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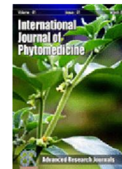

Original Research Article

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C-glycosyl flavones and a comparative study of the antioxidant, hemolytic and toxic potential of *Jatropha multifida* leaves and bark

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Abstract

The ethyl acetate extract from *Jatropha multifida* (Euphorbiaceae) leaves yielded two C-glycosyl flavones. Their structures were elucidated through spectroscopic methods, including UV, IR, 1D and 2D NMR, and compared with the related known compounds. The structures of the two flavonoids were determined as Vitexin (1) and Isovitexin (2). The ethanol extracts of leaves and bark and their fractions did not interfere in the integrity of erythrocytes, not even 1 and 2. In the Brine shrimp lethality method, bark extracts showed greater toxic potential than the leaf extracts. Both flavonoids are not toxic. The Phosphomolybdenum and DPPH assays were used in order to investigate the antioxidant activity of both compounds and fractions of leaf and bark extracts. The ethyl acetate fraction of bark showed excellent activity, with IC50 17.23 µg/mL-1, equivalent to the standard values, Vitamin C and Rutin. Compounds 1 - 2 demonstrated good activity with IC50 values of 54.37 and 87.27µg/mL-1. In the Phosphomolybdenum test, the ethyl acetate fraction of bark showed 86.18% of antioxidant activity compared with Rutin, and the chloroform fraction of leaves, 103.29%. In all tests the bark extracts were more bioactive than the leaf extracts.

Keywords: Jatropha, Euphorbiaceae, Vitexin, Isovitexin, Antioxidant, Flavonoid.

Introduction

The *Jatropha multifida* L. (Euphorbiaceae) species is being studied because of its use in popular medicine, especially for the treatment of infected wounds, skin infection and as a cicatrizant [1-2]. Preliminary phytochemical studies of the different species of Jatropha revealed the presence of alkaloids [3], cardiac glycosides, flobatannins, flavonoids, tannins and saponins [4]. Known in the traditional medicine as Coral Plant, Coral, Flower Coral, Blood Flower, Balsam and Metiolate Balsam [1] J. multifida is distributed in tropical areas like Brazil, Central America, Africa and India [5-7]. Chemical studies of this plant have led to the isolation of acylfloroglucinols [3] and diterpenes [8)]. In this study we investigated the phytochemical composition as well as the toxic, antioxidant and hemolytic potential of the J. multifida leaves and bark, which were collected in the Brazilian tropical forest. Through this study it was possible to compare the biological properties of the leaves and of the bark.

Materials & Methods

General Experimental Procedures

UV (Ultraviolet): Shimadzu UV-1601 in MeOH. IR (Infrared): Bomem-Hatmann & Braum MB-Serie. NMR (Nuclear magentic resonance): 1D Bruker® 200MHz, 2D Bruker Avance 400 MHz. HPLC (Hihg-performance liquid chromatography): MERCK Elite LaChrom employing for pumps L-2130, UV detector DAD L-2455 under a reversed phase LC_{18} column (250 mm x 4,6 mm x 5 µm) and (H_2PO_4 0,2%, H_2SO_4 0,02N) as solvent A, CH_3CN as solvent B : 1 min. 100% solvent A, 30 min. 47%, 31-35 min. 20%, 36 min. 100%, 1mL/min.

Plant material

The species was collected in the city of Alta Floresta, State of Mato Grosso, Brazil, in September 2009. The botanical determination was done by the biologist Osmar dos Santos Ribas, in the Municipal Botanical Museum of Curitiba, Paraná. The voucher specimen was deposited in the Municipal Botanical Museum of Curitiba under the number 226619, in Curitiba, Paraná, Brazil.

Extraction and isolation

The bark and leaves were separated, dried and grinded (429g of bark and 979g of leaves). The material was exhaustively extracted with ethanol in a Soxhlet apparatus. The ethanolic extract was concentrated to 1/3 of the initial volume and partitioned, originating the hexane, chloroform, ethyl acetate and the remaining extracts

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which were used in the biological tests. From the ethyl acetate fraction of leaves, a yellow precipitate, which has been denominated compound **1** (331mg), was obtained. This fraction was concentrated (2,06g) and submitted to liquid chromatography on silica gel, to which mixtures of hexane/ethyl acetate and ethyl acetate/water with increasing polarity were eluted. As a result, compound **2** was isolated (138mg).

Vitexin (**1**): Yellow amorphous powder. UV (MeOH) $_{max}$ (log) 270 (4.07), 300 (4.11) (sh), 336 (4.16), (NaOH) 280, 329, 394, (NaOAc) 281, 299, 385, (NaOAc/H_3BO_3) 272, 328 (sh), 344, ($AlCl_3$/HCl) 278, 304, 344, 381; IR (KBr): 3382, 3245, 1652, 1616, 1363, 1179, 1042, 833 cm^{-1}; ^1H and ^{13}C NMR 1D and 2D spectral data (DMSO-d_6), see Table 1.

Isovitexin (**2**): Caramel amorphous powder. UV (MeOH) $_{max}$ (log) 272 (4.07), 335 (4.16), (NaOH) 279, 331, 399, (NaOAc) 279, 298, 384, (NaOAc/H_3BO_3) 273, 340, ($AlCl_3$/HCl) 280, 303, 346, 257(s)h, 379; IR (KBr): 3397, 1652, 1610, 1355, 1247, 1082, 832 cm^{-1}; ^1H and ^{13}C NMR spectral data (DMSO-d_6), see Table 1.

Brine shrimp lethality assay

The general toxicity of flavonoids and extracts of leaves and bark were evaluated by the Brine Shrimp Lethality assay [9]. The lethal concentration of 50% (LC_{50}) was determined by counting the dead nauplii after a 24-hour incubation period. Data were analyzed by the statistical program Probits with a 5% confidence interval.

Hemolysis assay - Dilution Method

Sheep blood (Newprov®) was washed once with a phosphate buffer solution and then resuspended to give a 2% diluted suspension. A 1mL aliquot of the blood suspension was mixed with dilutions of extracts (0.1, 0.2, 0.5, 1.0mL) and a volume of phosphate buffer pH 7.4 to complete 2mL. The solutions were shaken, left to rest for 30 min. and shaken again. After a 130min. rest, the solutions were centrifuged at 3000 rpm for 5 min. and the hemolysis verification was made. Saponin R was used as positive control and distilled water as negative control [10].

Blood Agar plates method

Sterile paper discs impregnated with 1000 μg of extracts and fractions were placed on blood Agar plates (Newprov®). After incubation at 36ºC for 24 hours, the verification of the hemolysis halo was made. For positive controls, Saponin R and Triton solutions were used [11].

Phosphomolibdenum spectrophotometric method

The pure compounds **1** and **2**, fractions and ethanol extracts of bark and leaves were submitted to the Phosphomolibdenum spectrophotometric method [12]. Results were expressed as percentage of antioxidant activity in relation to Vitamin C and Rutin.

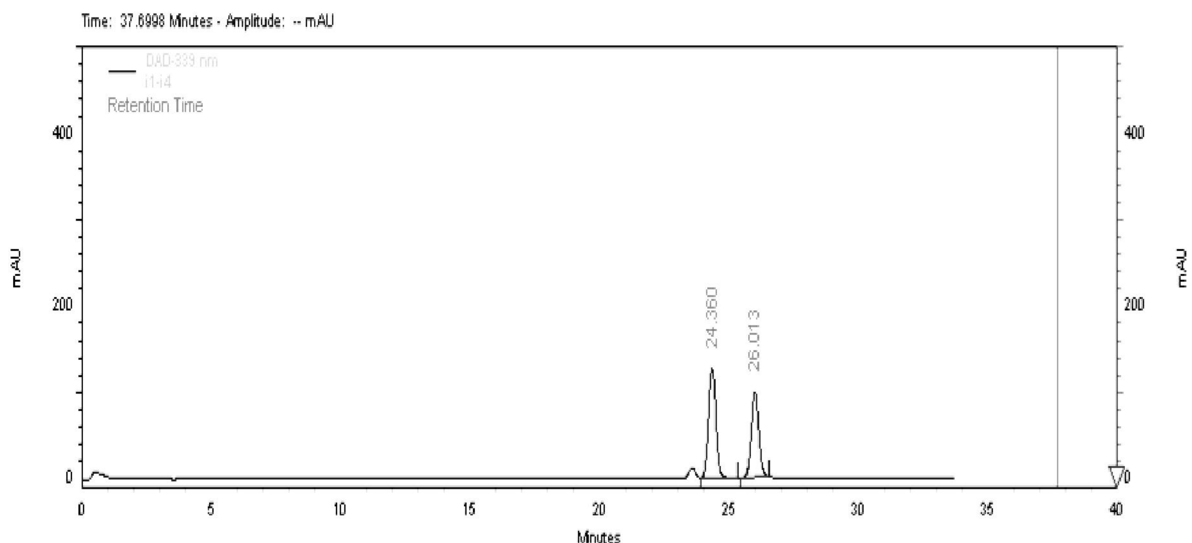

Figure-1 Cromatogram of compounds 1 and 2 injected on HPLC with methanol as solvent, wavelength 339 nm. The vitexin showed retention time at 24.360 min and isovitexin at 26.013. min.

Scavenging activity against DPPH radicals

The pure compounds, fractions and ethanol extracts of bark and leaves were submitted to the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay. Reaction mixtures containing dissolved test samples and a 300μM DPPH solution were left in tubes to react at room temperature for 30 min. The absorbance values were measured at 518 nm against the solvent without DPPH as the blank reference. The percentage of DPPH scavenging effect was determined and

IC_{50} values denote the concentration of sample required to scavenge 50% of DPPH free radicals. The obtained results of samples were compiled on Tukey test ($p<0.05$) [13-15]. All assays were done in triplicate.

Results and Discussion

The phytochemical analysis of the bark extract revealed the presence of coumarins, steroids, triterpenes, heterosides anthocyanin, tannins, gums and amino groups. In the leaf extract, the same groups were detected and also flavonoids and athraquinones.

From the ethyl acetate fraction of leaves, it was obtained a spontaneous precipitate, compound **1**, and by liquid chromatography on silica gel, compound **2**.

The UV spectrum of compounds **1** and **2** showed characteristic flavone absorptions at 270 and 336 nm. UV shift reagent studies along with sodium hydroxyde produced a bathochromic shift of band at 394nm, without a decrease in intensity, indicated that the 4′-hydroxy position is free on the B ring. The shift band of 281 nm with NaOAc suggested that the C-7 hydroxyl group was not substituted. The lack of a shift band with H_3BO_3 indicated the absence of ortho-dihydroxyl groups. A bathochromic shift with $AlCl_3$/HCl suggested that the 5-hydroxy position is free. The IR spectrum showed bands at 3300 cm^{-1}, typical of axial deformation of OH in intermolecular hydrogen bonds. The presence of the carbonyl group is characterized by the presence of bands at 1610 cm^{-1}. These data suggested that the compounds **1** and **2** were 4′, 5, 7 hydroxyl flavones.

The ^1H NMR spectrum of compound **1** (Table 1) showed a singlet at 6.8 consistent with the H-3 of flavones and this was supported by the observation of carbon signal at 105 associated with the C-3 in their ^{13}C NMR spectra. The B ring moiety was oxygenated only at C-4′ on account of the two doublet signals at 6.9 and 8.6 (2H, d, J=8.7 Hz) assigned to H-3′, H-5′ and H-2′, H-6′, respectively, characteristic of para-disubstituted rings. The proton singlet at 6.28 in the ^1H NMR spectrum was assigned to C-6 at 98.0. The position of this proton was determined by HMBC correlations. This signal showed correlations with C5, C7 and C10, confirming the location of the aromatic proton at 6.28 bonded to a carbon atom at 98.0 (Figure 2).

Table 1. NMR Spectroscopic data for Vitexin (1) and Isovitexin (2).

	Vitexin (1)			Isovitexin (2)	
Position	$_{C}$, mult.	$_{H}$ (J in Hz)	HMBC[a]	$_{C}$, mult.	$_{H}$ (J in Hz)
2	164.4, C			163.5, C	
3	102.0, CH	6.8, s	2, 3, 4, 1’	102.7, CH	6.81, s
4	182.0, C			182.0, C	
5	160.8, C	13.18, s	5, 6, 10	160.6 C	13.56, s
6	98.0, CH	6.28, s	5, 7, 10	108.8, C	
7	163.0, C			163.6, C	
8	104.5, C			93.5, CH	6.52, s
9	156.0, C			156.2, C	
10	105.0, C			103.3, C	
1’	122.0, C			121.0, C	
2’6’	129.4, CH	8.6, d (8.7)	2, 2’, 4’	128.4, CH	7.94, d (8.8)
3’5’	116.2, CH	6.9, d (8.7)	1’, 3’, 4’	116.0, CH	6.94, d (8.8)
4’	161.6, C			161.1, C	
1”	79.0, CH	4.66, m	7, 9, 10, 1”, 2”, 5”	78.9, CH	4.59, d (9.8)
2”	73.0, CH			70.5, CH	
3”	71.3, CH			72.9, CH	
4”	71.0, CH			70.1, CH	
5”	82.0, CH			81.5, CH	
6”	61.5, CH_2			61.4, CH_2	

[a]HMBC correlation, optimized for 6 Hz, are from proton (s) stated to the indicated carbon (200 MHz for ^1H NMR and ^{13}C NMR, 400 MHz for HMBC, DMSO-d_6).

The C-5 hydroxyl proton at 13.18 is chelated by a vicinal carbonyl group and also showed cross-peaks with C-5, C-6 and C-10. The presence of one anomeric sugar proton at 4.66 attached to carbon 79.0 confirmed that this sugar is C- attached. The glycosyl linkage was determined to be C-C on the basis of the relatively upfield anomeric carbon resonances at 79.0 in contrast with the anomeric carbons of O-glycosyl which normally resonate at 100.0 [16-17]. The glucose was determined to be bonded to C-8 of compound **1**, directly. This linkage was confirmed by the observation of the HMBC correlations between H-1″ and C-7, C-9, C-10, C-1′, C-2″ and C-5″ [18].

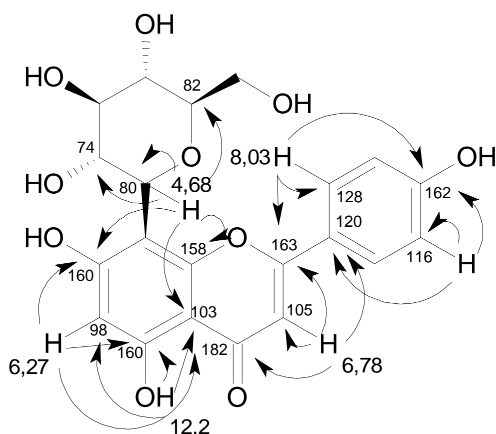

Figure 2. HMBC correlations of compound 1.

Given the similarities between the spectroscopic data of UV, IR and NMR of compounds **1** and **2**, an experiment was performed in HPLC: the compounds were injected separately and then co-injected. The retention times were different, confirming that they are two different compounds.
With the confirmation by HPLC experiment that compounds **1** and **2** are different and the comparison of chemical shifts in literature, it was concluded that the compounds **1** and **2** are the flavonoids Vitexin and Isovitexin (Figure 3) respectively [19]. The isolation of Vitexin in J. multifida species was previously reported [20].

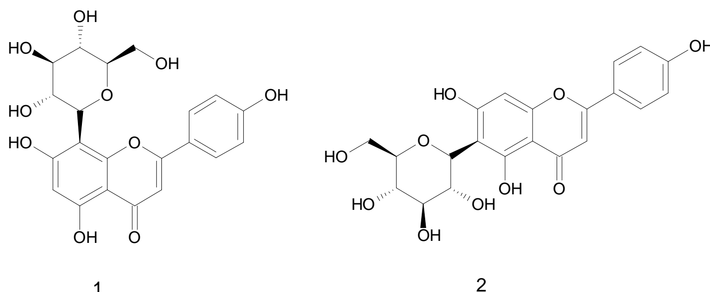

Figure 3. Flavonoids of *Jatropha multifida* L. 1. Vitexin, 2. Isovitexin.

Ethanol extracts, fractions of bark and leaves and compounds **1** and **2** were submitted to Brine shrimp lethality test with quinidine sulfate as positive control. The chloroform fraction of bark demonstrated higher toxicity with LC_{50} 57.59 $\mu g.mL^{-1}$, in a 5% confidence interval, a value which is close to quinidine sulfate LC_{50} 50.1 $\mu g.mL^{-1}$. The extracts of leaves showed lower toxicity, the higher data among these samples was from chloroform fraction with LC_{50} 252.96 $\mu g.mL^{-1}$.
The hemolytic capacity of ethanol extracts and fractions of bark and leaves were evaluated. For both methods, all samples did not cause hemolysis.

The antioxidant activities of ethanol extracts, fractions and compounds 1 and 2 were evaluated through scavenging activity assay against DPPH radical and phosphomolybdenum test with Vitamin C and Rutin as positive controls. The Phosphomolybdenum test results were expressed in Relative Antioxidant Activity of extracts and fractions in relation to Vitamin C and Rutin. In the phosphomolybdenum test, although the chloroform fraction of leaves was more active than Rutin (103.29%), the ethanol extract, chloroform and ethyl acetate fractions of bark showed antioxidant activity next to Rutin. The IC_{50} of the DPPH assay results were calculated through the equation of straight line obtained in the graph of Percentage Antioxidant Activity versus sample concentration. In this test, ethyl acetate fraction of bark showed great activity with IC_{50} 17.23 µg, equivalent to the value of the current standards, according to the Tukey test ($p<0,05$). Ethanol extract of bark was the second better result with IC_{50} 40.57 µg, more active than Vitexin and Insovitexin with IC_{50} 54.37 µg and IC_{50} 87.27 µg, respectively. For both used methods the bark extracts were more active than the leaf extracts.

Conclusions

These data allow us to conclude that the extracts of bark demonstrated more bioactivity than the extracts of leaves. The results of Brine shrimp lethality and antioxidant activity were coherent, for, according to Meyer and colleagues, biological activities of known active compounds are manifested as toxicity to the shrimp. There are studies about the biological activities of the latex, roots and leaves of this species [20-22], but there are few studies about its bark. The negative results in the hemolysis test and the better performance of the bark in relation to the leaves are an incentive for more studies toward isolation and identification of bioactive substances from the bark of *Jatropha multifida* L.

Author's contributions

BCKH carried out the biological assays and isolation of compounds, performed the statistical analysis and wrote the manuscript, CMSM carried out the RMN assays, CAM helped to draft the manuscript and translation, MCV carried out the hemolysis assays, MK carried out the HPLC assays, CG performed the method used on HPLC assays and helped with data interpretation, ALLL assisted in the interpretation of RMN data, MDM revised and helped to draft the manuscript, OGM helped with isolation and identification of compounds, conceived of study and participated in its design and coordination.

Acknowledgements

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