.* (2002), *Cassia hirsute* and *Portulaca pilosa* leaf extracts showed 10% and 93% inhibition, respectively at 1000 µg/mL for both extracts (concentration in the reaction medium). In the same study, *Morus alba* extract was used as a positive control with 97% inhibition at same concentration (Baurin *et al.*, 2002). In the study by

Ohran *et al.* (2015) an ethanolic extract of *Viola odorata* L. inhibited tyrosinase activity by 80% at 100 µg/mL.

Souza *et al*. (2012a), studying with Brazilian Savannah species, the ethanolic extracts of *Genipa americana* (fruit), *P. torta* (leaf), and *P. ramiflora* (leaf) had higher IC$_{50}$ values of approximately 300, 200, and 200 µg/mL respectively, compared to that determined for *M. chamissois.* The aqueous extracts of *P. camito*, *P. torta,* and *Eugenia dysenterica* showed IC$_{50}$ values of approximately 50.0, 30.0, and 11.9 µg/mL, respectively, indicating a higher activity in inhibiting the enzyme than AEMC. The ethanolic extract of *M. nigra*, studied by de Freitas *et al.* (2016), demonstrated a lower IC$_{50}$ (between 5 and 9 µg/mL) than AEMC, indicating more potent enzyme inhibition activity.

Nowaday, plant-based medicines modulating physiological effects in the prevention and cure of diabetes has been the subject of research. A strategy to reduce the post-prandial increase of blood glucose in type 2 diabetic is the inhibition of α-amylase, enzyme involved in the digestion of carbohydrates (Tundis *et al.*, 2010). Species from the Melastomataceae family, as *Memecylon* and *Myconia* species*,* have shown alfa-amylase inhibition. The ethanolic extract of *Memecylon pauciflorum* and the ethanolic extract of *Miconia* species showed inhibitory activity with IC$_{50}$ values of 270 µg/mL (Deo *et al.*, 2016) and 28.2 µg/mL (Ortíz-Martinez *et al.*, 2016), respectively.

For other family species from the Brazilian Savannah, the ethanolic extracts of the leaves of *E. dysenterica* and *P. ramiflora* exhibited IC$_{50}$ values of alfa-amylase inhibition of approximately 20 µg/mL and 40 µg/mL, respectively, (Souza *et al.*, 2012b). In a study by Zorzin *et al.* (2014), isopropanolic fractions and sub-fractions of *E. dysenterica*, showed a higher IC$_{50}$ (between 8 and 128 µg/mL) than *M. chamissois* extract (Zorzin *et al*., 2014). The aqueous extract of *Euphorbia hirta* demonstrated an IC$_{50}$ of 180.1 µg/mL (Sheliya *et al.*, 2016). Therefore, AEMC, although less effective than the standard, displayed considerable alfa-amylase inhibitory activity, showing great potential for other studies to elucidate the compound(s) responsible for this action. *Miconia chamissois* has higher inhibitory efficacy than other species from the Brazilian Savannah.

Studies have shown a relationship between alfa-amylase inhibitory activity and the presence of phenolic compounds (Xiao *et al.*, 2013; Tadera *et al.*, 2006). A strong correlation between inhibitory activity and polyphenol content was found for *Aristotelia chilensis* (Rubilar *et al.,* 2011). In a study described by Sayed *et al.* (2008), in which inhibitory activity was determined by another method, the inhibition of vitexin and isovitexin were found to be 65.7% and 30.1%, respectively, at a concentration of 5.0 mg/mL. So, compared to those results, AEMC was found to have a higher inhibition at lower concentrations. Thus, vitexin derivatives present in AEMC may contribute to alfa-amylase inhibitory activity, along with other compounds present in the extract.

De Freitas *et al.* (2016) observed that the ethanolic extract of *M. nigra* leaves showed cell viability of the HaCat cell line below 10%, at a concentration of 1000 µg/mL and an IC$_{50}$ of 324.2 µg/mL. Compared to AEMC, *M. nigra* extract has a lower cytotoxicity. In the study described by Souza *et al.* (2012a), 500 µg/mL of aqueous extract of *E. dysenterica* and *P. torta* promoted viability of approximately 30% and 60%, respectively, (Souza *et al.*, 2012a). At the same concentration, AEMC showed approximately 22 % of viability, suggesting that the extract was more cytotoxic in the HaCat cell line.

In a study by Forte (2012), methanolic extract of *M. minutiflora*, at a concentration of 100 µg/mL, showed cell viability for the L929 line below 40%. At 10 µg/mL, the observed cell viability was approximately 70%. At these concentrations, AEMC showed lower cytotoxicity, with 100 % cell viability at a concentration of 62.5 µg/mL.

In comparison with other plants, in the same cell strain (L929), AEMC presents low cytotoxicity. In a study described by Souza *et al.* (2012a), the aqueous extract of *E. dysenterica* and *P. torta*, plants from Brazilian Savannah, at a concentration of 500 µg/mL, promoted 45% L929 cell viability. At the same concentration of AEMC, the cell viability was 50%. De Freitas *et al.* (2016) found an IC$_{50}$ of 116.3 µg/mL in the same cell line (L929) for the ethanolic extract of *M. nigra*, the ethanolic extract (1000 µg/mL) induced death of more than 80% of cells (L929), while, at the same concentration of AEMC, the cell viability was close to 50%.

As mentioned before, there are few studies published on the biological activity and the phytochemical constituents of *M. chamissois* Naudin. Due to the biological potential observed in this work, other methodologies and different approaches can be investigated, in order to improve the knowledge about the activities presented in this work. Although this paper suggests the presence of some compounds in *M. chamissois* Naudin, a phytochemical study has

yet to be better developed. Preparative thin layer chromatography and analysis by nuclear magnetic resonance and other methods of separation, isolation and identification of substances, must be carried out in order to direct the elucidation of the compounds present in the species, as well as provide standardization and characterization of the extract.

CONCLUSION

The results indicate that the aqueous extract of *M. chamissois* Naudin has considerable antioxidant activity that can be attributed to the presence of significant amounts of phenolic compounds. Furthermore, rutin and isoquercitrin, as well as a derivative of vitexin, were identified in AEMC. Further, AEMC showed antimicrobial activity against *C. albicans* and *S. aureus*. Also, AEMC was able to inhibit tyrosinase and alpha-amylase enzymes and showed low cytotoxicity. Thus, the extract can be promising in the development of useful products for the treatment of oxidative stress- and inflammation-induced diseases, hyperpigmentation conditions such as melasma, and diabetes.

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