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Phytochemical analysis, antioxidant activity and *in vitro* ocular irritation of *Hibiscus rosa-sinensis* L. extracts

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***Abstract*— *Hibiscus ssp. is a source of phytochemicals, mainly anthocyanins, widely known for its medicinal properties. The aim of this study was to develop a microwave-assisted extraction (MAE) method to extract bioactive compounds from yellow and red petals of Hibiscus rosa-sinensis L. (HRS) and evaluate their antioxidant and eye irritation potential. For MAE, the effect of microwave power, irradiation time, solvent concentration and solvent volume was tested. The antioxidant activity and eye irritation potential were evaluated. The extracts were analyzed by LC-DAD-ESI-IT-MS/MS. The acidified aqueous extraction was the best choice for the recovery of anthocyanins, while hydroethanolic was best extractor solvent to recovery flavonol and flavanols. MAE extracts showed efficient recovery: total phenolic content (28.4 and 23.2 mg GAE/g), total flavonoids content (44.8 and 34.5 mg RE/g),total anthocyanins content (4.1 and 16.3 mg Cy-3-glu/100 g) and extraction yields (29.5 and 23.2 %) and remarkable antioxidant activity (IC_{50}=12.35 and 15.76 mg L^{-1}) for yellow and red petal extracts, respectively. Main compounds were cyanidin-3-sophoroside and cyanidin-3,5-diglucoside, besides catechin, epicatechin and rutin. The extracts were classified as non-irritating ingredients. The data revealed HRS as a source of bioactive compounds and significant variations in phytochemical content among yellow and red petals of HRS.*

Keywords*— *Hibiscus rosa-sinensis L; microwave-assisted extraction; liquid chromatography; mass spectrometry; HET-CAM.

I. INTRODUCTION

Since ancient times plants have acted as therapeutic remedies, being used for the treatment of infections or degenerative diseases in humans and animals.[1,2] The effectiveness in using plants as a treatment for diseases is justified mainly, by the presence of phytochemicals originated in the secondary metabolism of several species. Secondary metabolites generated by plants have the primary function of mediating their contact with the environment, being extremely sensitive to changes or external stimuli, such as high levels of UV radiation, extreme temperatures, environmental pollution, presence of pollinating insects, or herbivorous animals.[1,3,4] Most of secondary metabolites represent an important source of drugs due to their anti-inflammatory, anticancer, antibacterial, cardioprotective and antioxidant properties. [5]

As a result, these compounds are desired by several industry sectors, since they can be incorporated into a variety of products such as cosmetics, food, nutraceuticals, polymers, waxes, colorants, perfumes, etc.[4] In addition to this, due to the strong connection of synthetic products to toxicity and carcinogenicity, there is an emerging search for novel bioactive compounds originated from natural to replace synthetics products. [6]

Among the 'products' offered by nature; flowers have attracted the researcher's attention for the fact of presenting a large amount of phytochemicals with broad

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bioactive potential. [1]However, the incautious consumption of natural products or substances is not recommended. In opposition to what is often conveyed by media or popular knowledge, it is important to consider that undesirable side effects are not exclusive to synthetic drugs. Either, plants can present, in addition to beneficial compounds, some toxic substances.

Since 2009, there has been a considerable increase in the consumption of cosmetics, comprising shampoos, conditioners, moisturizers and hair colorants, which has led to a greater safety control of such products. [7] Several cosmetics have incorporated natural products in their formulations. [8]Thus, aiming at the safety and health of consumers, the chemical composition, toxicity and side effects of natural products must be elucidated. [9]The studyof natural products begins with the specimen identification, followed by matrix extraction, screening (scanning) of the compounds and then, the assessment of its possible cytotoxicity. [10]

Currently, *Hibiscus sabdariffa* is the main worldwide traded specie belonging to the genus *Hibiscus* (Malvaceae). The United States and Germany are the main importers of this plant. Its dried or fresh calyces are widely consumed in culinary, in the preparation of alcoholic beverages, fermented drinks, syrups, salads, jellies, cakes and infusions. It is also used as a component in hair care and anti-ageing skin care products being considered an active oxygen scavenger cosmetic. [11-13]

Hibiscus rosa-sinensis L. (HRS) is an ornamental Chinese shrub widely cultivated in tropical and subtropical climates. In India, HRS has a great commercial appealing, being extensively used in local herbal products. [14] Its flowers and leaves are known to be emollient. [15] Studies regarding extracts from different parts of this plant have demonstrated its most popular medicinal properties such as the regulation of glycemia, and fertility. [16] have reported hair growth activity(*in vitro* and *in vivo*) byHRS extracts, suggesting its use as a constituent in hair care cosmetics.

Although, despite the promising evidences demonstrated by previous studies, only conventional methodologies for extracting bioactive compounds from HRS have been applied to theplant. In addition, it is desirable the use of advanced analytical tools in order to better identify the compounds present in the extracts, such as high-performance liquid chromatography (HPLC) coupled to mass spectrometry techniques. Furthermore, there is a lack data concerning the assessment of the biological safety of HRS extracts.

Hence, the present study was aimed to obtain HRS extracts using microwave assisted extraction (MAE), and investigate the chemical composition, their main biological activities and the *in vitro* potential ocular irritation of the extracts by HPLC-DAD-ESI-IT-MS and Hen's Egg Test - Chorioallantoic Membrane (HET-CAM), respectively.

II. EXPERIMENTAL

2.1 Plant material and chemicals

Fresh yellow and red flowers from *Hibiscus rosa-sinensis* were collected in June 2019 from a community garden (GPS location 10°57'56.0"S 37°03'40.9"W). The specie was identified by a taxonomist from Tiradentes University. The yellow *Hibiscus rosa-sinensis* (YHRS) (n°1142) and the red *Hibiscus rosa-sinensis* (RHRS) (n°1176) were deposited in the herbarium - UNIT, Aracaju-Sergipe/Brazil. Petals of the flowers were selected and oven dried at 50 °C for 24 h. The dried petals were grounded, sieved (8-16 Mesh) and kept in dark flasks until analysis. According to the different types of analysis, the material was subjected to different extractions. All solvents, reagents and reference standards used were chromatographic grade (JT Baker and Sigma Aldrich).

2.2. Microwave assisted extraction (MAE)

Microwave assisted extraction (MAE) was carried out using a single mode, sequential microwave system (CEM®, Discover SP). Each experiment was made with 0.5 g of powdered petals. Firstly, hydroalcoholic and acidic aqueous extraction by MAE were made withYHRS to determine the better extraction conditions. Then, these conditions were applied to RHRS.

2.2.1.Hydroalcoholic extraction by MAE.

These experiments were made to enhance the extraction of phenolic compounds from HRS. The experimental matrix was a 2^4 factorial design with central points to measure the experimental error. The independent variables used to evaluate the effi*ciency of MAE were*: microwave power (100 and 200 W), irradiation time (4 and 12 min), solvent concentration (25 and 75% of ethanol in water) and solvent volume (20 and 30 mL). The generated extracts were filtered and stored in dark flasks at −20 °C until the analysis. The dependent variables evaluated were: total phenolic content (TPC), total flavonoid content (TFC), % inhibition in DPPH (I%) and extract yield.

2.2.2. Acidic aqueous extraction by MAE.

These experiments were made to enhance the extraction of anthocyanins from HRS. The experimental matrix was a 2^3 factorial design with central points and the independent variables were: microwave power (100 and 200W), irradiation time (4 and 12 min) and, solvent volume (20

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and 30 mL). The generated extracts were filtered and stored in dark flasks at −20 °C until the analysis. The dependent variable was total anthocyanin content (TAC).

2.3. Exhaustive extraction

Exhaustive extraction was carried out in order to compare the performance of MAE. The compounds were extracted according to a method previously reported, [17] with some modifications. Briefly, 0.5g of each yellow and red powdered flowers were extracted with successive additions of 10 mL of methanol: water (8:2, v/v) acidified (1% HCl, w/v) in a Falcon tube. The tube was vortexed for 5 min and centrifuged at centrifuged at 4000 rpmfor 5 min. The extract was filtered (filter paper) and the residue was further extracted (14 more times) until no longer reacted with the Folin-Ciocalteu reagent. The supernatants were pooled for the quantification of TPC, TFC, I%, TAC and, extract yield. The experiments were made in triplicate.

2.4. Phytochemical analysis

2.4.1. Total phenolic content (TPC)

TPC was determined using the Folin–Ciocalteu assay with some modifications. [18] An aliquot (0.5 mL) from each extract was mixed with 9 mL of distilled water, 0.5 mL of Folin–Ciocalteu reagent and 5 mL of 7% Na_2CO_3. After 2.5 hours of incubation at 25 ºC, the absorbance was measured at 760 nm. A standard curve was prepared using 60 to 150 mg/L of gallic acid. TPC was expressed as mg of gallic acid equivalents per 1 gram of wet weight basis (mg GAE/g).

2.4.2. Total flavonoid content (TFC)

TFC was determined using a methodology previously reported, [19] with some modifications. The extracts (0.25 µL) were diluted in distilled water (0.75 µL). The diluted extract (1 mL) was mixed with 4 mL of distilled water and 300 µL of 5% $NaNO_2$ was added. Five minutes later, 300 µL of 10% $AlCl_3$ was added in the mixture. Following that, 2 mL of 1 mol/L NaOH was added in the reaction tube and the absorbance was measured at 510 nm using a spectrophotometer. A standard curve was prepared using 20 to 200 mg/L of rutin. TFC was expressed as mg of rutin equivalents per 1 gram of wet weight basis (mg RE/g).

2.4.3. Total anthocyanin content (TAC)

TAC was determined by pH-differential method, [20] with some modifications. Each extract (400 µL) was diluted in two different buffer solutions; first in potassium chloride solution (0.025 M, buffer pH 1) (3600 µL) and second in sodium acetate solution (0.4 M, buffer pH 4.5) (3600 µL). Fifteen minutes later, the absorbance was measured at 530 and 700 nm using a spectrophotometer. The absorbance of each sample was calculated as the difference in the absorbance between pH values and wavelengths:

$$A = (A_{530\ nm} - A_{700nm})_{pH\,1.0} - (A_{530nm} - A_{700nm})_{pH\,4.5}$$

Therefore, TAC value was obtained:

$$TMAC\ (mg/L) = \frac{A\ x\ MW\ x\ DF\ x\ 1000}{\varepsilon\ x\ 1}$$

where A is the absorbance of the sample, MW is the molecular weight of cyanidin-3-glucoside (449.2 g mol^{-1}), DF is the dilution factor, ε is the molar absorptivity of cyanidin-3-glucoside (26,900 L cm^{-1} mol^{-1}), and 1 is for a standard 1 cm path length. TAC was reported as milligrams anthocyanins per 100 g dry weight (mg Cy-3-glu/100 g).

2.4.4. Scavenging activity of DPPH radical

The antioxidant activity was measured using a modified DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging method. [21] Solutions (100 µL) of various concentrations of the extracts in methanol (1, 2.4, 5 and, 10 % v/v) were added to 3900 µL of a methanol solution of DPPH (0.06 mM). The control solution was made of 100 µL of methanol added by DPPH solution (0.06 mM). After 45 min of incubation at 25 ºC, the absorbance was measured at 516 nm. Free radical DPPH inhibition in percentage (I%) was calculated as follows:

$$I\% = \left(\frac{(A_{control} - A_{sample})}{A_{control}}\right) x\ 100$$

Where $A_{control}$ is the absorbance of the control solution and A_{sample} is the absorbance of the tested extract. Extract concentration providing50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration. Tests were carried out using quercetin as positive control.

2.4.5. Total carotenoid contents (TCC)

Carotenoids were extracted according to a method previously reported by Rodriguez-Amaya,[22]with some modifications. Briefly, 0,5 g of each yellow and red powdered flowers were extracted with successive additions of 8 mL of acetone, using a vortex homogenizer. The extracts were filtered (filter paper) and the remaining residue was further extracted until its complete depigmentation. The organic fractions were pooled in a separatory funnel and mixed with petroleum ether (50 mL). The extract was washed with distilled water (100 mL) three times. The aqueous phase was discarded and the fraction of petroleum ether containing the carotenoids was collected and anhydrous sodium sulfate was added. The extract was transferred to a 50 mL volumetric flask and the

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volume was completed with petroleum ether. The absorbance was measured at 455 nm. The carotenoid content was calculated and expressed as microgram of β-carotene per 1 gram of dried weight basis (µg β-carotene /g) as follows:

$$TCC = \frac{A_{sample} \times \text{volume (mL)} \times 10^4}{A_{\beta carotene} \times \text{M (g)}}$$

Where A_{sample} is the absorbance of the test extract, V is the total volume of the sample (mL), $A_{\beta\text{-carotene}}$ is the β-carotene extinction coefficient in petroleum ether (2592) and, M is the sample weight (g). The experiments were made in triplicate.

2.5. Extraction yield

The extraction yield was determined using 1 mL of each homogenizedextract. The solvent was eliminated by vacuum evaporation and the yield was calculated as the percentage weight of crude extract over the total weight of the powdered flowers (%m/m).

2.6. *In Vitro* Eye Irritation Potential Assessment

For the evaluation of the irritative potential of HRS extracts, the *in vitro* HET-CAM test was used. The methodology is based on the observation of irritative reactions such as hemorrhage, lysis or coagulation in the chorioallantoic membrane of embryonic chicken eggs on the tenth day of incubation after contact with the tested substance. [23 - 26] To perform the HET-CAM test, only extracts obtained under best conditions of hydroethanolic and acid extraction by MAE (from yellow and red petals) were used. Initially, the extracts were concentrated by vacuum evaporation at 60 °C and then, the dry extract was resuspended in 0.9% NaCl. The resuspended extracts were immediately used for HET-CAM test.

The test was carried out as follows: fertilized chicken eggs (weighing between 40 and 50 g), acquired from a local farm were incubated at 37.5 ° C for a period of 10 days. On the tenth day of incubation, the egg shell around the air chamber was removed with the aid of tweezers, exposing the outermost membrane. This membrane was hydrated with 0.9% saline solution. The eggshell membrane was removed to expose the innermost membrane, the chorioallantoic membrane (CAM). Each extract (300 µL) was deposited over the chorioallantoic membrane and the symptoms of irritation, such as: lysis, hemorrhage or coagulation, were observed after 300 seconds of exposure. A positive (300 µL of 1 $molL^{-1}$ NaOH solution) and a negative control (300 µL of 0.9% NaCl solution) were also evaluated. The irritation score was calculated as follows:

$$IS = \frac{(301 - H_{time}) \times 5}{300} + \frac{(301 - L_{time}) \times 7}{300} + \frac{(301 - C_{time}) \times 9}{300}$$

Where H_{time} is the time (in sec) of the first sign of hemorrhage, L_{time} is the time (in sec) of the first lysis signal and, C_{time} is the time (in sec) of the first coagulation signal. The positive and negative control and the extracts were evaluated according to the irritation classification designated by Luepke, [23] where 0.0 - 0.9 is nonirritant, 1.0 - 4.9 is slightly irritant, 5.0 - 8.9 is moderately irritant and 9.0 - 21.0 is severely irritant. The experiments were made in triplicate.

Due to its high degree of pigments, the CAM exposed to aqueous acid extracts was rinsed with 0.9% NaCl prior to the observing the vascularization.

2.7. LC-MS/MS analysis

The extracts obtained under best conditions of hydroethanolic and acid extraction by MAE (from yellow and red petals) were analyzed by HPLC-DAD-ESI-MS/MS. Prior to the injection, in order to remove unwanted compounds, the extracts were subjected to solid phase extraction (SPE). Thus, each extract generated two fractions: F_1 (non-anthocyanin compounds) and F_2 (anthocyanin compounds). The procedure used was as follows: SPE cartridges of C_{18} were activated with methanol and conditioned with water acidified (0.01% HCl). Then, the sample was applied to the cartridge. The unwanted compounds were eluted with water acidified (0.01% HCl) and discarded. After that, F_1 fraction (non-anthocyanin compounds) was eluted with ethyl acetate and recovered in dark flask. After that, F_2 fraction (anthocyanins) was eluted with methanol acidified (0.01% HCl). The fractions were concentrated by vacuum evaporation at 40 °C until 1 mL and transferred to dark flasks.

The HPLC system consist of a high-performance liquid chromatograph (Shimadzu, SPD-M20A, Japan) with a Diode Array Detector (DAD) connected in series to an ion trap mass spectrometer, model Esquire 6000 (Bruker Daltonics, USA), with an electrospray ionization (ESI) source. The separation of the compounds was performed using a C_{18} column (4.0 µm × 150 cm × 4.6 mm, Phenomenex, USA) the compounds were separated at flow rate at 0.7 mL min^{-1} at 29 °C. Mobile phase were solvent A: water/formic acid (99.5:0.5, v/v) and solvent B: acetonitrile/formic acid (99.5:0.5, v/v). The used elution gradient was beginning with A/B 99:1 (v/v) to 50:50 (v/v) in 50 min, then 50:50 (v/v) to 1:99 (v/v) in 5 min, at the end the ratio (1:99, v/v) was maintained for another 5 min. Ultraviolet-visible spectra were obtained between 200 and 800 nm and the chromatograms processed at 278, 354, and 515 nm. The column eluate was reduced to only 0.35 mL min^{-1} before enter the ESI interface. The mass

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spectrometer was operated under the following conditions: mass range from 100 to 800 Daltons, source in the positive and negative ionization modes, capillary voltage of 3000 V, nebulizer gas 30 psi, skimmer 72 V, temperature and drying nitrogen flow at 310 °C and 11 L min^{-1} and fragmentation energy of 1.6 V.

The extracts were diluted with solvent A and filtered (Millipore, 0.22 µm) prior to analysis.

The identification of phenolic compounds was based on:

(1) retention time and elution order in the chromatographic column;

(2) UV–Vis and MS spectra, compared to analytical standards analyzed under the same conditions; and

(3) data from the literature.

The identification was made by injection of external standard solution of gallic acid, chlorogenic acid, catechin, epicatechin,caffeic acid, rutin, *p*-coumaric acid,*m*-coumaric acid, ferulic acid and, quercetin, (25 mg mL^{-1}).

2.8. Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) using MATLAB software version 5.3 (The Math Works Inc, USA). Significance level was set at 5%.

III. RESULTS AND DISCUSSION

3.1. Extractions

3.1.1. Hydroalcoholic extraction microwave assisted extraction (MAE)

Table 1 shows the levels attributed to each independent variable and the obtained values for the dependent variables: % inhibition in DPPH (I% - y_1), total flavonoid content (TFC – y_2), total phenolic content (TPC – y_3) and, extract yield (y_4) obtained from yellow petals of HRS (YHRS).

As it is possible to note, the variables microwave power (x_1) (increasing from 100 to 200 W), irradiation time (x_2) (varying from 4 to 12 minutes) and the solvent concentration (x_3) (with amount of ethanol ranging from 25 to 75%) did not show any significant difference in the variation of TPC, TFC, I% and, extract yield ($p > 0.05$). Thus, considering the consume of energy, the degradation of the compounds at higher irradiation time and the cost of the experiment, these variables were kept at their lower levels (100 W, 4 minutes and 25%). However, it was not possible to evaluate this variable at higher levels because 30 mL was the limit of the microwave vessel. Thus, this variable was kept at 30 mL.Therefore, hydroalcoholic extraction conditions by MAE were: microwave power (100 W), irradiation time (4 min), ethanol concentration (25 %) and, solvent volume (30 mL). This set of conditions were also used to extract bioactive compounds from red petals of HRS (RHRS). **Figure 1** (red and yellow dotted lines) summarizes the results for TPC, TFC, I%, and extract yield for the two kind of flowers obtained hydroethanolic extraction by MAE.

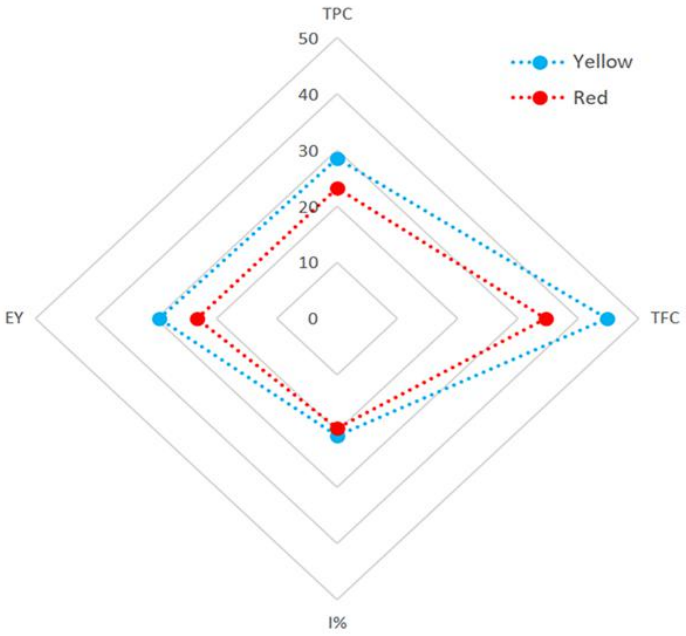

Fig.1: Total phenolic content (TPC in mg GAE/g), total flavonoid content (TFC in mg RE/g), % inhibition in DPPH (I%), extract yield (EY in %m/m) obtained by MAE from HRS petals.

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Table 1: The experimental matrix of the hydroethanolic extraction with the values for the responses.

Assay	Independentvariables				Dependentvariables			
	X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3	Y_4
1	-1	-1	-1	-1	27.13	76.37	23.34	28.80
2	1	-1	-1	-1	22.15	72.28	20.69	30.40
3	-1	1	-1	-1	23.76	73.53	22.92	28.80
4	1	1	-1	-1	46.07	86.51	23.85	29.60
5	-1	-1	1	-1	17.82	79.63	23.64	28.80
6	1	-1	1	-1	17.50	83.89	26.44	30.00
7	-1	1	1	-1	19.58	84.43	24.70	30.00
8	1	1	1	-1	24.40	91.09	27.85	31.80
9	-1	-1	-1	1	26.16	89.17	25.85	34.40
10	1	-1	-1	1	32.10	89.88	29.56	32.80
11	-1	1	-1	1	26.16	56.11	20.92	25.20
12	1	1	-1	1	22.63	65.35	20.63	24.00
13	-1	-1	1	1	21.83	90.83	29.88	34.80
14	1	-1	1	1	16.05	71.63	23.73	25.20
15	-1	1	1	1	20.71	91.36	28.43	32.40
16	1	1	1	1	29.05	92.16	30.02	33.60
R_1	0	0	0	0	26.97	95.02	28.65	31.50
R_2	0	0	0	0	27.93	101.91	28.17	31.50
R_3	0	0	0	0	25.36	88.13	24.74	28.00

As seen in **Figure 1**, the extracts obtained by MAE (yellow and red dotted lines, respectively) were rich in TPC (28.4 and 23.2 mg GAE(Gallic Acid Equivalent) per gram (GAE g^{-1}) and in TFC (44.8 and 34.5 mg RE (Rutin Equivalent) per gram (RE g^{-1}), besides exhibited inhibition in DPPH assay (20.9 and 19.4%) and produced high extract yield (29.5 and 23.2**%)** for YHRS and RHRS extracts, respectively. The IC_{50} value in DPPH of extracts were also determined (12.35 and 15.76 mg L^{-1}, respectively) and were in accordance with %I. However, the contents of all the evaluated responses were significant different among the yellow and red petals extracts (p<0.05), with YHRS showing higher levels than RHRS in all parameters.

3.1.2. Acidic aqueous extraction by MAE

Acidic aqueous extraction was used to enhance the extraction of anthocyanins from HRS. **Table 2** shows the levels attributed to each independent variable and the values of the dependent variable: total anthocyanin content (TAC – y_1) obtained from YHRS.

Table 2: The experimental matrix of the Acidic aqueous extraction with the values for the responses.

Exp.	variables			
	independents			dependents
	X_1	X_2	X_3	Y_1
1	-1	-1	-1	4.67
2	1	-1	-1	4.03
3	-1	1	-1	3.41
4	1	1	-1	4.59
5	-1	-1	1	4.07
6	1	-1	1	4.64
7	-1	1	1	4.42
8	1	1	1	4.77

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R_1	0	0	0	4.96
R_2	0	0	0	5.20
R_3	0	0	0	5.24

Microwave power, solvent volume and irradiation time did not show any significant difference in the variation of TAC ($p > 0.05$). Thus, the variables were kept at the lower levels: microwave power (100 W), irradiation time (4 min), and solvent volume (20 mL). These experimental conditions were used to extract anthocyanins from RHRS. **Figure 2** (red bars) shows TAC for the two type of flowers. RHRS have four times higher level of TAC than YHRS: 4.1 and 16.3 mg Cy-3-glu/100 g.

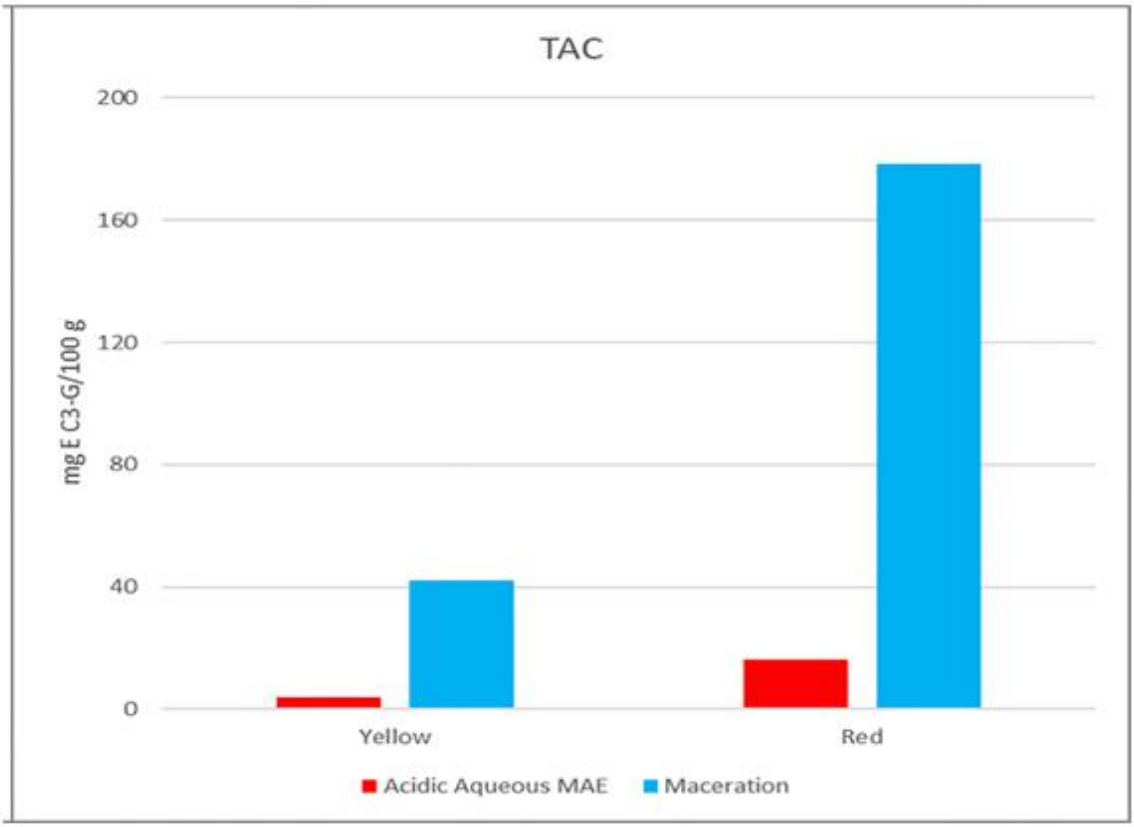

Fig.2: Total anthocyanins content (TAC in mg Cy-3-glu/100 g) obtained by MAE from HRS petals.

Microwave-assisted extraction has demonstrated advantages over conventional extraction strategies because since provides extraction yields comparable with the latter and requires less extraction time, once the microwaves rupture the plant structure to increase extraction power. In addition to similar recovery, MAE allows reduced solvent consumption, shorter extraction times and temperature control that ensuring stability of thermolabile components, among others advantages. [27]By comparing the results obtained here with those obtained by maceration, a conventional extraction technique, the proposed methodology showed similar recovery of TPC and TFC from HRS petals. [28-30] However, these values were significantly lower than those reported by Mak et al.[31]for aqueous and ethanolic extracts of *H. rosa-sinensis* petals. It's important to highlight that TPC and TFC values obtained in the present study were close to some varieties of *Hibiscus sabdariffa.*[32] Regarding to the TAC, the MAE performance was lower than that obtained by maceration. [29,33,34]

3.2. Total carotenoid contents (TCC)

The flower extract of HRSY had significantly higher TCC value than HRSR (295.99 ± 1.69 and 78.19 ± 0.89 µg β-carotene/g, respectively - $p<0.05$). The TCC value for YHRS was higher than that reported for *H. sabdariffa*flowers by Attaugwu andUvere[35] but lower than that reported by Gbadamosi, Abiade and Agbatutu[36] for *H. asper*flowers. Normally, fruits, vegetables, and vegetable oils (especially olive oil) offers the most amount of carotenoid intake in the diet. [37] According to the data, the yellow and red petals of *H. rosa-sinensis*present TCC value in the same order of

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magnitude of important sources of carotenoids, such as peach, watermelon, and apricot. [38]

Carotenoids are naturally occurring pigments in the chloroplasts of plants and algae, as well as, in egg yolk, fishes and crustaceans. This class of compounds plays a large role in the human diet. Studies demonstrated that the consumption of diets rich in carotenoids is associated with a lower incidence of cancer, cardiovascular diseases, age related macular degeneration and cataract formation. [39,40]

3.3. HPLC-DAD-ESI-MS/MS analysis

Flavonoids are a class of secondary metabolites of plants with powerful antioxidant and pharmacological activities. They constitute colored pigments of fruits, herbs, vegetables and medicinal plants and are divided into the subgroup's flavanones, flavanols, flavonols, isoflavons, flavons, and anthocyanidins. [41] The HPLC analysis of hydroethanolic extracts from YHRS and RHRS obtained by MAE revealed the presence of five flavonoids. Three peaks were identification based on comparison of their retention time (RT),UV–Vis and mass spectrometric data using standards. For these compounds, MS analysis carried out in the negative-ion mode $[M - H]^-$. At retention time 22.9 min showed the presence of catechin (m/z 289, MW 290) (λ_{max} 278 nm), at 25.2 min, epicatechin (m/z 289, MW 290) (λ_{max} 278 nm) and, at 29.4 min, rutin (m/z 610, MW 609) (λ_{max} 354 nm). Catechin and epicatechin are flavanols report for the first time in HRS extracts. **Figure 3** (**A** and **B**) present the HPLC profiles of three identified compounds in hydroethanolic extracts from YHRS.

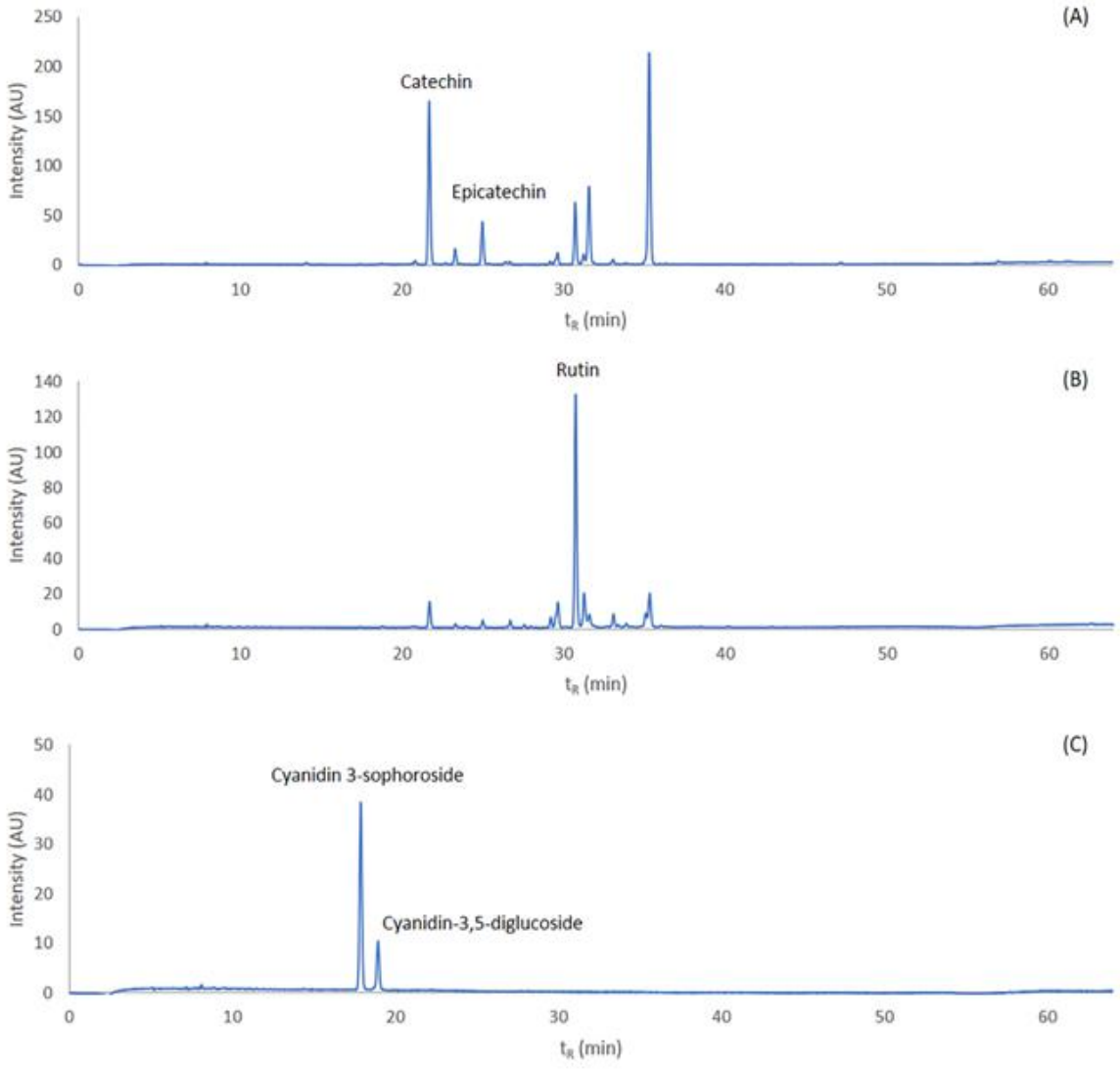

Fig.3: High-performance liquid chromatograms of the hydroalcoholic extracts from YHRS, processed in: 278 (A), 354 (B) and, 520 nm (C).

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Acid extracts from red petals of HRS were found to contain catechin and epicatechin, whereas none flavonoid was identified in acid extract from yellow petals. Thus, hydroethanolic extraction enhanced the yield of flavanols and the flavonol, when compared with acidic aqueous extraction. Rutin was identified in HRS extracts in former studies. [30,42,43] Furthermore, Pillai and Min[43] identified chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid and, quercetin in the HRS extracts. These polyphenolic compounds were not identified in the present study. Rutin, catechin and epicatechin are believed to exhibit significant biological activities, including antioxidant, anti-inflammation, anti-diabetic, anti-adipogenic, neuroprotective and cardioprotective activity. [44,45]

Two anthocyanins were tentatively identified in HRS extracts, these peaks represented the first and second predominant areas in mass spectra chromatogram. The identification was based on the information obtained from UV–Vis and mass spectra data and comparison with data reported in the literature. MS analysis carried out in the positive-ion mode that affect the dissociation of glycoside bonds at positions of C_3, C_5 and/or C_7 of the anthocyanidins.[46]

Peak at RT 18.0 min, the major compound, showed m/z 611 (λ_{max} 515 to 516 nm), which corresponded to the molecular cation tentatively identified as cyanidin-3-sophoroside. MS/MS fragmentation of m/z 611 produced a daughter ion at m/z 287, which was indicative of the cyanidin moiety, 287 [M-324].[47] While peak at RT 19.2 min was tentatively assigned as cyanidin-3,5-diglucoside. This peak showed a $[M]^+$ ion at m/z 611 (λ_{max} 515 to 518 nm) and m/z 449 - loss of glucose (162 Da) - and a predominant ion at m/z 287 in MS2, resulting from the consecutive losses of 2 glucoses. [48]

The acid solution enhanced the extraction of the anthocyanins, since acidic extracts from RHRS present the two anthocyanins, while hydroethanolic extracts from RHRS showed only cyanidin-3-sophoroside. **Figure 3C** shows the HPLC profiles of anthocyanins in hydroethanolic extracts from YHRS.

Studies concerning the composition of HRS petals identified cyanidin-3-sophoroside. [47] and cyanidin-3,5-diglucoside [48] in hydroalcoholic acid extracts. Anthocyanins are a subclass of water-soluble flavonoid pigments, important in the plant-derived food industry. [46] These bioactive compounds are responsible for the commercial and medicinal value of genus *Hibiscus* spp.[47]The interest in anthocyanins as functional ingredients has also been generated by their potential role in preventing chronic and degenerative diseases due to their antioxidant, [49-51] anti-inflammatory,[51] antiarteriosclerosis, [52] anticancer, [53] hyperlipidemia, [54,55] and hypoglycemic activities. [56]

3.4.*In vitro* eye irritation potential assessment

After applying the extract over the CAM, the images obtained were analyzed and the irritative ocular potential was determined (**Figure 4**).

Both, YHRS and RHRS extracts, as well as, negative control, presented no irritation (IS=0.00 ± 0.00). Based on the irritation scores of the control solutions, the analyzed extracts were classified as non-irritating ingredients, since no vascular events were observed during the period of 300 s. The positive control was classified as a strong irritant (IS= 18.07 ± 0.87).

The hair care, comprising of shampoos, conditioners, styling agents and hair colorants represents around 17% of the global beauty market. In addition to the advantages of these hair care cosmetics, they present a potential risk of eye irritation due to exposure during use.[7] Cosmetic industries are focused on explore alternative sources of raw materials, especially of plant origin, due to their content of biologically active compounds, the synergy of their action and the high degree of assimilation by the human body. [57] Leaves and flowers of *H. rosa-sinensis* exhibit hair growth *in vitro* and *in vivo*, as well as anti-greying properties,[16] suggesting that these proprieties could be used in hair growth formulations.

The HET-CAM test[17]allows the identification of irritating and non-irritating substances, which appear to be similar to those, which occur in the eye using the standard Draize rabbit eye test. It is applicable on shampoos as well as the ingredients used in their composition.

Since the HSR extracts presented here are consisted of several bioactive compounds and non-irritating ingredients were observed, this plant can be considerate as an important raw material for cosmetic formulations.

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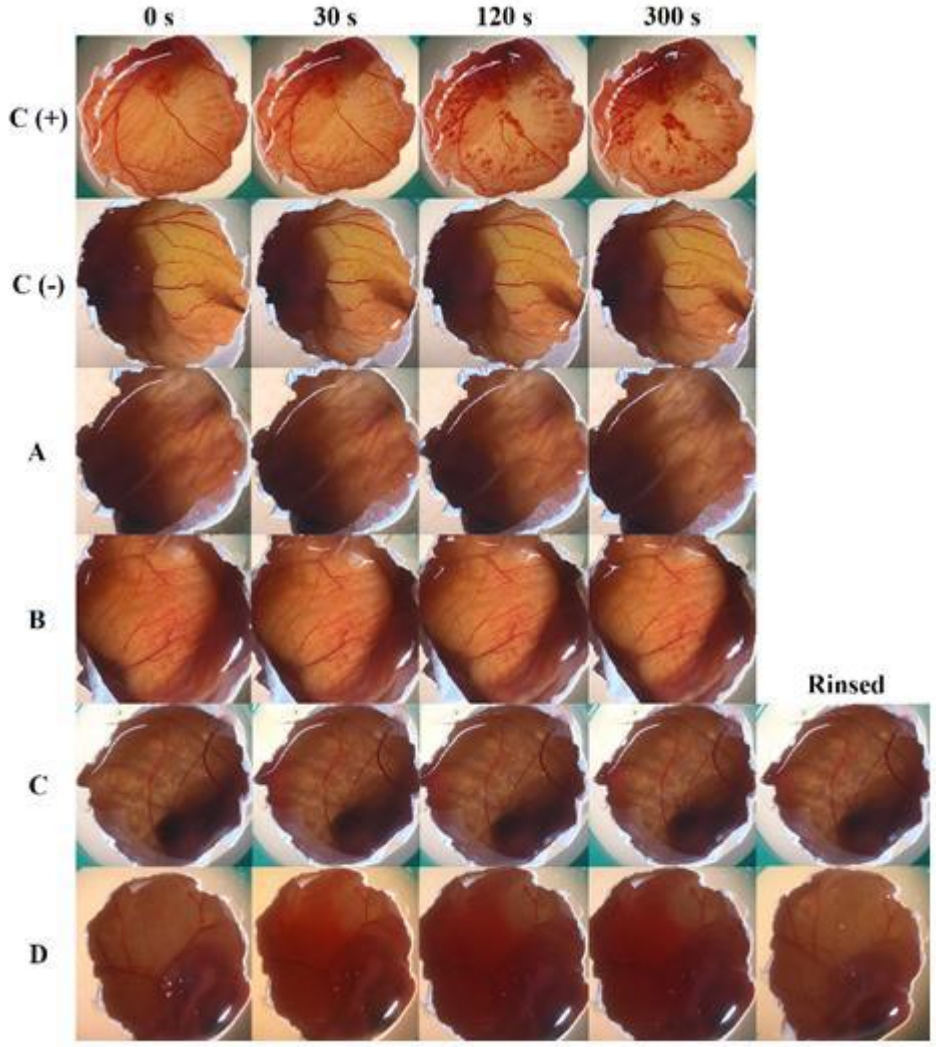

Fig.4: Representative images of the chorioallantoic membrane (CAM) after the application of: C (+) positive control; C (-) negative control; (A) hydroethanolic extract from YHRS; (B) hydroethanolic extract from RHRS; (C) acid aqueous extract from YHRS and (D) acid aqueous extract from RHRS observed during the period of 300 s.

IV. CONCLUSIONS

The two experimental designs allowed to identify which independent variables has an influence the extraction process of bioactive compounds from the petals of *H. rosa-sinensis*. In the specific experimental design for the extraction of phenolic compounds (hydroethanolic extraction), the only variable that proved to be significant was the solvent volume. The greater the amount of solvent used, the highest the amount of polyphenolic compounds extracted. Regarding acid extraction, no independent variable studied was found to be significant to the extraction process ($p > 0.05$). Then it was possible to continue the extraction under gentle conditions with no losses in its performance.

The antioxidant activity of the extracts obtained in the hydroethanolic extraction measured in terms of IC_{50} proved to be significant. The HRS extracts of both varieties showed to be a great source of carotenoids, suggesting its use as a nutraceutical, dye, or constituent of animal feed. The chromatographic analysis showed the presence of the compounds catechin, epicatechin, both for the first time in HRS, rutin, cyanidin-3-soforoside and cyanidin-3,5-diglycoside. Finally, the HET-CAM assay showed non ocular irritating potential of HRS extracts, thus being able to be safely incorporated into cosmetic formulations that may come into contact with the ophthalmic mucosa.

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