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CHEMICAL CONSTITUENTS AND IN VIVO PRELIMINARY EVALUATION OF THE TOXICOLOGICAL ACTIVITY OF *Ouratea spectabilis* (OCHNACEAE) AND *Clitoria guianensis* (FABACEAE) LEAVES

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Abstract

Clitoria guianensis and *Ouratea spectabilis*, found in the Brazilian Cerrado, are used in folk medicine, despite the few chemical and biological studies reported in the literature. The present study aims to investigate the toxicity and effect of extracts from both species on the microcrustacean *Artemia salina*, and to determine the chemical composition of the hexane extract of *O. spectabilis* leaves and the EtOAc fraction of *C. guianensis* leaves. Kaempferitrin, a flavonoid isolated from of the EtOAc fraction of *C. guianensis* leaves, was identified by chemical analysis. Analysis of the hexane extract of *O. spectabilis* leaves using gas chromatography-mass spectrometry (GC-MS) suggested the presence of twenty-five known substances. The Hex, EtOAc, and EtOH crude extracts of *C. guianensis* leaves exhibited high and moderate toxicity against *Artemia salina*, with median lethal dose values (LD_{50}) of 43.7, 25.4, and 233.4 mg.L^{-1}, respectively. The acetone extract of *O. spectabilis* leaves showed moderate toxicity against *Artemia salina* with an LD_{50} value of 115.13 mg.L^{-1}.

Keywords: *Artemia salina.* Cerrado. Kaempferitrin.

1. Introduction

The Cerrado is the second-largest biome in Brazil and has the richest biodiversity among the tropical savannahs on the planet. There are 11.627 species of cataloged native vegetation, with new species being discovered each year, corresponding to approximately 5% of all the world's diversity. The sources of the three largest hydrographic basins in South America, i.e., the Amazon Basin, Prata Basin, and São Francisco River Basin, are located in the Cerrado. Due to its natural wealth, the Cerrado is now considered a global biodiversity hotspot that is threatened by agricultural expansion (Estrabis et al. 2019; Colli et al. 2020; Neto et al. 2020).

Despite the great diversity present in the Cerrado, the chemical composition of many plant species remains underexplored, highlighting the potential of this biome. These species include *Ouratea spectabilis* from the Ochnaceae family and *Clitoria guianensis*, belonging to the Fabaceae family.

O. spectabilis is a tree species popularly known as saw leaf. The leaves are traditionally used in the treatment of gastric, stomach, and vermifuge-induced discomfort. Among the classes of metabolites found

in the genus *Ouratea*, terpenoids, lignoids, monosaccharides, triglycerides, depsides, and biflavonoids, which are abundant in these plants, are considered chemotaxonomic markers of this genus (Fidelis et al. 2014; Rocha et al. 2020).

The biflavonoids (6,6-)-bigenkwanin and (7,7")-dimethoxyagatisflavone isolated from *O. spectabilis* leaves exhibit inhibitory activity on the production of aflatoxins B_1 and B_2 in *Aspergillus flavus* fungi (Gonçalez et al. 2001). These biflavonoids also show inhibitory activity against aldose reductase, an enzyme associated with the onset of cataract formation in patients with diabetes (Simoni et al. 2002).

Assays performed with ethanol extracts of *O. spectabilis* leaves and bark showed antiviral activity against human herpes virus type 1 (HSV-1) and inhibitory activity against the production of TNF-α in monocytic cells activated by lipopolysaccharide (LPS) THP-1 (Brandão et al. 2011; Campana et al. 2015). Furthermore, the hydroethanolic extract and the ethyl acetate fraction of *O. spectabilis* showed phytotoxic activity, causing a decrease in the germination rate of lettuce seeds (Mecina et al. 2014).

The species *C. guianensis* grows as a sub-shrub and is commonly known as "vergateza". The roots are popularly used in the form of a decoction or "garrafada" (combinations of medicinal plants used in alcoholic beverages) as an aphrodisiac and to treat mental disorders (Verde 2003; Souza and Felfili 2006).

In a phytochemical study of *C. guianensis* roots, flavanones, isoflavones, rotenoids, and phenolic glycosides were isolated (Cunha et al. 2020). Ethyl acetate extracts of the leaves and roots of *C. guianensis* showed antioxidant activity, with a high capacity to inhibit 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and high toxicity against the microcrustacean *Artemia salina* (Cunha et al. 2020; Soares et al. 2020).

The present study aims to investigate the toxicity of extracts and fractions of *O. spectabilis* and *C. guianensis* against the microcrustacean *Artemia salina*. The chemical composition of extracts of both species, as well as that of the hexane extract of leaves of *O. spectabilis* and the EtOAc fraction of *C. guianensis* leaves, is analyzed by GC-MS.

2. Material and Methods

Plant material

Samples of *C. guianensis* were collected in Gurupi (11°43'S, 49°15'W), Tocantins, Brazil, in November 2013, and identified by Prof. Rodney Haulien Oliveira Viana as *Clitoria guianensis* (Aubl.) Benth var. *guianensis*. A voucher sample (10.637) was deposited at the herbarium of Tocantins (HTO), Porto Nacional, TO, Brazil. The materials were separated according to the plant parts and dried (at 45 °C).

O. spectabilis leaves were collected from previously identified species within the Gurupi campus of the Federal University of Tocantins (11°43 S, 49°15'W), Tocantins, Brazil, in November 2018. After collection, the leaves (252 g) were separated, washed with distilled water, and dried completely.

Extraction and isolation

The leaves (194.1 g) of *C. guianensis* were ground and exhaustively extracted (thrice) with ethanol (3 × 200 mL) at room temperature. The plant material remained immersed in the solvent for 7 d and was manually shaken every 12 h for 2 min. The crude EtOH extract (29.8 g) was solubilized in a mixture of MeOH:H_2O (250 mL, 1:1 v/v) and then subjected to liquid-liquid partitioning with Hex and EtOAc. The solvents were removed under reduced pressure to give the EtOAc (13.8 g) and Hex (3.6 g) fractions.

The EtOAc fraction (2.0 g) was subjected to column chromatography (CC) (2.3 × 29.0 cm, silica gel eluted with a gradient of $CHCl_3$ → 100% MeOH to give 17 subfractions (ca. 25 mL each; SFr1-SFr17)). The SFr13 subfraction (28.5 mg) was washed with $CHCl_3$ to produce flavonoids.

Dried samples of *O. spectabilis* leaves were extracted by maceration at room temperature in hexane, acetone, and ethanol (2 × ~ 200 mL, 48 h, and shaken manually every 12 h for 2 min for each extraction). The solvents were removed under reduced pressure to give the remaining acetone (1.1 g), hexane (0.6 g), and ethanol (0.57 g) fractions.

Characterization by GC-MS

The composition of the hexane extract was determined by gas chromatography-mass spectrometry (GC-MS). Portions (0.5 mg) of the dried hexane extract were dissolved in GC-hexane (1 mL), filtered through a PVDF 0.45 µm membrane, and analyzed by GC-MS (Shimadzu QP2010) coupled to an AOC-20i mass spectrometer (GC-MS) equipped with a Phenomenex ZB-5HT capillary column (30 m x 0.25 µm x 0.25 mm). The injector temperature was maintained at 260 °C, and the oven temperature was ramped from 140 °C to 320°C at a rate of 3 °C.min^{-1}. The GC-MS was operated in the electronic ionization mode at 70 eV, with the transfer line maintained at 250 °C. Helium (1.0 mL.min^{-1}) was used as the carrier gas. The retention indices for all compounds were determined according to the equation proposed by van Den Dool and Kratz (1963), using *n*-alkanes as standards. The components were identified by comparing their mass spectra and retention times with those in the NIST/EPA/NIH mass spectral database.

Structural elucidation of kaempferitrin

One-dimensional (^{1}H, ^{13}C, and TOCSY) and two-dimensional (HSQC and HMBC) NMR experiments were performed on a Bruker Avance™ III 600 spectrometer (14.1 T). The experiments were performed at 600 MHz (^{1}H) and 151 MHz (^{13}C). Deuterated solvents ($CDCl_3$, CD_3OD, and dimethyl sulfoxide (DMSO-d_6)) (99.98% D) were used as internal standards for calibrating the ^{13}C NMR chemical shifts, and the residual solvent was used as an internal standard for ^{1}H NMR. The δ values are reported relative to those of Me_4Si.

Toxicity testing using *Artemia salina*

The lethality test was performed according to the McLaughlin method (McLaughlin et al. 1998; Meyer et al. 1982). *Artemia salina* eggs were incubated with saline solution (NaCl = 38 g.L^{-1}) under constant oxygenation for 24 h.

After the eggs hatched, 10 units of nauplii were added to test tubes containing crude extracts of the EtOH, Hex, and EtOAc fractions of *C. guianensis* leaves and acetone extract of *O. spectabilis* leaves solubilized in saline solution containing 1% dimethyl sulfoxide (DMSO). Each sample was tested in triplicate. The crude EtOH extract of *C. guianensis* was prepared at concentrations of 100, 75, 35, and 20 mg.L^{-1}, EtOAc fractions were prepared at concentrations of 75, 50, 35, and 20 mg.L^{-1}, and the Hex fraction was prepared at concentrations of 500, 200, 50 and 10 mg.L^{-1}, while the acetone extract of *O. spectabilis* was prepared at concentrations of 150, 140, 120, 110, and 70 mg.L^{-1}.

For each concentration, triplicate samples of the control solution containing saline solution with 1% DMSO were made and the nauplii were added. The surviving nauplii were counted after 24 h of incubation. The collected data were computerized, and the median lethal dose (LD_{50}) was determined by probit analysis.

3. Results and Discussion

The crude EtOH extract of *C. guianensis* leaves was partitioned with *n*-hexane and ethyl acetate. Chromatographic fractionation of the EtOAc fraction resulted in isolation of the diglycosylated flavonoid, which was isolated for the first time in the genus *Clitoria*.

GC-MS analysis of the hexane extract of *O. spectabilis* leaves (Figure 1 and Table 1) suggested the presence of 25 substances out of the 55 detected, with palmitic acid (17.83 %), sitostenone (10.80 %), nonacosane (10.74 %), hentriacontane (8.23 %), tricosane (5.96 %), docosane (5.18 %), and heneicosane (5.05 %) as the main compounds.

Among the identified metabolites, fifteen *n*-alkanes were identified, which are known to confer hydrophobic properties to the epicuticular wax of plants. These waxes are responsible for reducing the dehydration of leaves by evaporation and increasing the efficiency of water use in water-deficient environments (Bush and McInerney 2013). Ketones, carboxylic acids, and esters were also observed.

Tocopherol, palmitic acid methyl ester, and tetracosane were previously isolated from *O. parviflora* and *O. nitida*, respectively (Estevam et al. 2005; Araujo et al. 2011). This study presents the first report of the chemical profile of the hexane extract of *O. spectabilis*.

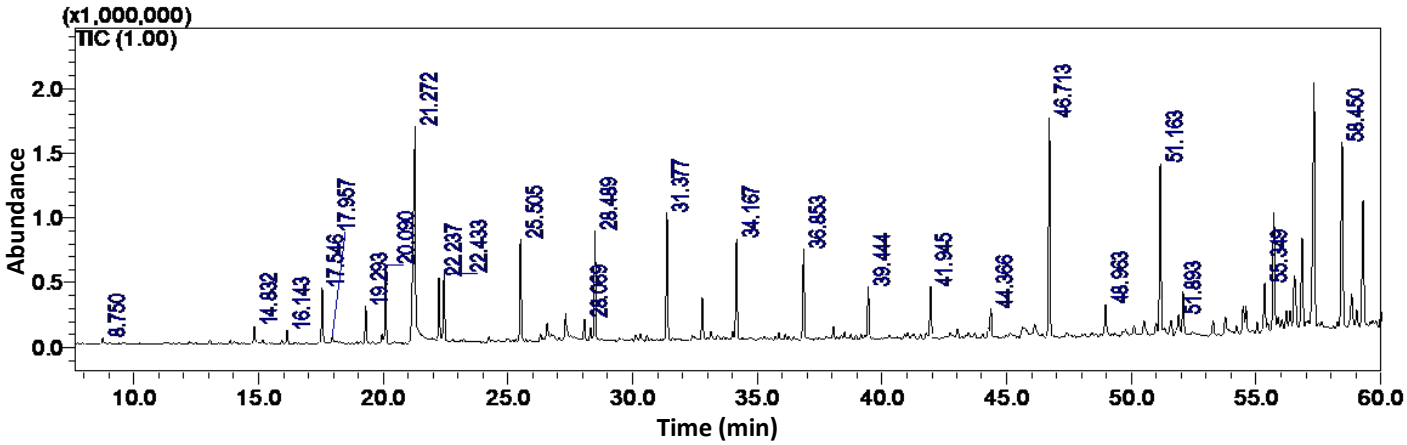

Figure 1. Total ion chromatogram (TIC) of hexane extract of *O. spectabilis* leaves.

Table 1. Phytochemical constituents identified in the hexane extract of *O. spectabilis* leaves using gas chromatography-mass spectrometry.

Peaks	Time (min)	Area %	Identification Proposal			
			IR_{cal}	IR_{lit}	Similarity %	Compound
1	8.750	0.25	1544	1548	85	Dihydroactinidioide
2	14.832	0.88	1757	1769	95	Tetradecanoic acid
3	16.143	0.61	1800	1800	91	Octadecane
4	17.546	2.41	1844	1846	96	Hexahydrofarnesyl acetone
5	17.957	0.35	1857	1857	88	Pentadecanoic acid
6	19.293	1.62	1900	1900	94	Nonadecane
7	20.091	3.41	1925	1926	96	Palmitic acid methyl ester
8	21.272	17.83	1963	1963	95	Palmitic acid
9	22.237	2.71	1994	1994	94	Ethyl palmitate
10	22.433	3.01	2000	2000	94	Eicosane
11	25.505	5.05	2100	2100	94	Heneicosane
12	28.069	1.06	2186	2180	85	Octadecanoic acid
13	28.489	5.18	2200	2200	95	Docosane
14	31.377	5.96	2300	2300	94	Tricosane
15	34.167	4.75	2400	2400	95	Tetracosane
16	36.853	4.27	2500	2500	94	Pentacosane
17	39.444	2.30	2600	2600	95	Hexacosane
18	41.945	2.21	2700	2700	95	Heptacosane
19	44.366	1.39	2800	2800	94	Octacosane
20	46.713	10.74	2900	2900	95	Nonacosane
21	48.963	1.70	3000	3000	95	Triacontane
22	51.163	8.23	3100	3100	94	Hentriacontane
23	51.893	0.88	3135	3138	80	Tocopherol
24	55.349	2.40	3300	3300	94	Tritriacontane
25	58.450	10.80	3458	3458	91	Sitostenone

By analyzing the ^{1}H NMR data and correlations in the HMBC contour map and comparing the experimental data with the spectroscopic data reported in the literature, the flavonoid kaempferol-3,7-*O*-dirhamnoside or kaempferitrin (Figure 2) was identified (Valente et al. 2009).

Kaempferitrin has been shown to have important biological effects, such as antibacterial, antiparasitic, antifungal, immunostimulant, hypoglycemic, anti-inflammatory, anticancer, antidepressant, and anti-osteoporosis activities (Hernández et al. 2017).

Bioassays with *A. salina* can be used to screen and discover natural compounds with bioactivity against human carcinomas, because the ED_{50} values (median effective dose) for cytotoxicity against cancer

cells correspond to approximately 1/10 of the LD_{50} values (mean lethal dose) obtained in bioassays with *A. salina* (Mclaughlin et al. 1998). The results of toxicity tests using *A. salina* are shown in Table 2.

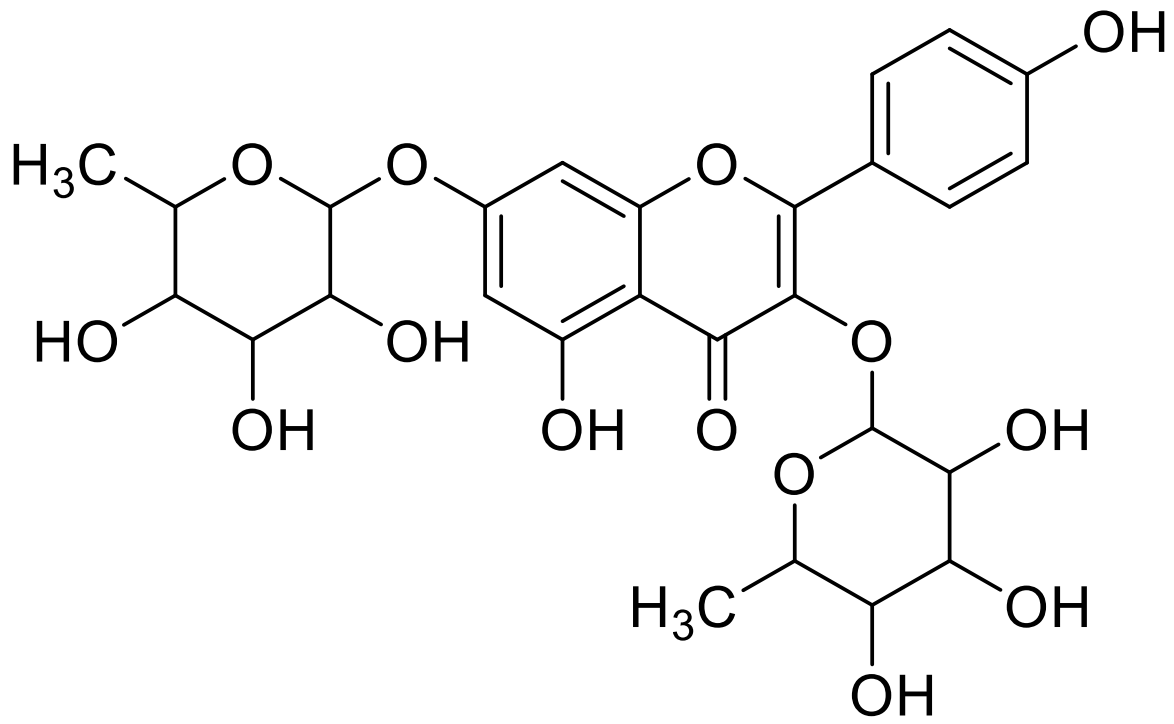

Figure 2. Chemical structure of kaempferitrin.

Table 2. Toxicity of crude EtOH extract and Hex and EtOAc fractions of the leaves of *C. guianensis* and acetone extract of the leaves of *O. spectabilis* towards *A. salina.*

Test solution	Concentration ($mg.L^{-1}$)	Log C	Mortality (%)	LC_{50} ($mg.L^{-1}$)
Crude ethanol extract of leaves of *C. guianensis*	20	1.301	3.7	233.42
	35	1.544	18.6	
	75	1.875	30.0	
	100	2.000	33.3	
Ethyl acetate fraction of leaves of *C. guianensis*	20	1.301	29.7	25.44
	35	1.544	75.0	
	50	1.699	96.3	
	75	1.875	100.0	
Hexane fraction of leaves of *C. guianensis*	10	1.000	29.2	43.67
	50	1.699	41.7	
	200	2.301	77.8	
	500	2.699	100.0	
Acetone extract of leaves of *O. spectabilis*	70	1.845	6.2	115.13
	110	2.041	45.8	
	120	2.079	50.0	
	140	2.146	62.4	
	150	2.176	81.8	

The LD_{50} values for the crude EtOH extract and the Hex and EtOAc fractions from *C. guianensis* leaves were 233.4, 43.7, and 25.4 $mg.L^{-1}$, respectively. The acetone extract of *O. spectabilis* leaves had a LD_{50} value of 115.13 $mg.L^{-1}$.

The Hex and EtOAc fractions from *C. guianensis* leaves are considered highly toxic (LD_{50} <100 $mg.L^{-1}$), whereas the crude EtOH extract from *C. guianensis* leaves has moderate toxicological activity (100 $mg.L^{-1}$<LD_{50} <500 $mg.L^{-1}$), according to the classification of Nguta et al. (2012), suggesting the potentially high bioactivity of the components. Compared with toxicity tests performed previously with *Clitoria* species, the results are promising, considering that in a study carried out with *C. guianensis* roots, the

crude EtOH extract and Hex and EtOAc fractions exhibited high toxicological activity, with LD_{50} values of 23.44, 41.16 and 8.53 $mg.L^{-1}$, respectively (Cunha et al. 2020). The crude MeOH extract and dichloromethane and methanol fractions from *C. ternatea* leaves had LC_{50} values of 25.82, 31.55, and 22.28 $mg.L^{-1}$, respectively, also demonstrating high toxicity against *Artemia salina*. The hexane fraction of the leaves and crude MeOH extracts from the seeds and stem bark of *C. ternatea* had moderate toxicological activity, with LC_{50} values of 115.24, 110.92, and 179.89 $mg.L^{-1}$, respectively (Rahman et al. 2006). The crude MeOH extracts of *C. fairchildianta* seeds and roots also had LD_{50} values of 315 and 158 $mg.L^{-1}$, respectively (Santos et al. 2016).

The acetone extract of *O. spectabilis* leaves had moderate toxicological activity, surpassing the result obtained for the species *O. nitida* (LD_{50} 2083 $mg.L^{-1}$) (Coe et al. 2010).

4. Conclusions

Twenty-five substances were identified via GC-MS characterization of the chemical composition of the hexane extract of leaves of the species *O. spectabilis*. Chromatographic fractionation of the EtOAc extract of the leaves of *C. guianensis* resulted in the isolation of the flavonoid kaempferitrin, which is the first report of isolation of this compound from the genus *Clitoria*. The Hex and EtOAc fractions from the leaves of *C. guianensis* showed high toxicity, whereas the crude EtOH extract of *C. guianensis* and the acetone extract of the leaves of *O. spectabilis* showed moderate toxicity against *A. salina*, suggesting the presence of bioactive compounds.

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