DOI: 10.22278/2318-2660.2023.v47.n4.a4018

ARTIGO ORIGINAL DE TEMA LIVRE

ANTIOXIDANT ACTIVITY, CYTOTOXICITY, PHENOLIC CONTENT, AND PHYTOCHEMICAL COMPOSITION OF PITHECELLOBIUM DIVERSIFOLIUM BENTH: A PILOT STUDY

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Abstract

Species of *Pithecellobium* (Fabaceae) are used in traditional medicine to treat diabetes, cough, bronchitis, and inflammation. This study aims to evaluate the content and determine the antioxidant activity, phenolic compounds content, and cytotoxicity of the extract and the fractions of *Pithecellobium diversifolium*. This is unprecedented research with an exotic species from the Caatinga, northeastern Brazil, using High-performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (HPLC-ESI-MS). The MeOH frac-

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tions of leaves and stem barks showed a high content of flavonoids (198.1 ± 106.50 and 542.7 ± 2.52 mg EqQ/g). The CH_2Cl_2 fraction of peels showed a high content of total phenolic compounds (516.7 ± 3.00 mg EqAG /g). The DPPH test showed that the CH_2Cl_2 fraction (leaves) held an EC_{50} of 0.08 ± 0.02, a higher value than that observed for the standards used in the test—Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), and ascorbic acid. The AcOEt and MeOH fractions of peels presented moderate cytotoxicity with values below 500 μg/mL. The MeOH fraction of leaves showed seven major compounds: myricetin, quercetin, quercetin-arabinofuranoside, apigenin-triglycosides, and apigenin-diglucoside, being the last three unpublished in studies involving the genus. The tests conducted in this study show the potential of *P. diversifolium* as a promising source of biomolecules with therapeutic applicability.

Keywords: *Pithecellobium*. Phytochemistry. Antioxidant potential. High performance liquid chromatography.

ATIVIDADE ANTIOXIDANTE, CITOTÓXICA, CONTEÚDO FENÓLICO E COMPOSIÇÃO FITOQUÍMICA DE *PITHECELLOBIUM DIVERSIFOLIUM* BENTH: UM ESTUDO PILOTO

Resumo

Espécies de *Pithecellobium* (Fabaceae) são usadas na medicina tradicional para tratar diabetes, tosse, bronquite e inflamação. Este estudo teve como objetivo avaliar o teor e determinar a atividade antioxidante, o teor de compostos fenólicos e a citotoxicidade do extrato e das frações de *Pithecellobium diversifolium*, uma pesquisa inédita com uma espécie exótica da Caatinga do Nordeste do Brasil, utilizando a instrumentação Clae-IES. As frações MeOH das folhas e cascas do caule apresentaram alto teor de flavonoides (198,1 ± 106,50 e 542,7 ± 2,52 mg EqQ/g). A fração CH_2Cl_2 das cascas apresentou um elevado teor de compostos fenólicos totais (516,7 ± 3,00 mg EqAG/g). O teste DPPH mostrou que a fração CH_2Cl_2 (folhas) apresentou um EC_{50} de 0,08 ± 0,02, valor superior ao observado para os padrões utilizados no teste – Butil hidroxianisol (BHA), Butil hidroxitolueno (BHT) e ácido ascórbico. As frações AcOEt e MeOH das cascas apresentaram citotoxicidade moderada com valores inferiores a 500 μg/mL. A fração MeOH das folhas apresentou sete compostos majoritários: miricetina, quercetina, quercetina-arabinofuranosídeo, apigenina-triglicosídeos e apigenina-diglucosídeo, sendo os três últimos inéditos em estudos envolvendo o gênero. Os testes realizados demonstram o potencial de *P. diversifolium*, uma promissora fonte de biomoléculas com aplicabilidade terapêutica.

Palavras-chave: *Pithecellobium*. Fitoquímica. Potencial antioxidante. Cromatografia líquida de alta eficiência.

ACTIVIDAD ANTIOXIDANTE, CITOTÓXICA, CONTENIDO FENÓLICO Y COMPOSICIÓN FITOQUÍMICA DE *PITHECELLOBIUM DIVERSIFOLIUM BENTH*: UN ESTUDIO PILOTO

Resumen

Las especies de *Pithecellobium* (Fabaceae) se utilizan en la medicina tradicional para tratar diabetes, tos, bronquitis e inflamación. Este estudio tuvo como objetivo evaluar el contenido y determinar la actividad antioxidante, el contenido de compuestos fenólicos y la citotoxicidad del extracto y de las fracciones de *Pithecellobium diversifolium*, un estudio inédito con una especie exótica de la Caatinga de la región Nordeste de Brasil, que utilizó la instrumentación HPLC-ESI. Las fracciones MeOH de hojas y cortezas de tallo mostraron un alto contenido de flavonoides (198,1 ± 106,50 y 542,7 ± 2,52 mg EqQ/g). La fracción CH_2Cl_2 de las cortezas presentó un alto contenido de compuestos fenólicos totales (516,7 ± 3,00 mg EqAG/g). El ensayo DPPH mostró que la fracción CH_2Cl_2 (hojas) tenía EC_{50} de 0,08 ± 0,02, valor superior a lo observado para los estándares utilizados en el ensayo –Butilhidroxianisol (BHA), butilhidroxitolueno (BHT) y ácido ascórbico. Las fracciones AcOEt y MeOH de las cortezas presentaron una citotoxicidad moderada con valores inferiores a 500 μ g/mL. La fracción MeOH de las hojas contiene siete compuestos principales: miricetina, quercetina, quercetina-arabinofuranosido, apigenina-triglucósidos y apigenina-diglucósido, de los cuales los tres últimos son inéditos en estudios sobre el género. Las pruebas realizadas demuestran el potencial de *P. diversifolium*, una fuente prometedora de biomoléculas con aplicabilidad terapéutica.

Palabras clave: *Pithecellobium*. Fitoquímica. Capacidad antioxidante. Cromatografía Líquida de Alta Eficiencia.

INTRODUCTION

Several plants species are sources for medicines and, for thousands of people, they are the only and main alternative in healthcare since they are easily accessible and culturally accepted in many communities[1]. Some of these species belong to the genus *Pithecellobium,* which encompasses 120 species[2] distributed across tropical regions of America, Asia, Oceania, and East Africa[3,4,5]. In Brazil, four species were catalogued: *Pithecellobium cochilocarpum*, *Pithecellobium racemosum*, *Pithecellobium roseum*, and *Pithecellobium diversifolium*[6,7,8,9].

Pithecellobium diversifolium is an endemic species of the Caatinga, popularly known as *carcarazeiro* and *brinco-de-sauim*, traditionally used to treat various diseases such

as diabetes, cough, bronchitis and inflammation[8,10]. However, the phytochemistry, biological activity, and cytotoxicity of the species are not widely investigated.

Several species of the genus *Pithecellobium* were previously studied, such as *P. cochliocarpum*, which presented saponins, flavonoids, and tannins in hydroalcoholic extracts from stem barks[7]. Leaves extracts of *Pithecellobium dulce* presented alkaloids, anthraquinones, flavonoids, tannins, and terpenoids[11]. In this context, phytochemical studies are necessary to identify the medicinally important compounds of *P. diversifolium*, improving data on abundance and diversity of chemicals present in the species. We highlight that the study of natural products is a starting point for the development of new drugs, offering several advantages such as richness in the variety of chemical structures, thus providing the opportunity to use these products as raw material in the synthesis of candidates for new drug prototypes. Moreover, this type of study contributes to the environmental preservation of an endemic species being studied in the state of Bahia and the semi-arid regions of Brazil.

Therefore, we performed, for the first time, a study on the extracts and fractions of *P. diversifolium* content to describe the chemical constituents and investigate the biological activities of the species.

MATERIAL AND METHODS

COLLECTION AND PROCESSING OF PLANT MATERIAL

Leaves and stem barks of *P. diversifolium* were collected from a 5 m tall tree in the municipality of Chorrochó, state of Bahia, Brazil. The botanical identity of the species was confirmed by a biologist, Dr. Daniel Salgado Pifano. A specimen of the plant was deposited at the *Núcleo de Ecologia e Monitoramento Ambiental* (NEMA – Ecology and Environmental Monitoring Center), under registration number 13598. The present study was registered at the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen – National System for the Management of Genetic Heritage and Associated Traditional Knowledge) under registration number A230F9E.

CRUDE ETHANOLIC EXTRACT (CEE) COLLECTION

The plant material was dried separately in an oven with circulating air at 40 °C for eight days, following the parameters suggested for plant material drying process[12]. Then, leaves and stem barks were pulverized.

The leaves and stem barks were placed in a percolator with 95% ethanol, replacing the ethanol every 72 h. The extractive solutions were filtered using a Solab® (SL-126) rotary evaporator at reduced pressure and at an average temperature of 50 °C to obtain the crude ethanolic extracts of leaves and stem barks.

PHYTOCHEMICAL SCREENING

Qualitative and preliminary phytochemical investigation was conducted to characterize the main groups of secondary metabolites present in the ethanolic extracts and fractions (hexane, dichloromethane, ethyl acetate, and methanol) of leaves and stem barks. The chromatographic profiles were obtained by thin-layer chromatography, using silica gel 60 G/UV 254 (5×6 cm) with aluminum support (Macherey-Nagel). The compounds were isolated from samples with highest levels of phytochemical classes.

QUANTIFICATION OF TOTAL PHENOLS

Spectrophotometric determination of phenolics using the Folin-ciocalteu reagent[13] (Sigma Aldrich®) was adopted to assess the antioxidant capacity. The extracts, fractions, and the gallic acid standard were evaluated in a concentration range of 50-100 mg/mL. Then, 200 μL of sodium carbonate solution were added and left to rest for 2 h. Absorbance was read at 765 nm on the UV-VIS spectrometer Shimadzu® (UV 1601). Results were expressed in milligrams of gallic acid equivalent per gram of extract or fraction (mg EAG/g). The experiments were performed in triplicate.

QUANTIFICATION OF TOTAL FLAVONOIDS

The total flavonoid content was determined by the colorimetric method[14] with a few adaptations. The experimental procedure consisted of dissolving the samples and the quercetin standard (Sigma Aldrich®) in methanol at concentrations ranging from 50 to 1000 mg/mL and adding 90 μL of a 5% $NaNO_2$ solution. The solutions were put to rest for 6 min, then 180 μL of a 10% $AlCl_3$ $6H_2O$ solution was added and left to rest again for 5 min. The volume was completed with 600 μL of 1 M NaOH and 330 μL of distilled water, followed by homogenization. The absorbance reading was measured against the blank, with description of absorbance versus concentration, in a ultraviolet-visible (UV-VIS) spectrophotometer. Analyses were performed in triplicate.

IN VITRO ANTIOXIDANT ACTIVITY

The quantitative evaluation of antioxidant activity was performed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich®) free radical scavenging method. It is an *in vitro* test used to evaluate the sequestering capacity of substances present in the extracts and fractions of leaves and stem barks. All experiments were performed in triplicate.

2,2-DIPHENYL-1-PICRYLHYDRAZYL FREE RADICAL SCAVENGING METHOD (DPPH)

Fractions and standards were diluted in ethanol at concentrations of 243, 81, 27, 9, 3, and 1 μg/mL. An ethanol solution of DPPH at 50 μg/mL was prepared and DPPH and ethanol were used as negative control. The standards used were L- ascorbic acid, butylhydroxytoluene (BHT), and butylhydroxyanisole (BHA). Absorbance values were measured at 518 nm in a UV-VIS spectrophotometer[13].

ANALYSIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION-MASS SPECTROMETRY (HPLC-ESI-MS)

The methanolic fraction of stem barks of *P. diversifolium* was analyzed by HPLC (Shimadu, Kyoto, Japan), coupled to an electrospray ionization (ESI) of a MicroToF II (Bruker Daltonics, Billerica, MA, USA). The parameters applied were: 4.5 kV capillary, negative mode ESI, 500 V final plate displacement, 40 psi nebulizer, dry gas (N_2) with an 8 mL/min flow rate, and a temperature of 200 °C.

The chromatographic method used acidified water (0.1% formic acid) (solvent A) and methanol (solvent B). Chromatographic separation was performed on a C-18 5 μm 100Å, 250×4.6 mm column (Kromasil, Bohus, Sweden). Injections (20 μL) were made using an automatic injector (SIL-10AF). Elution consisted of a linear exploratory gradient (5×100% B) for 60 min.

IN VITRO CYTOTOXIC ACTIVITY

The cytotoxic activity of the stem bark fractions was evaluated using an adapted version of the lethality test against *Artemia salina*[15].

A total of 4500 μg/L of fractions at concentrations of 1, 50, 100, 250, 500, and 1000 μg/L + artificial sea water were added to the test tubes. Paracetamol was used for positive control. After hatching of *A. salina cysts*, 10 nauplii were captured using a Pasteur pipette and transferred to tubes containing samples and saline water. The counting of live and dead nauplii occurred after 24 and 48 h. The experiments were performed in triplicate.

STATISTICAL ANALYSIS

The tests were performed in triplicate and the results were expressed as mean ± standard deviation, analyzed using the GraphPad Prism® 8.0.1 software program.

RESULTS AND DISCUSSION

PHYTOCHEMICAL SCREENING

The qualitative phytochemical screening of the crude ethanolic extract and fractions of leaves and stem barks identified the presence of phenolic compounds, alkaloids, coumarins, monoterpenes, sesquiterpenes, and diterpenes, as shown in **Table 1**. The classes of metabolites reported corroborate the phytochemistry presented for the genus. Flavonoids are the most investigated metabolites at genus level, with 23 substances isolated and identified in previous research, including quercetin, (+) – catechin, luteolin, and naringenin[16]. Flavonoids have aroused interest due to its various pharmacological properties, currently used as pigments, also presenting nutritional value, antitumor, anti-inflammatory, antioxidant, and antiviral properties, among others[12]. In a previous study, (+)-catechin, quercetin, luteolin, naringenin, quericitrin, and myricitrin were extracted from *P. clypearia*[9]. In the study, catechin-3'-*O*-gallate, catechin-4'-*O*-gallate, and gallocatechin-7,3'-di-*O*-gallate were isolated from *P. lobatum* leaves[9]. Quercetin is one of the most abundant flavonoids in vegetables and one of the most studied due to its biological activities. It has been demonstrated that quercetin induces a dose-dependent reduction in blood pressure, administered in several models of hypertension in mice[17].

Table 1 – Phytochemical characterization obtained from the crude ethanolic extract of fractions of leaves and stem barks of *P. diversifolium*.

Classes of metabolites	Stem barks					Leaves				
	CEE	Hex	CH_2CL_2	AcOEt	MeOH	CEE	Hex	CH_2CL_2	AcOEt	MeOH
General alkaloids	-	++	++	+	+	+++	+++	++	+	+
Anthocyanins	++	-	-	+	++	+	-	-	-	-
Phenolic compounds	+++	+	++	+++	++	+++	++	++	++	+++
Anthracene derivatives	+++	+	+	++	++	++	+	+++	++	+++
Cumarins	+	+++	+++	-	-	++	-	-	-	-
Lignanas	+++	++	++	++	++	++	-	-	-	-
Mono, sesqui ans diterpenes	-	-	-	-	-	-	+	-	-	-
Naphtoquinones	++	-	-	-	-	++	-	-	-	-
Saponins	+	+	+	+	-	+				
Triterpenes and steroids	-	-	-	+++	-	+	+	+	++	+++
Anthraquinone	+	-	-	+	+	+	-	-	-	-
Aglycones										
Condensed tannins	+	-	-	+	+	+	-	-	-	-
Hydrolysable tannin	+	+	+	+	++	+	-	-	-	-
Xanthines	-	-	-	-	-	+	-	-	-	-

Source: Elaborated by the authors.

CEE: crude ethanolic extract; Hex: hexanic fraction; CH2Cl2: dichloromethane fraction; AcOEt: ethyl acetate fraction; MeOH: methanolic fraction. (-) Not detected; (+) Low presence, (++) Moderate presence; (+++) Strong presence.

QUANTIFICATION OF TOTAL PHENOLS AND TOTAL FLAVONOIDS

The methanol fraction found a total phenol content of 108.5 ± 23.1 mg EqAG/g in leaves and a CH_2Cl_2 fraction of 516.7 ± 3.00 mg EqAG/g in stem barks (**Table 2**). These results indicate a high phenolic compounds content in stem barks, values that are more expressive or equivalent to the standard (gallic acid) at the same concentration. These values corroborate

results of the phytochemical screening, which found moderate to strong presence of phenolic compounds in the fractions.

The CH_2Cl_2 fraction of leaves (255.5 ± 5.29 mg EqQ/g) and methanol fraction of stem barks (542 ± 2.52 mg EqQ /g) showed the highest values of total flavonoids. These values confirm the presence of phenolic compounds in the CH_2Cl_2 fractions of leaves and in the methanol fraction of stem barks, detected in the phytochemical screening. The higher content of phenols and flavonoids in CH_2Cl_2 and MeOH fractions may be associated with moderate to high polarity of these fractions. The antioxidant activity of polyphenols and phenolic acids is associated with the capacity to donate a proton from the OH bond to form stable radicals by the effect of hyperconjugation or resonance. A pharmacological study confirmed that phenolic compounds from the hydroalcoholic extract of *P. dulce* fruits present gastroprotective effect by inhibiting the enzyme H+, K + -ATPase[18]. Furthermore, pelargonidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside were associated with a lower risk of diabetes[4]. Previous studies with rodents attested that polyphenols present in species of the genus *Vitis* delayed the onset of rheumatoid arthritis symptoms, reduced inflammatory mediators, and blocked signaling pathways[19]. The significant level of polyphenols in *P. diversifolium*, an endemic species located in the territory of Bahia, highlights the importance of the state's biodiversity, with potential for development of patents, drug prototypes, and dietary and food sources that can provide quality of life and well-being to the population.

Table 2 – Determination of total phenol content (mg EqAG/g) and total flavonoid content (mg EqQ/g) of the fractions obtained from the leaves and stem bark of *P. diversifolium*.

Samples	Stem barks	Leaves	Stem barks	Leaves
	TP (mg EqAG/g)	TP (mg EqAG/g)	TF (mg EqQ/g)	TF (mg EqQ/g)
Hex	-	41.8 ± 76.0	-	-
CH2CL2	516.7 ± 3.00	59.2 ± 26.1	455 ± 4.14	255.5 ± 5.29
AcOEt	216.0 ± 4.46	74.5 ± 35.5	534 ± 5.28	161.9 ± 3.67
MeOH	206.8 ± 5.07	108.5 ±23.1	542 ± 2.52	198.1 ± 10

Source: Elaborate by the authors.
TF: total flavonoids. TP: Total phenolics. EqQ: Quercetin equivalent. EqAG: gallic acid equivalent. Values are presented as mean ± SD (n=3). -: not determined.

ANTIOXIDANT ACTIVITY ANALYSIS

2,2-diphenyl-1-picrylhydrazyl free radical scavenging method (DPPH•)

The CH_2Cl_2 fractions of leaves and the methanol fraction of stem barks showed EC_{50} values of 0.08 ± 0.02 and 0.60 ± 0.27, respectively, meaning that the values of antioxidant activity are higher than the standards used in the tests (**Table 3**). In comparison with the data presented, a study[20] indicated that the aqueous and methanol extracts of *Pithecellobium dulce* seeds can neutralize radicals at a concentration of 1000 μg/ml, with values of 81.95% and 85.41%, respectively, showing more expressive results than the BHT (78.48%). We analyzed the main biological activities reported for the genus. Treatment with aqueous extract of *P. dulce* showed a protective effect on kidney tissue after exposure to toxins, in addition to the capacity to eliminate free radicals[21]. Exogenous antioxidants are fundamental for combating oxidative stress due to their capacity to donate electrons and hydrogen[22].

Table 3 – *In vitro* antioxidant activity of Hex, CH_2Cl_2, AcOEt, and MeOH fractions from leaves and stem barks of *P. diversifolium*

Samples	Stem DPPH (IC50, μg/ml)	Leaves DPPH (IC50, μg/ml)
Hex	34.91 ± 0.00	-
CH_2CL_2	-	0.08 ± 0.02
AcOEt	0.76 ± 0.21	0.59 ± 0.31
MeOH	0.60 ± 0.27	77.5 ± 4.61
BHA	3.93 ± 0.12	3.93 ± 0.12
BHT	12.82 ± 0.27	12.82 ± 0.27
Ascorbic acid	4.21 ± 0.44	4.21 ± 0.44

Source: Elaborated by the authors
DPPH: 2,2-diphenyl-1-picrylhydrazyl free radical. Values are presented as mean ± SD (n=3). -: not determined.

Analysis by High-performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (HPLC-ESI-MS)

HPLC-ESI-MS identified five major compounds in the methanol fraction of *P. diversifolium* leaves. Identification was based on retention time and on comparison with literature data.

The chromatogram of the methanol fraction showed 13 peaks (**Table 4** and **Figures 1** and **2**), making it possible to identify five substances. Compounds 2-7 were identified as being glycosylated flavonoids. Substances 2 (rt. 30.4 min m/z 755.1582), 3 (rt. 32.4 min m/z 739.1626),

and 7 (rt. 38.5min m/z 723.4554) are, respectively, myricetin, quercetin, and apigenin-triglycoside. Flavonoids present in the genus present diverse structures that are subdivided into six main skeletons: flavones, flavanones, flavans, flavan-3-ol, flavan-3-ol derivatives, and anthocyanidins. There is a certain substitution pattern for glycosylated flavonoids in species of the genus. These conjugated forms can be called heterosides, comprising O-heterosides, when the bond is established via the hydroxyl group, and C-heterosides, when the bond is established via carbon[12]. A study described the isolation of myricetin and quercetin extracted from leaves and branches of *P. clypearia*[16]. Quercetin is a potent antioxidant polyphenol, with pharmacological and economic importance[23]. In absence of a glycidic group, the flavonoid is called an aglycone[24]. Glycosylation influences the absorption of flavonoids by cell membranes, as well as the number of hydroxyls in the molecule.

Compound 4 (rt. 33.4 min m/z 433.1406) was attributed to quercetin-arabinofuranoside ($C_{20}H_{18}O_{11}$) and compound 6 (rt. 37.3 min m/z 593.1118) as being apigenin-diglucoside ($C_{27}H_{30}O_{15}$). Compounds 8-13 presented ions with m/z greater than 900 Da and, considering the chemistry reported for the Fabaceae family and the molecular formulas compatible with substances composed of C56 (or more), these compounds are believed to be saponins. Previous reports present no data on isolation of saponins in leaves and stem barks for *Pithecellobium* species. However, several studies identified saponins in seeds and roots of the genus species[2, 25, 26, 27]. Geographic distribution and seasonality can influence the distribution of metabolites between species[12].

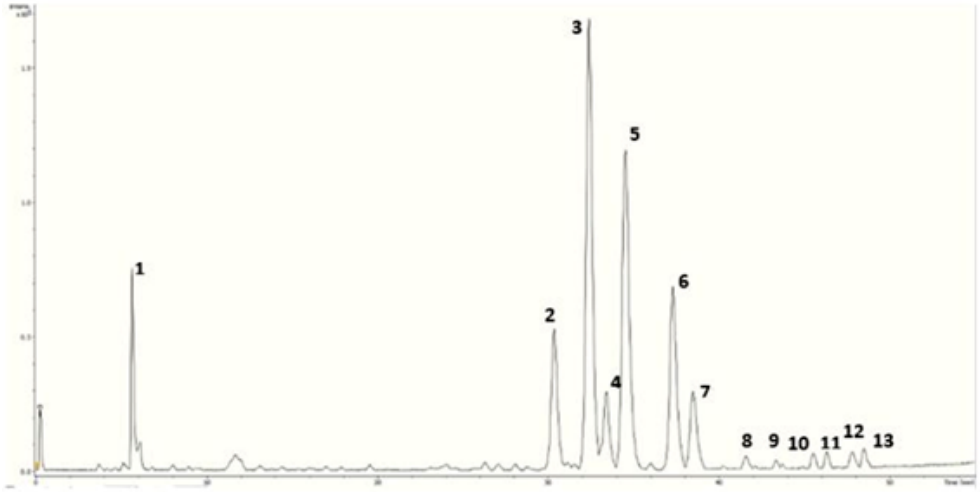
The major substance present in the methanol fraction of leaves was identified as quercetin. At genus and at species level, this study describes, for the first time, the isolation of glycosylated flavonoids: apigenin-triglycoside (7), apigenin-diglucoside (6), and quercetin-arabinofuranoside (4).

Table 4 – Substances identified by HPLC-ESI-MS

Peak	Substance identified	Retention time (minutes)	Mass/Charge	Molecular Formula
1	-	-	-	-
2	Miricetin	30.4 min	755.1582	$C_{15}H_{10}O_8$
3	Quercetin	32.4 min	739.1626	$C_{15}H_{10}O_7$
4	Quercetin-arabinofuranoside	33.4 min	433.1406	$C_{20}H_{18}O_{11}$
5	-	-	-	-
6	Apigenin-diglucoside	37.3 min	593.1118	$C_{27}H_{30}O_{15}$
7	Apigenin-triglycoside	38.5 min	723.4554	$C_{30}H_{34}O_{22}$

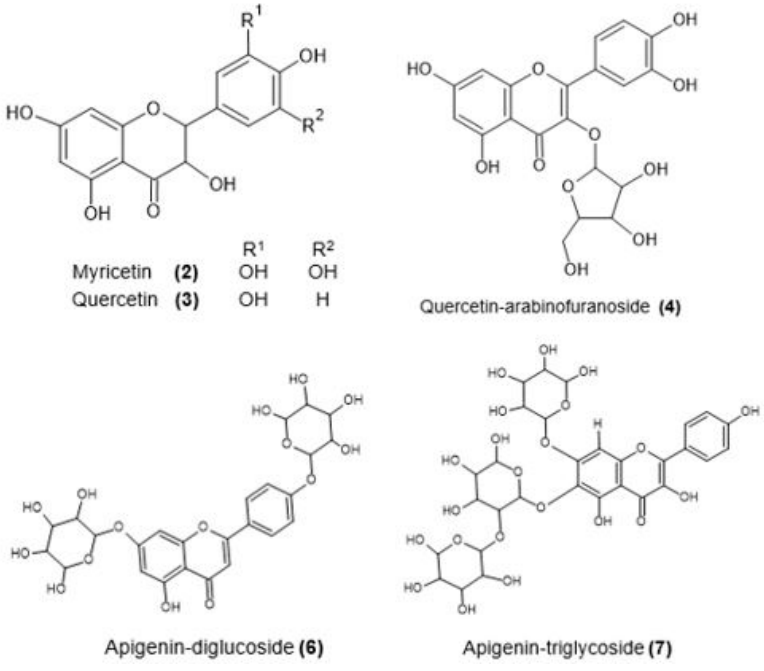
Source: Elaborated by the authors.
-: not determined; min: minutes.

Figure 1 – Chromatogram of the base peak, acquired in negative mode, of the methanolic fraction of the *P. diversifolium* leaf extract



Source: Federal University of Paraíba (UFPB).

Figure 2 – Molecules identified by HPLC-ESI-MS.



Source: Elaborated by the authors.

In vitro cytotoxic activity

The toxicity was measured by counting the number of dead nauplii in the period of 24 and 48 h. The LC_{50} values for the CH_2Cl_2, ethyl acetate, and methanol fractions in the period of 24 h were 2823, 210.1, and 364.4 μg/ mL, respectively. After 48 hours, the values were 1742, 206.2, and 342.1 μg/ ml, respectively (**Table 5**). Therefore, according to the tests, the dichloromethane fraction is non-toxic with a LC_{50} > 1000 μg/ mL. The ethyl acetate and methanolic fraction presented moderate toxic activity. The moderate cytotoxicity against *A. salina* suggests that AcOEt and MeOH fractions may have trypanosomicidal, antifungal, and antibacterial properties[28]. The genus *Pithecellobium* is little investigated in Toxicology. However, a study indicated that *P. dulce* leaves extract exhibited moderate cytotoxicity with an EC_{50} of 126 μg.mL^{-1} against the Caco-2 enterocyte cell line[29]. In the lethal concentration test (LC_{50}) against *Artemia salina*, the ethanolic extract of *P. cochliocarpum* stem barks exhibited weak toxicity, with a LC_{50} of 543.5 μg.mL^{-1} [7]. Studies report a good correlation between the assay with *A. salina* and cytotoxicity tests against human cell lines[30]. In recent years, the use of this microcrustacean has been increasing as an alternative method to the use of animal models, such as mice. It is a preliminary method of general toxicity, as required by the scientific community.

Table 5 – Lethal concentration 50 (LC_{50}) in the 24- and 48-hour period

CL_{50} (μg/ml)		
Fractions	**24 hours**	**48 hours**
CH_2CL_2	2823	1742
AcOEt	210.1	206.2
MeOH	364.4	342.1

Source: Elaborated by the authors.
Values are presented as mean ± SD (n=3).

CONCLUSION

This report is a lead study of *Pithecellobium diversifolium,* species from the Brazilian semi-arid region. The study contributes with theoretical foundations for ongoing pharmacological research and quality control of the species, as well as ethnobotanical and environmental conservation work.

In this study, five flavonoids (myricetin, quercetin, apigenin-triglycoside, quercetin-arabinofuranoside, and apigenin-diglucoside) were isolated from methanol extract

of *P. diversifolium* leaves and were identified by HPLC-ESI-MS. This result confirms the content of flavonoids and total phenols presented in the methanol extract.

The antioxidant activity indicated that the CH_2Cl_2 fractions of leaves and methanol fraction of stem barks have a good capacity to scavenge the DPPH free radical, with EC_{50} values greater than the adopted standards. The total phenolic content test conducted with the methanol fraction of leaves and CH_2Cl_2 fraction of stem barks revealed a strong presence of total phenols, with more expressive values than standard (gallic acid) at the same concentrations, indicating a possible potential to reduce the damage caused by free radicals. The CH_2Cl_2 fraction of leaves and methanol fraction of stem barks showed the highest levels of total flavonoids. The cytotoxicity assay with *Artemia salina* indicated that the CH2Cl2 and methanol fractions presented moderate toxic activity.

Therefore, *P. diversifolium* species is a promising source of biomolecules with therapeutic potential.

ACKNOWLEDGMENTS

The authors would like to thank the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES – Coordination for the Improvement of Higher Education Personnel) – Financing Code Number 88887.499311/2020-00 and the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq – National Council for Scientific and Technological Development) for the financial support. We would also like to thank teacher Jackson Roberto Guedes da Silva Almeida for his contributions to this study and Cristiane Cerqueira for the grammar review of the manuscript.

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Received on: 5.30.2023. Approved on: 12.4.2023. Published on: 31.01.2024