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Saline extract from *Malpighia emarginata* DC leaves showed higher polyphenol presence, antioxidant and antifungal activity and promoted cell proliferation in mice splenocytes.

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Abstract: Currently, the research of new natural compounds with biological potential demonstrates great ethnopharmacological importance. In this study, we evaluated the biological properties promoted by saline extract from *Malpighia emarginata* DC leaves, whose objective is to evaluate the antioxidant, antimicrobial and cytotoxicity potential. Phytochemical characterization was performed by UPLC-MS chromatography to identify the chemical compounds. For the antioxidant potential, DPPH, ATT and FRAP methods were used. The antibacterial and antifungal tests were performed evaluating the MIC50, MIC90, CMB and CMF parameters. Moreover, antibiofilm action was evaluated. Cytotoxicity and proliferation were performed using splenocytes from Balb/c mice and were evaluated by cytometry. We found a list of phenolic compounds among other bioactive compounds in the *M. emarginata* saline extract. In addition, higher antioxidant profile and antifungal activity against different strains of *Candida* spp. was promoted by the saline extract. Splenocytes showed greater cell viability (more than 90%) and showed higher proliferate index in 24 and 48 hours of incubation with the extract. Saline extract from *Malpighia emarginata* DC has potential action like antioxidant and antifungal agent without promote animal cell damage.

Key words: bioactive compounds, cytotoxicity, *Malpighia emarginata* DC, phytochemical.

INTRODUCTION

Human culture has been profoundly influenced by the diversity of plant species, particularly those used for therapeutic purposes. Currently, the continuous knowledge about the medicinal properties of plants has been the focus of several scientific investigations around the world. Is important and necessary the

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study of phytochemical constituents, therapeutic action mechanism and identify active principles responsible for many biological activities observed in medicinal plants (Lin et al. 2018). Moreover, many authors have been publishing evidences about the efficacy of medicinal plants and their constituents on the improvement of diseases as cancer (Shirzad et al. 2011), atherosclerosis (Asgari et al. 2012) and diabetes (Asgary et al. 2011).

Malpighia emarginata DC. is a plant found throughout Central and South America. It is a tropical specie originating in the Caribbean region, being known as the Antillean cherry (Oliveira et al. 2012, Moreira et al. 2016). In Brazil the fruit is known as acerola and possess high content of ascorbic acid (vitamin C), carotenoids, anthocyanins, thiamine, riboflavin, niacin, proteins and mineral salts, mainly iron, calcium and phosphorous, being considered a functional food (Mezquita and Vigoa 2000, Rosso et al. 2008, Alvarez-Suarez et al. 2017). This plant also has other phytochemical constituents such as flavonoids, phenolic acids and polyphenols (Bataglion et al. 2015, Malegori et al. 2017). All these compounds are able to minimize oxidative damage caused by the reactive oxygen and nitrogen species in animal organism (Oliveira et al. 2012) and prevent some chronic diseases such as cancer (Mezadri et al. 2008, Leffa et al. 2014). Moreover, these molecules also exhibit potential therapeutic effect as anti-inflammatory, radiation-protective, chemoprotective, vasoprotective, inhibition of LDL oxidation and decrease the risks of cardiovascular diseases (Wang et al. 1997, Seeram and Nair 2002, Rosso et al. 2008).

Local communities, especially in northeast of Brazil, use the juice of acerola mainly for respiratory diseases as flu and bronchitis. However, some accounts showed that leaves of plant were used to make tea associated with garlic and honey to flu symptoms. In addition, recent biological studies have shown that acerola consuming is not dangerous to human cells.

Many researches show important biological actions promoted by different organic extracts (like phenolic and ethanolic fractions) made using leaves of *M. emarginata* (Silva 2014, Oliveira 2015, Rezende et al. 2018). However, is known that the true use of *M. emarginata* in local communities is like teas or infusions using water as the solvent.

Although the studies about *M. emarginata* fruit are extensive, is very important to study the phytochemical composition and biological activities promoted by other organs of this plant. Thus, the present study aimed investigates the phytochemical composition of the saline extract from *Malpighia emarginata* DC leaves and shows the initial biological activities promoted by this compound.

MATERIALS AND METHODS

VEGETABLE MATERIAL AND EXTRACT PREPARATION

The leaves of *Malpighia emarginata* were collected in the city of Recife, state of Pernambuco, Brazil. The botanical identification was performed by the Professor Marlene Barbosa, on May 30, 2017, at the Herbarium Geraldo Mariz, located at the Center of Biosciences of the Federal University of Pernambuco through exsiccate deposited under registration number 82.615. After collection, the leaves were kept at room temperature for drying during 4 days. The dried leaves were crushed in Reverse Black Oster and the pulverized material (20g) was diluted in 0.15 M NaCl (200 mL) in the proportion of 10% (w/v). Therefore, using an orbital and alternative incubator shaker (Lab Companion™ IS-971) the material was kept under agitation (400 rpm) in constant temperature (28 ºC) for 16 hours. After the stirring time, the material was filtered using paper filter and centrifuged (15 min, 10,000 *x* g). The obtained supernatant was collected and called saline extract from *Malpighia emarginata* leaves. After this, the extract was lyophilized and

9.2 g of crude material was obtained. Material was stored at -20 °C until use.

DETERMINATION OF TOTAL PHENOLS

The total phenols were determined by the Folin-Ciocalteu method according to Li et al. (2008) with some modifications. Extracts diluted at 1 mg/mL were dissolved in distilled water. In 0.1 mL of Folin solution (1:10 v/v) was added 0.02 mL of the diluted extracts. After 3 minutes of incubation (in the dark), 0.08 of sodium carbonate (7.5%) was added and incubated for 120 minutes in the dark at room temperature. After this period, the absorbance of the samples was measured at 765 nm against a blank (reagent added to distilled water). A calibration curve was prepared by plotting the absorbance as a function of the concentration of gallic acid (0 - 500 μg/mL) was prepared and the linear equation ($y = 0.0048x + 0.0016$ $R^2 = 0.9999$). Phenols are expressed in gallic acid equivalent (mg EAG/g of extract).

DETERMINATION OF FLAVONOID CONTENT

The determination of flavonoids followed the methodology in accordance with Woisky and Salatino (1998) and Santos et al. (2017) with some modifications. The experiment consists of pipetting 1 mL of extract (1 mg/mL) in test tube. After this, 1 mL of aluminum chloride ($AlCl_3$, 5%) prepared in methanol (2 mL) was added in tubes. The blank was prepared using 3 mL of methanol and 1 mL of aluminum chloride. After 30 minutes of incubation (in dark) the absorbance was measured at 425 nm. A standard curve with quercetin (0 - 500 μg/mL) was performed to obtain the equation $Y = 0.023x + 0.1509$, $R^2 = 0.9956$. The assays were performed in quintuplicate. Flavonoids are expressed in quercetin equivalent (mg QE/g of extract).

ANALYSIS OF PHYTOCHEMICAL COMPOUNDS THROUGH OF ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY – UPLC-MS

Ultra-performance liquid chromatography (UPLC) was performed with an Acquity H-Class (Waters) employing a 2.1 x 100 mm BEH column with a particle size of 1.7 μm. The column was maintained under a constant temperature of 40 ºC and the auto injector at 10 ºC. We use an aqueous solution (eluent A) containing 2% methanol (MeOH), 5Mm ammonium formate and 0.1% formic acid and a methanol solution (eluent B) containing 0.1% formic acid, which were pumped at a flow rate of 0.3 mL/min. Ten microliters of the *M. emarginata* extract was injected. Elution was performed in gradient mode and the initial condition (98% A / 2% B) was maintained for 0.25 minutes. The B ratio increased linearly to 99% in 8.5 minutes, remaining at 99% B for one minute, followed by immediate decreased to 2% B, where it was maintained for up to 11 minutes. The UPLC system was coupled to a single quadrupole mass spectrometer SQ Detector 2 (Waters®). The data were obtained in fullscan mode, analyzing masses between 100 and 1000 Da, in negative ionization. The acquisition of the chromatograms and mass spectra was obtained through MassLynx ™ software (Waters®).

EVALUATION OF ANTIOXIDANT ACTIVITIES IN VITRO

Total Antioxidant Activity

The total antioxidant activity promoted by saline extract from *Malpighia emarginata* leaves was determined as a function of ascorbic acid, according with (Pietro et al. 1999). Saline extract (100μL at 500 μg/mL) was mixture with ascorbic acid (1 mg/mL) and 1 mL of phosphomolybdenum solution (600 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). This mixture was incubated in water at 95 °C for 90 minutes.

After returning to room temperature, the absorbance are measured at 695 nm against a blank (1 mL of solution and 0.1 mL of water). A standard curve with ascorbic acid (0 - 500 µg/mL) was performed to obtain the equation Y = 0.019x + 0.0723, R^2 = 0.9937. Assays were performed in quintuplicate. Total antioxidant activity was calculated by the formula ATT (%) = [(As - Ac) / (Aaa - Ac)] 100, where: Ac = Control Absorbance, As = Sample Absorbance and Aaa = Ascorbic Acid Absorbance.

Free radical sequestration by DPPH

The antioxidant activity of saline extract was measured by the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), as described by (Blois 1958). Using 0.04 mL of different concentrations of the saline extract (3.9; 7.8; 15.6; 31.3; 62.5; 125; 250 and 500 µg/mL) it was added 0.25 mL of the DPPH solution (1 mM and OD_{517} = 0.650 ± 0.50). After incubation time (25 minutes) at room temperature, protected from light, the absorbance was measured at 517 nm. We used as control the DPPH solution added to distilled water. Assays were performed in quintuplicate. The sequestration of DPPH radicals was calculated by the formula: SRL [$DPPH^{\bullet}$] (%) = [(As - Ac) / Ac] x 100. When: As = sample Absorbance and Ac = control Absorbance. The antioxidant activity of saline extract was measured by the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Reduction of Ferric Ion (FRAP assay)

The stock solution of the FRAP assay was prepared with 300 mM acetate buffer (3.1 g CH_3COONa and 16 mL CH_3COOH) at pH 3.6, 10 mM TPTZ (2.4, 6-tripyridyl-s-triazine) solubilized in 40 mM HCl, and 20 mM $FeCl_3$ solution. The working solution was prepared by mixing the acetate buffer, TPTZ and $FeCl_3$ in a ratio of 10:1:1 (v/v/v) and incubated for 5 min at 37 ºC. Saline extract (25µL at 1 mg/mL of concentration) was added in 180 µL of FRAP reagent and this solution was incubated for 30 minutes at 37 °C in the dark. Subsequently the absorbance was measured at 593 nm. A standard curve with $FeSO_4$ (0 - 1000 µg / mL) was performed to obtain the equation Y = 0.0024 x + 0.0019, R^2 = 0.9953. The results were expressed as mg EFeSO4 (II)/g of extract.

ANTIMICROBIAL ASSAYS

Bacterial and fungal strains, culture conditions and preparation of samples

Fungal strains were obtained from Micoteca of Mycology Department (URM) and the collection of microorganisms from the Department of Antibiotics (UFPEDA), both departments belonging to Biosciences Center of Federal University of Pernambuco. Stock cultures were kept under refrigeration (-20 °C) in sterilized skimmed milk containing 10% (v/v) glycerol or 30% (v/v) Mueller Hinton glycerol broth. Bacterial and fungal strains used in this study are: *Candida albicans* (URM 5901), *Candida krusei* (URM 6391), *Candida tropicalis* (URM 6551), *Candida parapsilosis* (URM 6951), *Candida glabrata* (URM 4246), *Escherichia coli* (UFPEDA 224), *Klebsiella pneumoniae* (UFPEDA 396), *Pseudomonas aeruginosa* (UFPEDA 416), *Salmonella enteritidis* (UFPEDA 414), *Staphylococcus aureus* (UFPEDA 02), *Staphylococcus saprophyticcus* (UFPEDA 833). For antimicrobial activity assays, bacterial and fungal species were cultured in Mueller Hinton and Sabouraud Dextrose agar medium, respectively, overnight at 36 °C, and subsequently the colonies were resuspended in sterile saline solution (0.15 M NaCl) and adjusted turbidimetrically at a wavelength of 600 ηm (DO_{600}) to obtain the suspension equivalent to 10^6 colony forming units (CFU) per mL. For the assay, the samples were filtered on the sterile polyvinylidene difluoride (PVDF) syringe filter of 13 mm x 0.22 µm.

Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

The minimum inhibitory concentration (MIC) promoted by saline extract from *Malpighia emarginata* leaves was determined by the microtiter test proposed by the Clinical and Laboratory Standards Institute (CLSI). In 96-well microtiter plates were added (80 μL) into the fourth well from which a serial dilution in sterile Milli-Q water was performed to the twelfth well of the same row. Subsequently, 40 μL of the medium Mueller Hinton broth (bacteria) or Sabouraud Dextrose (yeast) were added to all but the first wells, where 200 μL of the culture medium was added, corresponding to the sterility control. The antibiotics ampicillin and tetracycline (8 μg/mL) and antifungal fluconazole (64 μg/mL) were used as positive controls in the second well. Finally, the bacterial and fungal suspension (80 μL, 106 CFU/mL) was added in the second well to the last well in the row. The third well (containing microorganisms in the absence of the sample) corresponded to the 100% growth control. The plates were incubated at 36 ºC and the optical density was measured at time zero and after 24 h of incubation using a microplate reader. The Minimal Inhibitory Concentration MIC90 and MIC50 corresponded to the lowest concentration of the sample capable of promoting a reduction of ≥90% or ≥50%, respectively, in optical density, as compared to the 100% growth control. For determination of Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC), aliquots (10 μL) of the wells containing concentrations of ≥MIC50 samples were inoculated into petri dishes containing Mueller Hinton or Sabouraud Dextrose agar medium, which were subsequently incubated at 36 °C for 24h. The MBC and MFC corresponded to the lower concentration of the sample able to reduce the number of CFU in 99.9% in relation to the initial inoculum. Each assay was performed in triplicate using three independent experiments.

Antibiofilm Activity Analysis

Biofilm formation was evaluated by the crystal violet method in flat bottom polystyrene microtiter plates. Then 80 μL of Milli-Q water, 40 μL of the Sabouraud Dextrose medium and 80 μL of the fungal suspension (10^8 CFU/mL; sterile saline) were added, respectively. A DO_{600} was also launched using a microplate reader and as plates were incubated at 36 °C for 24h. After this period plates were read again to determinate the bacterial and/or fungal growth rate in 600 nm. A sequential stage corresponds to the analysis of biofilm that is after sequencing of planktonic cells (unbound cells). In addition, the samples were washed with 0.15 M NaCl in pre-fixed methanol for 20 min, followed by a setting at 50 °C for 60 min and then labeled with 0.4% (w/v) crystal violet for 25 min at 25 °C. The wells were washed with water to remove unaccompanied violet crystal and subsequently the dye adhered to the biofilm was solubilized in absolute ethanol (20 min) and the absorbance was measured at 570 ηm. All experiments were performed in triplicate.

VIABILITY AND PROLIFERATION ASSAYS AGAINST MICE SPLENOCYTES

Animals

Female BALB/c mice (6-8 weeks old; 5 animals) were raised and maintained at the animal facilities of the Keizo Asami Immunopathology Laboratory - LIKA located in Federal University of Pernambuco, Brazil. Mice were kept under standard laboratory conditions (20-22 °C and 12 h day and night cycle) with free access to standard diet (Labina/Purina, Campinas, Brazil) and water. All experimental procedures were performed according to the Ethics Committee of Animal Use (CEUA) of Federal

University of Pernambuco (protocol number: 0048/2016).

Preparation of splenocytes

This procedure was performed in accordance with Melo et al. 2011. After the inoculation of anesthetics (10mg/Kg of xylazine and 115mg/Kg of ketamine i.p. injection) followed of cervical displacement, spleen of each mouse was removed aseptically and placed in a Falcon tube containing RPMI 1640 with fetal calf serum (complete medium). In a vertical flow, each spleen was transferred to a petri dish where they were soaked. The cell suspensions obtained from each spleen were transferred to Falcon tubes containing approximately 10 mL of incomplete medium. Spleen homogenates were overlaid onto Ficoll-Paque™ PLUS layer, with the density adjusted to 1.076 g/mL, and centrifuged at 1000 *x* g at room temperature for 25 min. The interface cell layer containing immune cells was recovered by Pasteur pipette, washed twice in phosphate-buffered saline (PBS) and centrifuged twice at 500 *x* g for 10 min. Cells were counted in a Neubauer chamber, and cell viability was determined by the trypan blue exclusion method. Cells were only used when viability was> 98%.

Analysis of cell viability by Annexin V-FITC and Propidium Iodide Staining

Mice splenocytes (10^6 cells) were treated with saline extract from *Malpighia emarginata* leaves at 50, 25, 12.5, 6 and 3 µg/mL and were maintained in 24-well plates for 24 hours to analyze their cell viability. Untreated cells (control) were used as a negative control. Following this, splenocytes were centrifuged at 4 °C, 450 *x* g for 10 min. After discarding the supernatant, 1 mL of PBS 1X was added to the precipitate and centrifuged at 4 °C, 450 *x* g for 10 min. After discarding the supernatant, the pellet was resuspended in a binding buffer of cell viability kit (Becton Dickinson Biosciences) and Annexin V conjugated with fluorescein isothiocyanate (FITC): 50 µg/mL) and propidium iodide (PI, 20 µg/mL) were added to each labeled cytometer tube. Flow cytometry was performed in a FACS Calibur (Becton Dickinson Biosciences) and analyzed using Cell Quest Pro software (Becton Dickinson). Result of the analysis was performed in graphs by dot plot. Annexin-FITC negative/PI positive cells were considered necrotic cells and Annexin-FITC positive/PI negative represented splenocytes in the early stage of apoptosis. Double negatives were considered viable cells.

Cell proliferation analysis using CFSE staining

Same protocol to obtained splenocytes was used for proliferation assay. After splenocytes obtainment, cell solution was centrifuged at 300 *x* g at room temperature for 5 min with sterile PBS 1X added with SFB 5% (pH 7.2). After this, the cell solution was adjusted to10×10^6 cells/mL and received 5mM of 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE). Cells were incubated for 10 min at room temperature in the dark. Cells were centrifuged twice at 300 *x* g / 5 min with sterile PBS 1X. Cells stained were cultured for 24 and 48 hours with 12.5 µg/mL of saline extract from *M. emarginata* leaves and only culture complete RPMI 1640 medium (negative control). After culture time cells were centrifuged (300 *x* g / 5 min), were carry to acquisition on FACSCalibur platform (Becton Dickinson Biosciences) and results were analyzed using Cell Quest Pro software (Becton Dickinson).

STATISTICAL ANALYSIS

To test the normal hypothesis on the variable involved in this study the Shapiro–Wilke test was applied. Means of samples were analyzed using non-parametric tests. The statistical difference between two groups was analyzed by Wilcoxon test and that among more than three groups by one-way analysis of variance (ANOVA). All the conclusions

were considered with a significance level of 5%. For statistical analysis was used GraphPad Prim 5.01 software.

RESULTS

PREVALENCE OF PHENOLIC COMPOUNDS IN PHYTOCHEMICAL SCREENING OF SALINE EXTRACT FROM *M. emarginata* LEAVES

Results of analysis in UPLC-MS about the phytochemical characterization of saline extract from *M. emarginata* leaves (Figure 1) showed that was found many phenolic compounds being some compounds belonged flavonoid (Table I).

In addition, we found 18 compounds in the saline extract in accordance with the area, retention time, molecular weight, molecular formula and negative mass, identifying other phenolic compounds, like Protocatechuic acid, Apigenin-7-O- glucoside flavonoid and terpenoids, like Isotriptophenolide (see Table II).

SALINE EXTRACT FROM *M. emarginata* LEAVES SHOWED SIGNIFICANT ANTIOXIDANT POWER

Saline extract from M. emarginata leaves showed higher antioxidant power being five times superior to butylated hydroxytoluene (BHT) standard (20.32±0.89 / 4.12±0.10 to saline extract and BHT, respectively). Saline extract also was able to

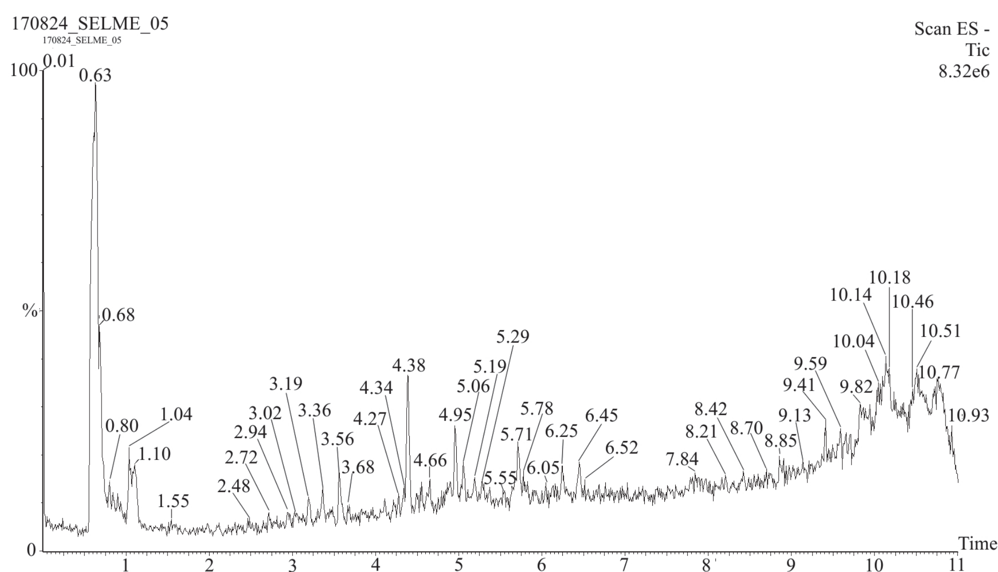

Figure 1 - UPLC-MS Chromatogram of saline extract from *M. emarginata* leaves.

TABLE I
Result of antioxidant activities and phenol amounts found on saline extract from *M. emarginata* leaves.

	M. emarginata	**BHT**	**Ascorbic acid**
Total phenolic compounds (mg GAE/g)	51.14 ± 0.21	-	-
Total of flavonoids (mg QE/g)	9.67 ± 0.08	-	-
Free radical sequestration by DPPH (% mg/mL)	38.59 ± 1.20	93.13 ± 0.58	90.04 ± 0.16
Total antioxidant capacity (mg AAE/g)	20.32 ± 0.89	4.12 ± 0.10	100
Reduction of Ferric Ion (EFeSO4(II)/g)	416.11 ± 0.46	679.17 ± 25.98	1215 ± 48.55

TABLE II
UPLC-MS spectral analysis of compounds found in saline extract from *M. emarginata* leaves.

COMPOUNDS	RETENTION TIME (min)	MOLECULAR FORMULA	AREA	MASS	[M-H]-
Rhinocerotinoic acid	0.63	$C_{20}H_{30}O_3$	5.756.062	318.457	317.211
Quinic Acid	1.06	$C_7H_{12}O_6$	105.468.063	192.063	192.063
Dimethoxycurcumin	2.72	$C_{23}H_{24}O_6$	5.840.279	396.439	395.149
Protocatechuic Acid	3.36	$C_7H_6O_4$	16.625.830	154.026	153.018
Tolypodiol	3.60	$C_{28}H_{40}O_5$	6.356.449	456.623	455.279
Pauciflorol A	3.90	$C_{20}H_{34}O_2$	73.426.766	306.255	305.248
Gentisic acid	4.25	$C_7H_6O_4$	5.425.569	154.026	153.018
Matricin	4.38	$C_{17}H_{22}O_5$	73.426.766	306.358	305.138
Gallocatechin	4.38	$C_{15}H_{14}O_7$	7.342.677	306.27	305.066
11a-hidroxi-3,7-dioxo-5a-lanosta-8,24 (E)-dien-26-oic Acid	4.49	$C_{30}H_{44}O_5$	7.038.835	484,318	483,311
Protocatechuic acid	4.65	$C_7H_6O_4$	5.425.569	154.121	153.018
Cicutoxin	5.19	$C_{17}H_{22}O_2$	6.584.033	258.355	225.164
Salicylic acid	5.71	$C_7H_6O_3$	31.334.580	138.031	137.023
2,5 Dihydroxybenzaldehyde	5.71	$C_7H_6O_3$	31.334.580	138.031	137.023
Apigenin-7-O- glucoside	8.86	$C_{21}H_{20}O_{10}$	6.690.703	432.105	431.097
Magnosalicin	8.87	$C_{24}H_{32}O_7$	6.690.703	432.513	431.207
Apigenin-8-O- glucoside	8.87	$C_{21}H_{20}O_{10}$	6.690.703	432.381	431.097
Isotriptophenolide	9.85	$C_{20}H_{24}O_3$	50.121.777	312.409	311.164

increase of free radical sequestration (38,59 %) and promoted higher ferric ion reduction, also like BHT standard (see Table I).

HIGHER ANTIFUNGAL ACTIVITY WAS PROMOTED BY SALINE EXTRACT

Six bacterial species tested showed resistance to the extract. However, the saline extract from *M. emarginata* leaves inhibited 90% of growth capacity of *Candida albicans*, *Candida parapsilosis*, *Candida krusei* and *Candida tropicalis* in lower concentrations (Table III). In addition, was also possible observe that saline extract presented more significant results as antifungal against *C. albicans* and *C. parapsilosis*, comparing to the other species. Only the *C. glabrata* species was resistant to saline extract.

Antibiofilm activity was evaluated with the saline extract following the concentrations of MICs: 64 MIC, 16 MIC, 4 MIC, MIC, 1/4 MIC, 1/16 MIC and 1/64 MIC; according to the graphs in figure 2 and Table IV.

TABLE III
Antifungal activity against *Candida* spp. Promoted by saline extract from *Malpighia emarginata* leaves.

Strains	Fluconazole (µg/mL)	Saline extract from *M. emarginata* leaves (µg/mL)		
	MIC_{50}	MIC_{50}	MIC_{90}	MFC
C. albicans	0.25	0.51	0.52	ND
C. parapsilosis	ND	0.51	0.51	0.51
C. krusei	32	8.36	8.36	ND
C. tropicalis	4	0.13	0.26	1.05
C. glabrata	64	ND	ND	ND

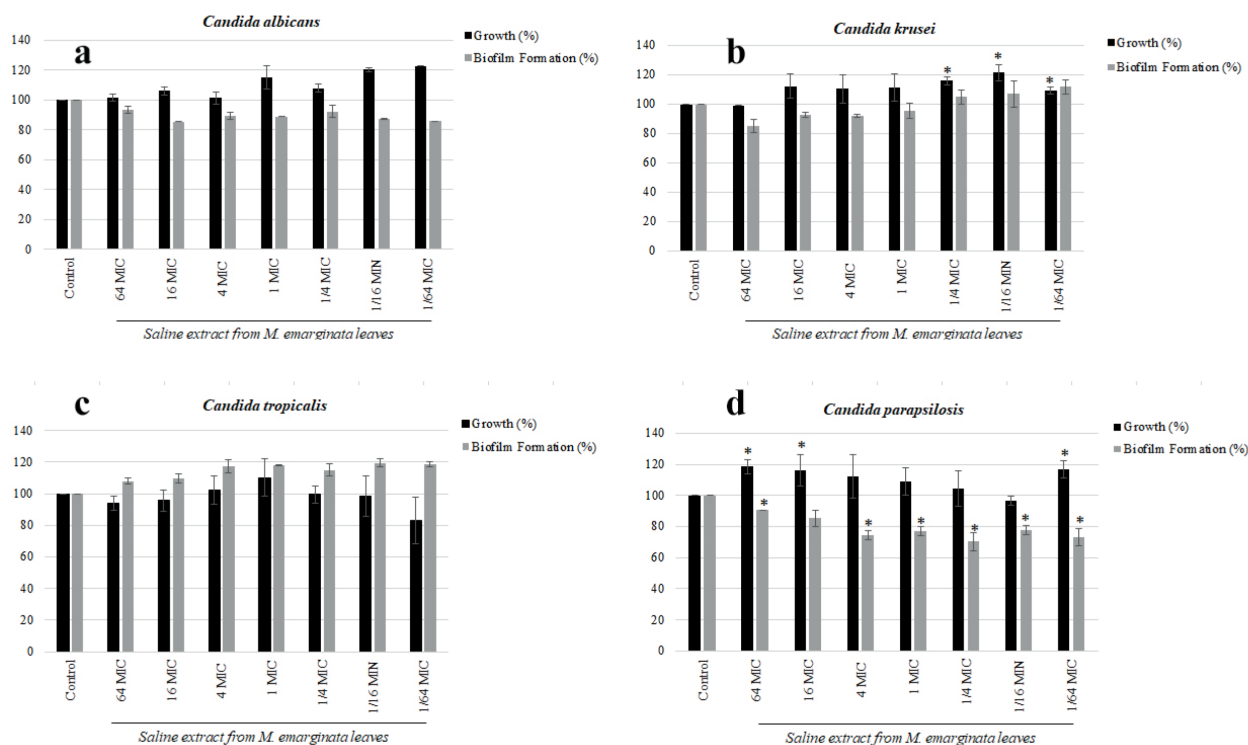

Figure 2 - Analysis of the antibiofilm activity of *Malpighia emarginata* saline extract to species of *Candida albicans* **(a),** *Candida krusei* **(b),** *Candida tropicalis* **(c)** and *Candida parapsilosis* **(d).** Assays performed in triplicate. $p < 0.002$.

TABLE IV
Results on the antibiofilm activity of *Malpighia emarginata* saline extract, in percentage, of *Candida* ssp. tested.

Value% of growth (DO_{600}) of *Candida* ssp. (mean) DO_{600}								
	Control	**64 MIC**	**16 MIC**	**4 MIC**	**1 MIC**	**1/4 MIC**	**1/16 MIC**	**1/64 MIC**
C. albicans	100	104.3	105.5	105.1	109.1	105.3	120.5	122.6
C. krusei	100	99	112.4	110.4	111.3	116.1	121.4	109.3
C. tropicalis	100	94	95.6	102.5	110.2	99.7	98.4	83.1
C. parapsilosis	100	118.7	116.4	112.2	109	104.3	96.6	116.8
Value% of biofilm biomass of *Candida* spp. (mean)								
	Control	**64 MIC**	**16 MIC**	**4 MIC**	**1 MIC**	**1/4 MIC**	**1/16 MIC**	**1/64 MIC**
C. albicans	100	93.4	85.5	87.7	88.7	89.2	87.2	85.8
C. krusei	100	85	92.7	92	95.4	104.9	107	111.9
C. tropicalis	100	107.9	109.7	117.3	118	115	119,6	118.6
C. parapsilosis	100	90.5	85.3	74.3	77.2	70.4	77.5	73.3

The results of the antibiofilm activity confirm the antimicrobial tests performed. The saline extract of *Malpighia emarginata* presented a considerable reduction in the biofilm formation of *C. albicans, C. krusei* and *C. parapsilosis* species, as described in table IV.

SALINE EXTRACT FROM *M. emarginata* LEAVES NOT INDUCED CELL DAMAGE AND PROMOTED HIGHER CELL PROLIFERATION IN MICE SPLEEN CELLS

Mice Balb/c spleen cells treated with saline extract in different concentrations (50, 25, 2.5, 6 and 3 µg/mL), did not suffer significant cell death, for both apoptosis and necrosis, in none concentrations evaluated (Figure 3). In fact, saline extract from *M. emarginata* leaves promoted a higher cellular viability, superior to 90% of surviving. For measuring if saline extract was able to activate immune spleen cells, we performed other assay to investigate the proliferation index of these cells challenged with only one concentration of extract (12.5 µg/mL) during two days of cell culture. Results showed that saline extract from *M. emarginata* leaves induced higher proliferation indices in both times evaluated (Figure 4).

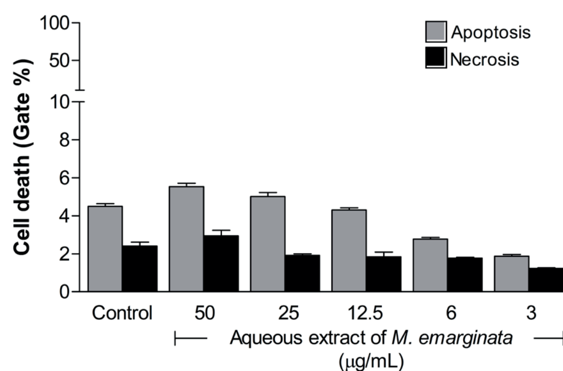

Figure 3 - Mice splenocytes viability evaluated using annexin V and propidium iodide staining. Saline extract from *M. emarginata* leaves did not promoted cell death in none concentration. Horizontal bars represent the average of three independent experiments performed in duplicate. Results were evaluated in the significance level of 5%.

DISCUSSION

Popular culture collaborates with the indiscriminate use of medicinal plants within the context of self-medication, requiring the study of its constituents, the mechanism of therapeutic action and the identification of active principles responsible for the various biological activities reported by the population (Ford et al. 2014). Here, we used a saline extract made from *M. emarginata* leaves. The proposal was investigated if an extract of plant,

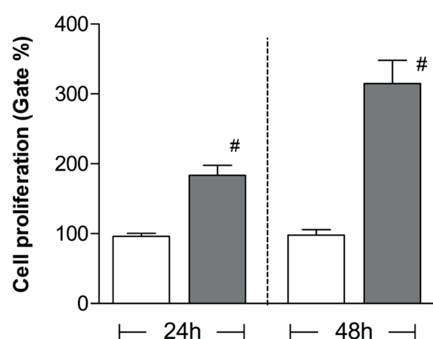

Figure 4 - Mice splenocytes proliferation index evaluated using CFSE staining. Saline extract from *M. emarginata* leaves promoted proliferation in mice splenocytes in both times evaluated (24 and 48 hours of assay). Vertical white bars represent negative control cells (cells + culture medium) and vertical gray bars represent saline extract from *M. emarginata* leaves at 12.5 µg/mL concentration. This assay was performed using three independent experiments performed in duplicate. #P= 0.003.

made with standard physiological solution (NaCl 0.15M), is cytotoxic to cells and microorganisms and if this extract is also able to be antioxidant.

In similarity to the results found by Wong et al. (2008) and Nowotarska et al. (2017), the saline extract from leaves of *M. emarginata*, as described in Table II prepared in its phytochemicals 18 characterized compounds, being a larger number of a class of phenolic and terpenoid compounds.

Among the compounds described are the apigenin-7-O-glucoside and Apigenin-8-C-glucoside flavonoids extensively studied and described in the literature for their potential applicability as anti-inflammatory agents (Kowalski et al. 2005) and antitumor agents (Bankeu et al. 2017, Smiljkovic et al. 2017, Zhao et al. 2017). In addition, Apigenin-8-C-glucoside is a biologically active molecule with potential antihypertensive, antidepressant, antispasmodic and antioxidant effect (Ele et al. 2016).

As described in Table I, saline extract from *M. emarginata* leaves showed a potential antioxidant capacity, especially when compared to butylated hydroxytoluene (BHT) a known antioxidant compound used as standard (Pasa et al. 2016, Nascimento et al. 2017). We suggest that this antioxidant potential is due to higher amounts of phenolic compounds, previously described in several studies as antioxidants molecules (Zengin et al. 2018). Similar to our results, other studies also showed that plants rich in phenolic compounds have antioxidant potential as well as the extracts of *Ficus beecheyana* (Yen et al. 2018), *Punica granatum* (Russo et al. 2018) and *Saccharum officinarum* (Abbas et al. 2014). Moreover, Nascimento et al. (2017) analyzed the antioxidant activity of *Malpighia glabra* fruits at various stages of maturation, thus finding high levels of antioxidant activity and, consequently, the presence of phenolic compounds.

Organic extracts from various organs of *M. emarginata* presented potential antimicrobial activity against different pathogenic bacteria species as described by Silva (2014) and Motohashi et al. (2004). Although such solvents (hexane and ethyl acetate) have high toxicity to microorganisms (Yung et al. 2016), the indiscriminate use of these solvents is also associated with intoxication, either by ingestion or inhalation, causing serious damage to the individual, such as severe lung injury and hepatotoxicity (Ford et al. 2014, Tormohelen et al. 2014, Connellan, 2017).

The results obtained in this study did not show action against the species of bacteria used. However, the saline extract of *M. emarginata* leaves presented a potential fungicidal, fungistatic and antibiofilm action, suggesting this capacity, once again, to the presence of phenolic compounds (Haghdoost et al. 2016, Barral et al. 2017, Jara et al. Wang et al. 2018).

As described by Khan et al. (2014) and Glorybai et al. (2015) plants are commonly used by traditional medicine as a source of cure for various diseases. However, the irrational use or the conception that a natural product does not cause harm to the health can be harmful to the individual,

being associated to diverse problems like poisoning and anaphylactic shocks (Lin et al. 2018), being the toxicological studies of medicinal plants, of great relevance to the security of the individual (Saleh-e-In & Staden, 2018).

Here we also investigate the cytotoxicity profile promoted by the saline extract from *M. emarginata* leaves in mice splenocytes and the results showed that, same in higher concentrations, this extract is not toxic. Moreover, *M. emarginata* extract also promoted lymphocyte proliferation in both 24 and 48 hours of incubation, suggesting immunostimulatory properties. Recent study showed that extract of several species of *Byrsonima* spp. (Malpighiaceae) in different concentrations (12.5 and 50 µg/mL) were not cytotoxic against RAW 264-7 cells (Fraige et al. 2018). In 2016, Düsman et al. evaluated the cytotoxicity of the aqueous extract from *Malpighia glabra* Linnaeus fruits *in vivo* and *in vitro* in Wistar rats, and against bone marrow derived cells from these animals. Their results demonstrated that the extract also was not cytotoxic.

The activation and cellular proliferation are directly associated with healing processes and with the replacement of dead cells of the tissues that are in constant development in the organism. For this, is very important investigate if a compound can promote cell proliferation, especially if these cells are immunological because the major activation mechanism of lymphocytes is the clonal expansion after it activation.

Plants extracts belonging to the Malpighiaceae family also present other potential pharmacological properties, such as antimicrobial, wound healing and antitumor (Düsman et al. 2016, Specian et al. 2016).

Recent studies with different species belonging to the genus *Byrsonima* (family Malpighiaceae) showed no cytotoxic action against different human cell lines (monocytes and cells of primary gastric epithelium) but demonstrated a potential cytotoxic capacity against colon cancer cell lines (HT29) and hepatocellular carcinoma (HepG2) (Specian et al. 2016, Verdam et al. 2014). Our results associated with these findings suggest the possibility of future investigations about antitumor, wound healing and immunological responses promoted by saline extract from *Malpighia emarginata* leaves.

CONCLUSIONS

Saline extract from *Malpighia emarginata* leaves showed the presence of a considerable concentration of total phenolic compounds, among them some flavonoids and phenolic acids. Moreover, showed antioxidant and antifungal properties and was not cytotoxicity against mice splenocytes, inducing high proliferation in these cells. These results are promisors, demonstrate the safe use of this plant against normal cells and stimulate new investigations to use this extract like antifungal and immunostimulant compound in future assays.

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AUTHOR CONTRIBUTIONS

B. Barros and B. Barboza performed the antioxidant, antimicrobial and immunological assays, as well as writing of the manuscript; B. Ramos and M. Moura performed the antimicrobial assays; L. Coelho, T. Napoleão, M. Correia, P. Paiva and C. Lima made available different reagents and equipment

to investigate both leaves and saline extract; I. Cruz Filho and T. Silva performed the structural and phytochemical analysis; C. Melo helped in experimental design, immunological assays and writing of the manuscript.

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