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ANTI-INFLAMMATORY, ANTINOCICEPTIVE AND ANTIOXIDANT ACTIVITIES OF THE HYDROMETHANOLIC FRACTION FROM *ANNONA NUTANS* LEAVES

ATIVIDADE ANTI-INFLAMATÓRIA, ANTINOCICEPTIVA E ANTIOXIDANTE DA FRAÇÃO HIDROMETANÓLICA DAS FOLHAS DE ANNONA NUTANS

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ABSTRACT: *Annona nutans* (Annonaceae) is a plant species found in Bolivia, Paraguay, Argentina, and the Brazilian Cerrado, specifically in the states of Mato Grosso and Mato Grosso do Sul (Brazil). Its common names are *Araticû-Mi* and *Araticû-Ñu.* The research contributions regarding the chemical composition and biological activities of extracts from *A. nutans* are rare, with only four articles being found in the literature. Therefore, the present study evaluated the anti-inflammatory and antinociceptive activities of the hydromethanolic fraction (FHMeOH) using carrageenan-induced paw edema and hot-plate tests. In addition, the antioxidant activity was evaluated by DPPH radical scavenging, total phenolic, flavonoid and tannin content assays and quantification of the major metabolites by LC-MS were performed. Oral treatment with the FHMeOH (at a dose of 300 $mg.kg^{-1}$) significantly reduced paw edema 2 h and 4 h after the inflammatory stimulus. The intraperitoneal (i.p.) treatment with the FHMeOH (50 and 100 $mg.kg^{-1}$) proved to be most effective, and the inhibition of acute inflammation was still visible 6 h after carrageenan injection. At doses of 50 and 100 $mg.kg^{-1}$ (i.p.), FHMeOH exhibits central antinociceptive effects by increasing the latency of the reaction in the hot-plate model. The FHMeOH showed antioxidant potential, and the metabolites quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-galactoside, quercetin-3-*O*-*β*-D-apiofuranosyl-(1→2)-galactopyranoside, and chlorogenic acid were identified and quantified by LC-MS. Our results indicate, for the first time, that FHMeOH has anti-inflammatory, antinociceptive and antioxidant potential, and it is a promising source of studies for new herbal medicines

KEYWORDS: *Annona nutans*. Flavonoids. Anti-inflammatory. DPPH. Antinociceptive. HPLC quantification.

INTRODUCTION

Annona is among the 27 most important genera of the Annonaceae family. This genus, containing approximately 162 species distributed among the tropics, is represented mainly in South and Central America, with 110 native tropical species in the Americas and Africa (CHATROU et al., 2012; COUVREUR et al., 2011). Initially, plants belonging the Annonaceae family were believed to predominantly contain alkaloids (LEBOEUF et al., 1982); however, a great diversity of chemical constituents was recently acknowledged. Studies of species belonging to the Annonaceae family intensified following the isolation of a chemical group known as "annonaceous acetogenins", which presented a wide variety of biological activities such as cytotoxic, antitumor, pesticidal, and antimicrobial (BERMEJO, 2005; CAVÉ et al., 1997). For many years, the interest in *Annona* species, and other genera in the Annonaceae family, was a result of the presence of acetogenins, which showed promising pharmacological activities (BERMEJO, 2005). However, epidemiological data from the island of Guadalupe (Caribbean) have associated the consumption of *Annona* species (*A. muricata, A. reticulata*, and *A. squamosa*) with the development of atypical parkinsonism, suggesting that acetogenins and quinoline alkaloid derivatives are directly related to its etiology, because the

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acetogenin annonacin was shown to cause neurodegeneration in rats (CAPARROS-LEFEBVRE; STEELE, 2005). Apart from acetogenins, plants belonging to the Annonaceae family possess a wide variety of metabolites that are responsible for important pharmacological activities, such as anti-inflammatory, antinociceptive, and antioxidant (BENITES et al., 2015; FORMAGIO et al., 2013a, 2013b).

Annona nutans (or *Annona spinescens* var. *nutans*) is a plant species found in Bolivia, Paraguay, Argentina, and the Brazilian Cerrado, specifically in the states of Mato Grosso and Mato Grosso do Sul (Brazil). Its more common names are *aratico, chirimoya del campo,* and *sinini de la pampa*; and those in the Guarani language are *Araticû-Mi* and *Araticû-Ñu* (CORRÊA, 1926; TROPICOS.ORG, 2017). The research contributions regarding the chemical composition and biological activities of the extracts from *A. nutans* are rare, with only four articles found in the literature (GLEYE et al., 2000, 1998; GONÇALVES et al., 2014; SILVA et al., 2015). The acetogenins were found in *A. nutans* roots (GLEYE et al., 2000, 1998), with studies in the literature showing the absence of acetogenins in the leaves (SILVA et al., 2015; SILVA, 2013). Thus, the present study identified and quantified some metabolites by LC-MS and quantitated the total phenolic, tannin, and flavonoid content, in addition to assessing the antioxidant potential as DPPH radical scavenging ability from the hydromethanolic fraction (FHMeOH) of *A. nutans*. Moreover, the anti-inflammatory and antinociceptive efficacy of the FHMeOH was evaluated *in vivo*.

MATERIAL AND METHODS

Plant material

The leaves of *A. nutans* were collected in Porto Murtinho, the state of Mato Grosso do Sul, Brazil. The plant was previously identified by Renato de Mello-Silva, and a voucher specimen was deposited at the CGMS Herbarium (MS, Brazil) under number 27648. The present study obtained a Certificate of Registration from the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (A90D499).

Preparation of fractions

Dried and powdered leaves (160 g) were percolated with methanol:water (9:1) (5.0 L) for 72 h at room temperature, yielding 32 g hydromethanolic extract. 28 g was dissolved in methanol:water (9:1) (500 mL), followed by sequential partitioning with n-hexane, chloroform, and ethyl acetate (200 mL, 5x of each solvent). The final hydromethanolic fraction (FHMeOH) was dried under a vacuum in a rotatory evaporator at 45 °C, and subsequently lyophilized, yielding 21 g. For the determination of total phenolic, tannin, and flavonoid contents, a stock solution in ethanol:water (1:1) was initially prepared from the FHMeOH solution at a concentration of 5 $mg.mL^{-1}$, and successive dilutions at concentrations of 5.0; 2.5; 1.25; 0.625; 0.3125 $mg.mL^{-1}$ were prepared.

Determination of the total phenolic content

The total phenolic content was determined by spectrophotometric quantitation using the Folin-Ciocauteu reagent in 96-well microplates, as described previously (ZHANG et al., 2006).

Briefly, a 20 µL sample (prepared as previously described) and 100 µL Folin-Ciocauteu reagent (Imbralab®) were added to each well, shaken, and incubated for 5 min. Subsequently, 80 µL 7.5% Na_2CO_3 (Alphatec®) solution was added.

A calibration curve was generated using gallic acid (Cromato Produtos Químicos Ltda®) at the standard at concentrations of 1; 0.5; 0.25; 0.125; 0.0625; and 0.0312 $mg.mL^{-1}$. For the reagent blank, 20 µL methanol, 100 µL Folin-Ciocauteu reagent, and 80 µL 7.5% Na_2CO_3 were used. The plates were incubated in the dark at room temperature for 2 h. Readings were performed using a microplate spectrophotometer (SpectraMax®Plus384, Molecular Devices, Sunnyvale, CA, USA, Gen5 software) at λ = 750 nm. For the instrument blank, 20 µL CH_3OH:H_2O (1:1) and 180 µL distilled water were used. The total phenolic content (TPC) was determined by interpolation of the absorbance of the samples against the calibration curve obtained for the standard and are expressed as µg of gallic acid $equivalents.mg^{-1}$ for respective fractions. All analyses were performed in triplicate.

Determination of the total tannin content

Sample solutions were prepared at the same concentrations as described for the total phenolic quantitation; however, prior to the addition of Folin-Ciocauteu reagent, 0.01 $g.mL^{-1}$ hide powder (Sigma-Aldrich) was added, and the solution was shaken for 60 min on an orbital shaker. The filtrates (20 µL) were added to each well of a 96-well plate with 20 µL methanol:water (1:1) and 100 µL Folin-Ciocauteu reagent, shaken, and incubated for 5 min. Subsequently, 80 µL of 7.5% Na_2CO_3 solution was added, and the plates were incubated in the dark at room temperature for 2 h. Readings were performed using a microplate spectrophotometer at λ = 750 nm.

For the instrument blank, 20 µL of methanol:water (1:1) and 180 µLof distilled water were used.

The total tannin content (TTC) was determined from the standard curve by subtracting the calculated content of the sample solution for non-adsorbed polyphenols from the calculated TPC content (sample solution for total polyphenols). The expression is shown below. The results are expressed as $\mu g.mL^{-1}$ of sample. All analyses were performed in triplicate.

TTC = content of the sample solution for total polyphenols – content of the sample solution for non-adsorbed polyphenols in hide powder (BRASIL, 2010a; VERZA et al., 2007).

Determination of the total flavonoid content

The total flavonoid content (TFC), equivalent to quercetin, present in the fractions was determined using a previously described method (BANOV et al., 2006; BRASIL, 2010b), and expressed as µg quercetin . mg^{-1} fraction.

For the calibration curve, quercetin (Sigma-Aldrich) was used as the standard reference flavonoid, of which a stock solution of 0.01 $\mu g.mL^{-1}$ was prepared. From this stock, dilutions were prepared at concentrations of 20, 10, 5, 4, 3, 2, 1, 0.75, 0.5 and 0.3 $\mu g.mL^{-1}$, to which 500 µL 5% (w/v) $AlCl_3$ solution was added. Following a 30-min incubation, the absorbance was read at 425 nm using a UV-Vis spectrophotometer (QUIMIS®) and the calibration curve was constructed.

To 100 µL 50% methanol, 100 µL FHMeOH and 500 µL 5% (w/v), $AlCl_3$ solution was added. Following a 30-min incubation, the absorbance was read at 425 nm using a UV-Vis spectrophotometer (QUIMIS®). All analyses were performed in triplicate.

Determination of the DPPH radical-scavenging capacity

The radical-scavenging capacity of FHMeOH was determined according to the method described by Burda and Oleszek (2001) (BURDA et al., 2001). BHT (2,6-di-tert-butyl-4-methylphenol) was used as the reference compound. FHMeOH was prepared in triplicate for each concentration (1, 10, 100, 250, and 500 $\mu g.mL^{-1}$). Each sample (75 µL) was added to three wells of a 96-well plate containing 150 µL 0.002% (w/v) DPPH-methanol solution, shaken vigorously, and incubated in the dark for 30 min. The control was prepared as above without any extract or BHT. The absorbance was measured at λ = 517 nm using a UV-Vis spectrophotometer (Biotek Power Wave XS2/US, U.S.A) and methanol was used for baseline correction. The radical-scavenging activity is expressed as the inhibition percentage and was calculated as:

$$[1 - (Abs_{control} - Abs_{sample})] \times 100$$

where $Abs_{control}$ = absorbance of DPPH radicals in methanol and Abs_{sample} = absorbance of fraction in methanol + DPPH. The scavenging activity is expressed as $\mu g.mL^{-1}$. IC_{50} values ($\mu g.mL^{-1}$) were calculated using Probit analysis (FINNEY, 1980).

Identification and quantitative UFLC-DAD- ESI-QTOF-MS analysis

The identification and quantification of phytocompounds was performed on a Shimadzu Prominence UFLC TM system, equipped with a LC20AD, coupled to a diode array detector and mass spectrometer (MicroOTOF-Q III Bruker Daltonics, Billerica, USA) with an electrospray ion source. The analyses were monitored between 210 and 800 nm and the mass spectrometer operated in a negative ionization mode (*m/z* 120-1300). Chromatographic analyses were performed in a Kinetex C-18 column (Phenomenex, 2.6 µ, 150 x 2.1 mm). For quantification, FHMeOH was dissolved in methanol and water (1:1, v/v) at a concentration of 1 $mg.mL^{-1}$. Standard solutions at a concentration of 1 $mg.mL^{-1}$ were prepared with chlorogenic acid (5-*O*-caffeoylquinic acid), the flavonoids quercetin-3-*O*-galactopyranoside, quercetin-3-*O*-glucopyranoside, and isorhamnetin-3-*O*-galactopyranoside (Sigma Aldrich®). For the analytical curve, dilutions were prepared at the concentrations from 0.0488 to 200.00 $\mu g.mL^{-1}$ (0.0488, 0.0976, 0.195, 0.390, 0.781, 1.56, 3.12, 6.25, 12.50, 25.00, 50.00, 100.00, and 200.00 $\mu g.mL^{-1}$). For the isorhamnetin-3-*O*-galactopyranoside, concentrations of 12.5, 25.0, 50.0, 100.0, and 200.0 $\mu g.mL^{-1}$ were prepared. About 3 µL of sample was injected in to the column by the auto sampler. The samples were eluted through the column with a gradient mobile phase consisting of A (water 0.1% (v/v) formic acid) and B (acetonitrile: formic acid 0.1% (v/v)). The gradient elution was programmed as follows: 0–2 min B (3%); 2–25 min B (25%); 25–26 min B (80%); 26–28 min B (80%); 28–29 min B (3%); and 29–35 min B (3%). The analyses were carried out in triplicate at a flow rate of 0.3 $mL.min^{-1}$, at a temperature of 50 °C, with the detector set at λ= 340 nm. Calibration curves were plotted showing a linear relationship between concentrations *versus* peak areas for all reference compounds. The

attribution of the chromatographic peak was based on the retention times and confirmed by the injection of standards. The concentration of each peak was calculated from the experimental peak areas by analytical interpolation in a standard calibration line. Peak areas were calculated at 340 nm. The limit of detection (LOD) was determined as a signal-to-noise ratio of 3:1 and the limit of quantification (LOQ) was determined as a signal-to-noise ratio of 10:1 (GARCÍA-SALAS et al., 2015). The precision was calculated by relative standard deviation (%RSD), and the selectivity was evaluated by comparing the chromatograms of the individual reference standards and the degree of interference between the peaks when injected simultaneously; the degree of purity of these peaks was also investigated (BRITO, 2014; RIBANI et al., 2004).

In vivo assays

Chemicals

Indomethacin (Ind) and carrageenan λ type IV were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Fentanyl citrate (Fent) was purchased from Cristália (SP, Brazil). DMSO 2% in physiological saline was used as the control, and FHMeOH was prepared in this vehicle for oral or intraperitoneal treatments to mice.

Animals

Adult male Swiss mice (28–30 g) were obtained from the Bioterium of Universidade Federal de São João del-Rei, Brazil, and were housed in temperature-controlled rooms (22–25 °C), under a 12–12 h light–dark cycle, with access to food and water *ad libitum*. The mice were acclimated for one week prior to the experiment. Twelve hours prior to the beginning of oral treatments, the mice were fasted and received only water *ad libitum*. For the intraperitoneal experiment, the food and water were retained. The number of mice and the intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments. All procedures were carried out in accordance with the guidelines set forth by the Brazilian National Council for the Control of Animal Experimentation and International Association for the Study of Pain and were approved by the Ethics Committee in Animal Experimentation of the Federal University of São João Del-Rei, Brazil (CEUA/UFSJ, protocol 034/2015).

Assessment of the anti-inflammatory activity

The anti-inflammatory activity was assayed using the carrageenan-induced paw edema model (LEVY, 1969). Mice were randomly divided into five groups (n=6), and orally (p.o.) received vehicle (10 $mL.kg^{-1}$, control group), FHMeOH (at doses of 30, 100, and 300 $mg.kg^{-1}$), or indomethacin (10 $mg.kg^{-1}$). Moreover, FHMeOH, at doses of 25, 50 and 100 $mg.kg^{-1}$, were intraperitoneally (i.p.) administered. Following 30 (i.p.) or 60 (p.o.) min following treatments, carrageenan (400 µg, 30 µL) was injected into the plantar side of the left hind paw. Paw volume was measured using a plethysmometer (Insight®, Brazil) prior to treatment (basal value) and at 1, 2, 4, and 6 h after the injection of the inflammatory stimulus. The volume of edema was calculated by the difference between the prior basal paw volume and the one after carrageenan injection.

Evaluation of the antinociceptive activity

Mice were tested on a hot-plate (Insight®, Brazil) kept at a constant temperature of 55 ± 0.50 °C for 24 h before the assay, and animals that remained on the apparatus for less than 15s were selected. Thus, the selected animals were randomly divided into five groups (n=7) and received (i.p.) vehicle (10 $mL.kg^{-1}$, control group), FHMeOH (at doses of 25, 50, and 100 mg. kg^{-1}), and Fentanyl (Fent 200 $\mu g.kg^{-1}$). Reaction times were recorded when the mice licked their paws or jumped at intervals of 30 min up to 120 min after treatments. A cut-off of 30s was chosen to avoid tissue lesions (MUHAMMAD; SAEED; H., 2012).

Statistical analysis

Microsoft Excel 2010 (Microsoft Corporation) was used for the quantitation of the total phenolic, tannin, and flavonoid content. In the evaluation of the anti-inflammatory and antinociceptive activities, results are expressed as the mean ± SEM. The statistical significance between groups was assessed using one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison *post-hoc* test. All calculations for anti-inflammatory, antinociceptive activities and DPPH radical-scavenging capacity were performed using GraphPad Prism™ version 5.01 (GraphPad® Software Inc., San Diego, CA). A level of significance ($p < 0.05$) was considered for each experiment.

RESULTS

Total phenolic, tannin and flavonoid content

The total phenolic, tannin, and flavonoid content of the FHMeOH extract from *A. nutans* was calculated based on the interpolation of the absorbance values of the samples from the calibration curve of gallic acid (TPC and TTC), which presented the following equation of the line, y = 0.2352x + 0.0044, R^2 = 0.9982, and from the calibration curve of quercetin (TFC), which presented y = 0.0228x + 0.0027, R^2 = 0.9986. The total phenolic, tannin, and flavonoid content was 62.96 ± 3.73 μg.mg^{-1}, 31.14 ± 3.11 μg.mg^{-1}, and 18.07 ± 0.10 μg.mg^{-1}, respectively.

Determination of DPPH radical-scavenging capacity

The DPPH radical-scavenging activity of the FHMeOH extract from *A. nutans* is presented in Figure 1. FHMeOH showed a dose-dependent inhibitory effect with an IC_{50} of 4.89 μg.mL^{-1}, which was comparable to that of the commercial antioxidant, BHT (IC_{50} = 16.36 ± 3,63 μg.mL^{-1}). The FHMeOH at doses of 1, 10, 100 and 250 μg.mL^{-1} presented a scavenging effect on the DPPH radical that was statistically significant compared to the BHT standard.

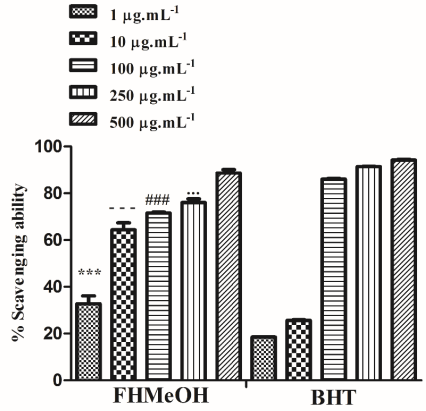

Figure 1. The DPPH radical-scavenging ability of the hydromethanolic fraction (FHMeOH) and 2,6-di-tert-butyl-4-methylphenol (BHT) at five different concentrations (μg.mL^{-1}). $p < 0.001$ as compared with BHT.

Identification and quantitative UFLC-DAD- ESI-QTOF-MS analysis

For the analytical curve and linearity, the regression equations were y = 12222x - 18557 (R^2 = 0.9992) for chlorogenic acid; y = 10087x - 13571 (R^2 = 0.9992) for quercetin-3-*O*-galactopyranoside; y = 9454.4x - 13865 (R^2 = 0.9991) for quercetin-3-*O*-glucoside; and y = 8597.5x + 12942 (R^2 = 0.9995) for isorhamnetin-3-*O*-galactopyranoside.

The limit of detection (LOD) for all flavonoids was 4.88 x 10^{-2} μg.mL^{-1} and chlorogenic acid was 9.77 x 10^{-2} μg.mL^{-1}. The limit of quantification (LOQ) was 1.95 x 10^{-1} μg.mL^{-1} for flavonoids and 3.91 x 10^{-1} μg.mL^{-1} for chlorogenic acid. Relative standard deviations (%RSD) were in the range of 0.11% to 3.49% and they were calculated as a mean of the three replications.

The estimated concentration of metabolites was calculated in μg.mg^{-1} of FHMeOH measuring 3.04 μg.mg^{-1} of chlorogenic acid, 6.74 μg.mg^{-1} of quercetin-3-*O*-galactopyranoside, 3.62 μg.mg^{-1} of quercetin-3-*O*-glucopyranoside, 2.12 μg.mg^{-1} of isorhamnetin-3-*O*-galactopyranoside, and 4.86 μg.mg^{-1} of quercetin-3-*O*-*β*-D-apiofuranosyl-(1→2)-galactopyranoside. Since the metabolite, quercetin-3-*O*-*β*-D-apiofuranosyl-(1→2)-galactopyranoside, had no reference standard, its content was calculated based on the analytical curve of quercetin-*3*-*O*-β-galactopyranoside with a correction factor based on the corresponding molecular weight, because they have the same aglycone and thus the same chromophore group. This compound (quercetin-3-*O*-*β*-D-apiofuranosyl-(1→2)-galactopyranoside) was identified in studies conducted in our laboratory using H^1 and C^{13} NMR, COSY and DEPT techniques (SILVA et al., 2015; SILVA, 2013)

The FHMeOH was also analyzed by UFLC-DAD-MS to identify its chemical constituents. The compounds were identified by the comparison of UV spectra and retention time with applied patterns and subsequent confirmation of their molecular weights and their fragmentation in MS^2. The compounds identified are listed in Table 1 and illustrated on the chromatogram in Figure 2.

Twenty-three compounds could be detected and identified from FHMeOH. Chlorogenic acids derivatives identified were 3-*O*-*E*-caffeoylquinic

acid (**3**) and 4-*O*-*E*-caffeoylquinic acid (**7**), as well as the O-glycosylated flavonols **14-22** and alkaloids **4**, **6**, **8-11** and **23**. These compounds were identified by comparing the spectral data reported for them in the literature (CLIFFORD et al., 2003; LIU et al., 2018) and data were also reported from *Annona* species for the alkaloids (FERRAZ et al., 2017; SHANGGUAN et al., 2018).

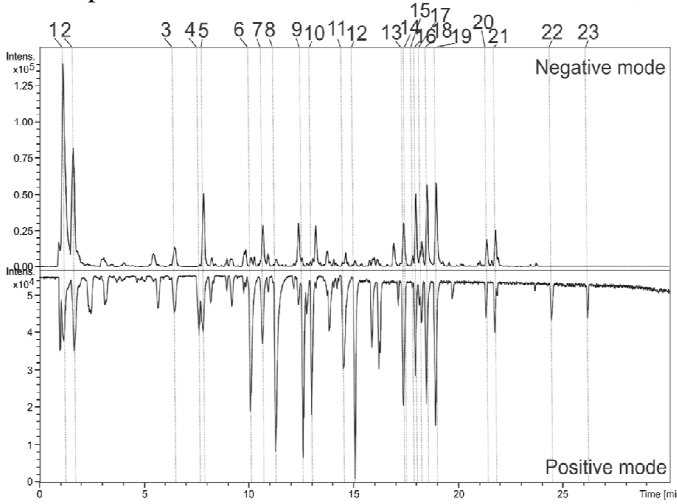

Figure 2. Base peak chromatogram obtained in negative and positive ion modes from FHMeOH of *A. nutans*.

Table 1. Constituents identified by UFLC-DAD-MS from FHMeOH

Peak	RT (min)	Compound	MF	UV (nm)	Negative mode (*m/z*)		Positive mode (*m/z*)	
					MS $[M-H]^-$	MS/MS	MS $[M+H]^+$	MS/MS
1	1.2	*O*-dihexoside	$C_{12}H_{22}O_{11}$	-	341.1086	191	365.1068^{Na}	
2	1.7	NI	$C_{11}H_{22}O_8$	-	279.1087	-	281.1252	
3	6.4	3-*O*-*E*-caffeoylquinic acid	$C_{16}H_{17}O_9$	299, 323	353.0885	191, 179	355.1043	163
4	7.5	norcoclaurine-hexoside	*O*-$C_{27}H_{35}NO_{12}$	-	-	-	566.2268	272, 255, 161
5	7.9	NI	$C_{19}H_{28}O_{11}$	275	431.1555	-	433.1728	205, 187, 175
6	10.1	NI	$C_{18}H_{19}NO_4$	288	312.1239	-	314.1406	178, 163
7	10.7	4-*O*-*E*-caffeoylquinic acidst	$C_{16}H_{18}O_9$	299, 325	353.0886	191, 173	355.1027	163
8	11.3	di-*O*-methoxyl di-hydroxyl aporphine alkaloid	$C_{18}H_{19}NO_4$	285	312.1254	-	314.1409	298, 284, 270, 151
9	12.6	NI	$C_{17}H_{19}NO_3$	285	-	-	286.1456	254, 237, 209, 191, 175, 165
10	13.0	Stepharine	$C_{18}H_{19}NO_3$	285	-	-	298.1451	254, 238, 223, 161, 146
11	14.5	Magnoflorine	$C_{20}H_{24}NO_4^+$	280	-	-	342.1712*	297, 282, 265, 237, 192
12	15.1	NI	$C_{19}H_{23}NO_4$	283	328.1566	-	330.1708	284, 267, 192, 177
13	17.4	NI	$C_{19}H_{32}O_8$	-	387.2021	-	389.2180	227, 209, 191, 173
14	17.5	Quercetin-*O*-hexosyl-deoxyhexoside	$C_{27}H_{30}O_{16}$	270, 350	609.1443	300, 271, 255	611.1605	303
15	17.8	Quercetin-*O*-hexosyl-	$C_{27}H_{30}O_{16}$	270,	609.1453	300, 271, 255	611.1623	303

		deoxyhexoside		350					
16	18.0	Quercetin-3-*O*-*β*-D-apiofuranosyl-(1→2)-galactopyranoside	$C_{26}H_{28}O_{16}$	265, 350	595.1305	300, 271, 255, 243, 179	597.1473	465, 303	
17	18.2	Quercetin-*O*-hexosyl-deoxyhexoside	$C_{27}H_{30}O_{16}$	270, 350	609.1441	300, 271, 255, 179	611.1630	465, 303	
18	18.3	Quercetin-*O*-pentosyl-hexoside	$C_{26}H_{28}O_{16}$	265, 350	595.1289	300, 271, 255, 243, 179	597.1485	465, 303	
19	18.5	Quercetin-3-*O*-*β*-galactopyranoside [st]	$C_{21}H_{20}O_{12}$	265, 352	463.0872	300, 271, 255, 243	465.1044	303	
20	19.0	Quercetin-3-*O*-*β*-glucopyranoside [st]	$C_{21}H_{20}O_{12}$	266, 348	463.0881	300, 271, 255, 243, 179	465.1044	303	
21	21.4	Quercetin-*O*-methyl-*O*-hexoside	$C_{22}H_{22}O_{12}$	268, 350	477.1031	314, 299, 285, 271, 257, 243	479.1196	317, 285	302,
22	21.8	Quercetin-*O*-methyl-*O*-hexoside	$C_{22}H_{22}O_{12}$	265, 350	477.1027	314, 299, 285, 271, 257, 243	479.1201	317, 285	302,
23	24.5	Xylopine	$C_{18}H_{17}NO_3$	284	-	-	296.1288	279, 249, 221, 178	264, 234, 206,
25	26.2	NI	$C_{59}H_{87}N_5O_{15}$	-	-	-	553.8154[**]	482, 359, 209	416, 237,

RT: retention time, MF: molecular formula; NI: non-identified; [Na]: $[M+Na]^+$; [*]: $[M]^+$; [**] $[M+2H]^{+2}$; [st]:confirmed by the injection of authentic standard

Anti-inflammatory effect of the FHMeOH

With respect to the anti-inflammatory activity, at 2 and 4 h post-carrageenan injection, the p.o. administration of 300 mg.kg^{-1} FHMeOH promoted a significant reduction in paw edema by 48.57% ($p < 0.05$) and 52.0% ($p < 0.05$), respectively, as compared with the control group (Figure 3).

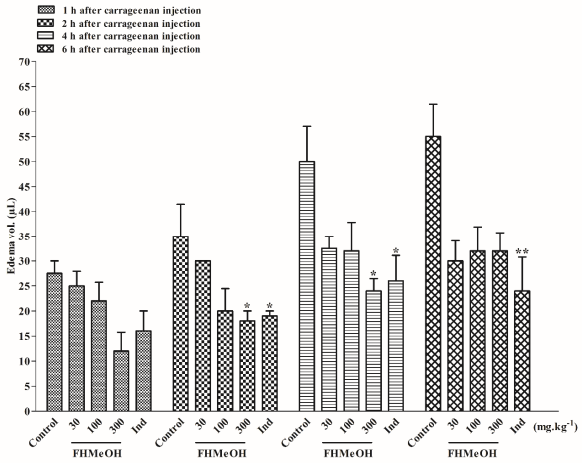

Figure 3. The effect of p.o. administration of the FHMeOH from *A. nutans* on carrageenan-induced paw edema in mice. Data were analyzed by ANOVA followed by Bonferroni's multiple comparison *post-hoc* test. Values are expressed as the mean ± SEM (n = 6). *$p < 0.05$ and **$p < 0.01$ compared with the control group.

In contrast, i.p. treatment with FHMeOH at minor doses (50 and 100 mg.kg^{-1}) exerted long-lasting anti-inflammatory effects that remained significant 6 h after inflammatory stimulus (Figure 4). The inhibitory values of paw edema at 2 and 4 h post-carrageenan-induced acute inflammation were 95.45% ($p < 0.001$) and 74.29% ($p < 0.001$), respectively, for 50 mg.kg^{-1} of the FHMeOH.

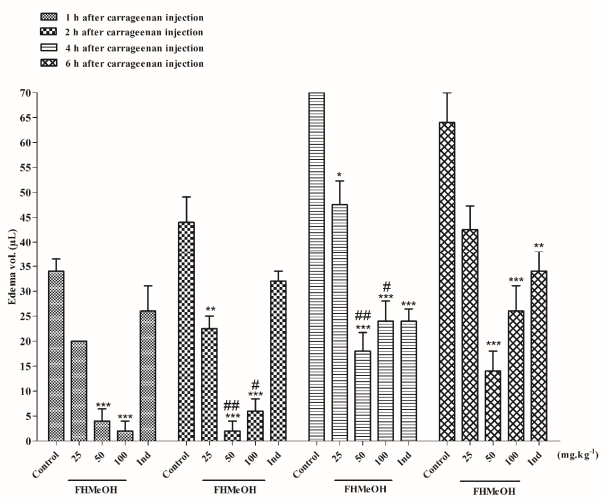

Figure 4. The effect of i.p. administration of the hydromethanolic fraction (FHMeOH) from *A. nutans* on carrageenan-induced paw edema. Data were analyzed by ANOVA followed by Bonferroni's multiple comparison *post-hoc* test. Values are expressed as the mean ± SEM (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared with the 25 mg.kg^{-1} FHMeOH group.

Antinociceptive activity of the FHMeOH

FHMeOH at doses of 50 and 100 mg.kg^{-1} (i.p.) induced a significant increase in the latency of reaction, and the central antinociceptive effects began 90 min post both treatments and were still observable after 120 min for the 50 mg.kg^{-1} (Figure 5).

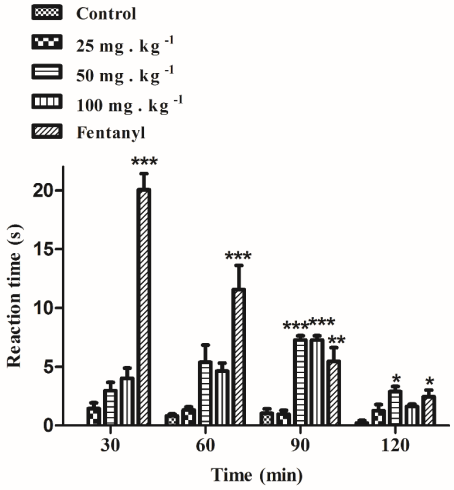

Figure 5. Effects of i.p. administration of the hydromethanolic fraction (FHMeOH) in the hot-plate model. Data were analyzed by ANOVA followed by Bonferroni's multiple comparison *post-hoc* test. Values are expressed as the mean ± SEM (n = 7). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the control.

DISCUSSION

The UFLC analysis in FHMeOH of *A. nutans* leaves revealed that quercetin-3-*O*-galactopyranoside is the identified phenolic compound which is the major constituent. The quantification by the UFLC-DAD method was validated and showed linearity, selectivity, and precision (BRASIL, 2003; DE AMORIM et al., 2014; LANDIM; FEITOZA; DA COSTA, 2013). From the Annonaceae family, reports including the quantitative determination of metabolites are uncommon, in particular for flavonoids (GARCÍA-SALAS et al., 2015), and most of the studies in the literature have evaluated the alkaloid and acetogenin content, because these two classes are the main ones in the species of the family (ALMEIDA, J. R. G. S.; JUNIOR, R. G. O; DE OLIVEIRA, 2015).

Currently, studies involving polyphenols such as flavonoids and chlorogenic acid derivatives have also gained great importance due to their diverse biological properties such as antioxidant, anti-inflammatory, antinociceptive, antimicrobial, and cardioprotective activity, among others (CALDERÓN-MONTAÑO et al., 2011; GALVÃO, STANLEY DE S. L. MONTEIRO et al., 2016; GARCÍA-LAFUENTE et al., 2009; GEORGIEV; ANANGA; TSOLOVA, 2014).

A direct relationship among antioxidant activity, phenolic compounds, and anti-inflammatory efficacy has been demonstrated in the literature (FORMAGIO et al., 2013a, 2013b; HIRANO et al., 2001), including in certain *Annona* species. The methanolic extract from A. *crassiflora*, for instance, has been shown to have a high total phenolic and flavonoid content (BENITES et al., 2015), and it effectively reduced paw edema and leukocyte recruitment induced by carrageenan at doses of 100 and 300 $mg.kg^{-1}$ (ROCHA et al., 2016). Similarly, the methanolic extract of *A. dioica* has high levels of total phenols and flavonoids, and in the concentration of 30 to 300 $mg.kg^{-1}$ p.o., it exhibited an anti-edematogenic effect in carrageenan-induced paw edema in a time- and dose-dependent manner (FORMAGIO et al., 2013b). *A. reticulata* has been also shown to possess strong antioxidant ability and dose-dependent inhibition of paw edema following carrageenan injection in rats (KANDIMALLA et al., 2016).

The present study demonstrated, for the first time, the antioxidant, anti-inflammatory and antinociceptive activities of the FHMeOH of *A. nutans* leaves. Moreover, the anti-inflammatory and antinociceptive activities of FHMeOH were demonstrated using acute inflammation and thermal hyperalgesia models in mice. Carrageenan injection into the paw provokes a biphasic response characterized by the initial phase (0 to 1 h) and the later phase (over 1 h). In the first phase, the release of histamine, serotonin, and bradykinins, and, to a lesser extent, prostaglandins occurs. The later phase is related to the overproduction of prostaglandins and polymorphonuclear leukocyte migration (CUZZOCREA et al., 1998). The p.o. treatment with FHMeOH failed to significantly inhibit paw edema formation with the lowest doses tested (30 and 100 $mg.kg^{-1}$). Only the highest dose of 300 mg/kg FHMeOH significantly inhibited paw edema 2 and 4 h after carrageenan injection. Since this inhibition was no longer observed at t = 6 h, this experiment suggests a low bioavailability by this route. On the other hand, the FHMeOH administered via i.p. exhibited significant anti-edematogenic activity in both phases. According to the literature, in the some studies of the pharmacological effects of natural products, using these models and the intraperitoneal route, the treatments with plant extracts showed anti-inflammatory and antinociceptive activities at higher doses (75-500 $mg.kg^{-1}$) (ALMEIDA et al., 2012; IBRAHIM et al., 2002; NARDI et al., 2003; SADANHA et al., 2016) than those used for FHMeOH in the present study.

Regarding the antioxidant activity, the DPPH radical-scavenging potential was used and is often compared with that of butylated hydroxytoluene (BHT), a commercial antioxidant used as a food additive (BURDA et al., 2001). As for measured by DPPH radical scavenging, FHMeOH presented an IC_{50} of 4.89 μg.mL-1, which was superior to that shown by BHT (IC_{50} = 16.36 $\mu g.mL^{-1}$). The IC_{50} of *A. nutans* is lower than the methanolic extract (17.84 $\mu g.mL^{-1}$) of *A. dioica* leaves (FORMAGIO et al., 2013b), and *A. dioica* presented high rates of flavonoids (FORMAGIO et al., 2013b). Although flavonoids present known antioxidant activity, it seems that the diverse metabolic profile of *A. nutans* has better antioxidant activity than other *Annona* species with higher content in flavonoids. A possible cause for these data is the presence of other metabolites which presented an antioxidant action such as proaporphine alkaloids stepharine (AVULA et al., 2018; COSTA et al., 2015), aporphine magnoflorine (KUKULA-KOCH et al., 2016; NASEER et al., 2015), and oxoaporphine xylopine (COSTA et al., 2010).

As soon as we identified the anti-inflammatory activity of FHMeOH, we tested its analgesic activity, because these properties are shared by several non-steroidal anti-inflammatory drugs. The hot-plate model is a specific central antinociceptive assay, and the nociceptive response to thermal stimulus is supraspinally integrated (JULIUS, D; BASBAUM, 2001; WOOLFE; MACDNOALD, 1944). The FHMeOH produced central antinociceptive effects, verified by the increase in reaction time. However, further studies are needed to establish the possible mechanisms of the antinociceptive action of FHMeOH (SALDANHA et al., 2016).

It can be stated that inflammation and nociception are correlated, because nociception is one of the cardinal signs of inflammation (LENARDAO et al., 2016; YIMAM et al., 2016). It is also known that flavonoids and cinnamic derivatives, for example, chlorogenic acids, have anti-inflammatory and antinociceptive activities

(GARCÍA-LAFUENTE et al., 2009; GEORGIADES et al., 2014; RATHEE et al., 2009; SERAFINI; PELUSO; RAGUZZINI, 2010; ZHAO, 2015; ZHU et al., 2013). These secondary metabolites may act via the inhibition of prostaglandin synthesis, neutrophil degranulation, and histamine, phosphodiesterase and protein kinases release (BASTOS, D. H. M.; ROGERO, M. M.; AREAS, 2009; RATHEE et al., 2009). The flavonoids and cinnamic derivatives play an important role because they present several biological actions besides anti-inflammatory and antinociceptive activities, such as antioxidant and anti-microbial effects, as well as the modulation of metabolic disorders (NAVEED et al., 2018). The antinociceptive and anti-inflammatory activities of FHMeOH can be attributed, at least in part, to the metabolites found in the fractions, such as flavonoids and chlorogenic acid derivatives.

CONCLUSION

The present study demonstrates, for the first time, that the FHMeOH fraction obtained from the leaves of *A. nutans* possesses *in vivo* anti-inflammatory and antinociceptive activities. Furthermore, the combination of phenolics present in this fraction can explain, at least partially, the effects observed. These activities raise interest in the therapeutic potential of the FHMeOH for the treatment and/or management of inflammatory and painful conditions.

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RESUMO: *Annona nutans* (Annonaceae) é uma espécie de planta encontrada na Bolívia, Paraguai, Argentina e no Cerrado brasileiro, especificamente nos estados de Mato Grosso e Mato Grosso do Sul (Brasil). Seus nomes mais comuns são *aratico* e *Araticû-Mi* e *Araticû-Ñu*. As contribuições da pesquisa em relação à composição química e atividades biológicas dos extratos de *A. nutans* são raras, com apenas quatro artigos encontrados na literatura. Portanto, o presente estudo avaliou as atividades anti-inflamatória e antinociceptiva da fração hidrometanólica (FHMeOH) utilizando edema de pata induzido por carragenina e testes de placa quente. Além disso, a atividade antioxidante foi avaliada por meio de sequestro de radical DPPH, e foram realizados ensaios de quantificação de fenóis, flavonoides e taninos totais e quantificação dos principais metabólitos por CL-EM. O tratamento oral com a FHMeOH (na dose de 300 $mg.kg^{-1}$) reduziu significativamente o edema da pata 2 e 4 h após o estímulo inflamatório. Por outro lado, o tratamento intraperitoneal (i.p.) com FHMeOH (50 e 100 $mg.kg^{-1}$) provou ser mais eficaz e a inibição da inflamação aguda foi ainda visível 6 horas após a injeção de carragenina. Nas doses de 50 e 100 $mg.kg^{-1}$ (i.p.), FHMeOH exibiu efeitos antinociceptivos centrais aumentando a latência da reação no modelo de placa quente. FHMeOH apresentou potencial antioxidante e os metabólitos quercetina-3-*O*-galactosídeo, quercetina-3-*O*-glicosídeo, isoramnetina-3-*O*-galactosídeo, quercetina- 3-*O*-*β*-*D*-apiofuranosil-(1 → 2)-galactopiranosídeo e ácido clorogênico foram identificados e quantificados por CL-EM. Nossos resultados indicam, pela primeira vez, que o FHMeOH possui efeitos anti-inflamatórios, antinociceptivos e antioxidantes, sendo uma fonte promissora de estudos para novos medicamentos fitoterápicos.

PALAVRAS-CHAVE: *Annona nutans*. Flavonóides. Anti-inflamatório. DPPH. Antinociceptiva. Quantificação por CLAE

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