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Artigo

PHYTOCHEMICAL STUDY, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *Stemodia maritima*

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Stemodinol, a new natural compound, together with known compounds including jaceidin, stemodin, stemodinoside B, isocrenatoside, verbascoside, crenatoside, and isoverbascoside, were isolated from *Stemodia maritima* Linn. The antioxidant (DPPH method) and antimicrobial activities of stemodin, stemodinoside B, and crenatoside were investigated. Among the components tested, only crenatoside isolated from the roots showed a high antioxidant power. Stemodin and stemodinoside B exhibited antibacterial activities.

Keywords: *Stemodia maritima* Linn; stemodinol; Scrophulariaceae.

INTRODUCTION

The genus *Stemodia* (Scrophulariaceae) is represented by annual herbs and shrubs that occur in Asia, Africa, Australia, and America.[1] The species *S. foliosa* is source of antibacterial labdane diterpenoids,[2] although diterpenes containing stemodane, an unusual tetracyclic skeletal compound, seem to be the most characteristic compounds of *Stemodia*.[3] Earlier chemical studies on *Stemodia maritima* Linn, which is used for the treatment of venereal disease, stomach aches, edema, and swelling,[1,2] reported the isolation of diterpenes, flavonoids, and other compounds showing antiviral, cytotoxic, and larvicidal activities.[4-6]

This paper describes the isolation and structural elucidation of a new natural compound named stemodinol (**2**) along with seven known compounds using 1D (^{1}H- and ^{13}C-NMR, DEPT-135) and 2D (^{1}H-^{1}H-COSY, ^{1}H-^{1}H-NOESY, HMQC, HMBC) NMR and HR-ESI-MS spectroscopies. In addition, the antioxidant and antimicrobial activities of stemodin, stemodinoside B, and crenatoside isolated from *Stemodia maritima* Linn are determined.

RESULTS AND DISCUSSION

Chemistry

The phytochemical study of *Stemodia maritima* Linn afforded a new natural compound, stemodinol (**2**), along with seven known compounds: jaceidin (**1**),[7] stemodin (**3**),[8] stemodinoside B (**4**),[5] isocrenatoside (**5**),[9] verbascoside (**6**),[10] crenatoside (**7**),[9] and isoverbascoside (**8**).[11] Stemodinol (**2**) was obtained as an amorphous white solid (Figure 1). The molecular formula $C_{20}H_{34}O_2$ was established by HR-ESI-MS, which showed a peak related to the sodium adduct of the molecule at *m/z* 329.2450 $[M+Na]^+$ (calculated *m/z* 329.2456 $[M+Na]^+$).

The presence of a hydroxyl absorption (ν_{max} 3370 cm^{-1}) band was inferred from its IR spectrum. Comparative analysis of the {^{1}H}- and DEPT 135°-^{13}C-NMR spectra (Table 1) revealed 20 signals corresponding to the presence of four non-protonated (all sp^3, including one oxygenated at δ_C 72.59, C-13), three methine (all sp^3), ten methylene (all sp^3, including one oxygenated at δ_C 71.94, $HOCH_2$-18) and three methyl carbon atoms; these results are compatible with the expanded molecular formula $(C)_3(C\text{-}OH)(CH)_3(CH_2)_9(CH_2OH)(CH_3)_3 = C_{20}H_{34}O_2$.

The absence of signals corresponding to sp^2 carbon atoms and the aforementioned data are in accordance with a tetracyclic stemodane-type diterpene containing two hydroxyl groups and some similarities with the diterpene aphidicolin.[1] This result is further corroborated by the compound's ^{1}H-NMR spectrum (Table 1), which showed the presence of three methyl group represented by singlet signals at δ_H 1.31 (3H-17), 1.02 (3H-20), and 0.87 (3H-19), two methylene groups at δ_H 2.75 (d, J = 11.3 Hz, H-16a) and δ_H 1.42 (m, H-16b), in accordance with the methylene envelope of the stemodane skeleton, an another oxygenated methylene group at δ_H 3.66 (dd, J = 12.0 and 3.0 Hz) and 3.32 (dd, J = 12.0 and 3.0 Hz). The last group was also confirmed by the analysis of its NMR ^{1}H-^{1}H-COSY spectrum.

The location of the hydroxyl groups was verified though HMBC analysis (Table 1), which revealed long-range correlations from methylene hydrogens at δ_H 3.32 and 3.66 ($HOCH_2$-18) with CH_3-19 (δ_C 18.9, $^3J_{CH}$), CH_2-3 (δ_C 36.8, $^3J_{CH}$), and CH-5 (δ_C 42.7, $^3J_{CH}$). Additional correlations involving the hydrogen signal at δ_H 1.31 (3H-17, methyl group) with the carbon signals at δ_C 40.5 (CH_2-12, $^3J_{CH}$), 49.6 (C-14, $^3J_{CH}$), and 72.6 (O-C-13, $^2J_{CH}$) were also observed, allowing the two hydroxyl groups to be placed at the C-18 and C-13 positions. Additional and important heteronuclear long-range couplings are summarized in Table 1.

The relative configuration of **2** was assigned based on the coupling constants of relevant hydrogen atoms along with the ^{1}H-^{1}H-NOESY analysis, which showed cross-peaks assigned to dipolar interaction (spatial proximity, as shown in Figure 2). Thus, NOESY dipolar interactions of the H-8 hydrogen (δ_H 1.75) with the 3H-17 (δ_H 1.31) and 3H-20 (δ_H 1.02) methyl groups and 3H-19 (δ_H 0.87) with 3H-20 (δ_H 1.02) indicated that these hydrogen atoms possess β-orientations. In contrast, the interaction of H-5 [δ_H 2.05, dd, J = 12.7 (axial-axial

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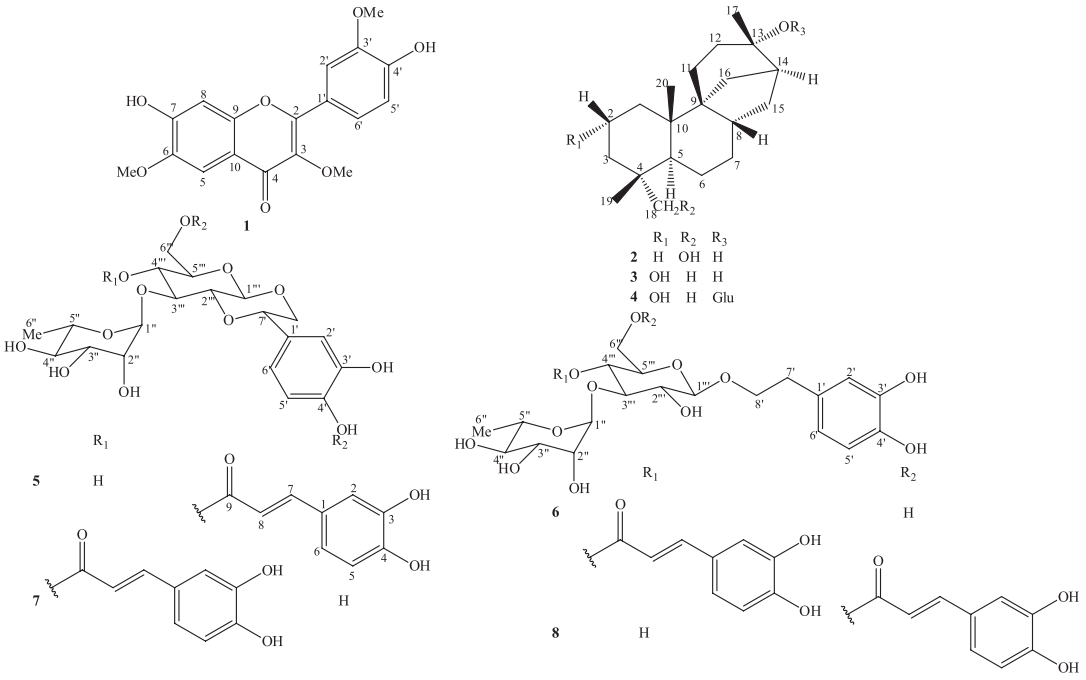

Figura 1. *Compounds isolated from Stemodia maritima*

coupling), 2.6 Hz] with H-16a (δ_H 2.75, d, J = 11.3 Hz) suggested spatial α-orientations for these atoms (Figure 2). Based on these correlations, all the methyl groups were established in β-orientations, which is consistent with the reported structure of natural stemodane in the literature.[4,12]

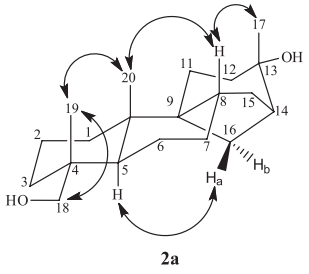

Figura 2. *Selected ^{1}H-^{1}H NOESY correlations for compound* ***2***

Thus, the structure of compound **2** was unambiguously established as stemodinol,[13] making this the first report of stemodinol as a natural product. The results of the extensive application of 1D and 2D NMR spectral techniques were also used to confirm the structure and establish the complete ^{1}H and ^{13}C chemical shift assignments of **2** (Table 1).

The other isolated compounds were identified on the basis of their spectral analyses and comparison with literature data.

Biological activities

Oxidative stress, which is the uncontrolled production of oxygen-derived free radicals in the body, is believed to be involved in the development of many pathological disorders.[14] Natural antioxidants that display few toxic side effects can be used as substitutes for synthetic antioxidants to provide protection against oxidative degradation by free radicals.[15] The use of plant products as antimicrobial drugs presents a low risk for the development of drug resistance due to their chemically complex structures.[16] As part of our ongoing search for natural bioactive compounds from Northwest Brazil,[6] we evaluated the antioxidant powers of stemodin (**3**)[8] and crenatoside (**7**).[9] In addition, the antibacterial activities of stemodin (**3**) and stemodinoside B (**4**)[5] were investigated; the results are summarized in Tables **2** and **3**, respectively.

Crenatoside (0.0014% in the roots; **7**) showed a high antioxidant potential, with 99.9% radical scavenging activity at a concentration of 1.0 mg/mL; at a concentration of 0.005 mg/mL, the activity was 93.7%, higher than those of the controls Trolox and Vitamin C. The results are in good agreement with a previous study that reported the antioxidant proprieties of crenatoside (**7**) using TLC autographic assays.[17] Stemodin (**3**) was also assayed but showed no activity. The results suggest that *Stemodia maritima* is a potential source of free radical scavengers, indicating its possible use in medicine, cosmetics or food industries.

Table **3** shows the minimum inhibitory concentration (MIC) of stemodin (**3**) and stemodinoside B (**4**). The observed MIC varied between 1024 to 256 μg/mL; the better results were obtained for the assays with stemodinoside B against *Klebsiella pneumonia* and *Listeria monocytogenes*. Many substances produced from stemodin by biocatalysis have demonstrated antiviral and cytotoxic activities as well as inhibitory activity of the lipids peroxidation and cyclooxigenase 1 and 2.[18] However, this is the first report indicating that stemodin and its derivate stemodinoside B may possess antibacterial activity.

CONCLUSIONS

This is the first report of stemodinol (**2**) as a natural product. In addition, the results demonstrated that the compound **7** has a high antioxidant activity. The compounds **3** and **4** showed bioactivity against some bacterial strains, with appreciable MIC values. These data are promising and could encourage further studies on the products of *S. maritima* to support their possible uses in antioxidant and antimicrobial therapies.

EXPERIMENTAL

General experimental procedures

Melting points were obtained from a Mettler FP82HT apparatus

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data assignments for the compound **2** (stemodinol), in C_5D_5N, including results obtained by heteronuclear 2D shift-correlated HSQC and HMBC. Chemical shifts (δ, ppm) and coupling constants (*J*, Hz, in parenthesis)

	HSQC		HMBC		^{1}H-^{1}H-NOESY
C	δ_C	δ_H	$^{2}J_{CH}$	$^{3}J_{CH}$	
4	38.9	-	H-3, H-5, 3H-19	-	
9	52.5	-	H-8, 2H-16	H-5, 2H-7, H-14, 2H-15, 3H-20	
10	39.4	-	3H-20	-	
13	72.6	-	H-14, 3H-17	H-11, 2H-16	
CH					
5	42.7	2.05 (dd, 12.7, 2.6)	H-6	H-3, H-7, H-18b, 3H-19, 3H-20	H-16a
8	39.9	1.75	2H-7, 2H-15	2H-16	3H-20
14	49.6	2.16 (t, 4.9)	2H-16	3H-17	
CH_2					
1	32.7	1.43	H-2	3H-20	
2	19.3	1.72, 1.56	2H-1	-	
3	36.8	1.84 (m), 1.45		2H-18, 3H-19	
6	23.4	1.73, 1.34	H-5, H-7a	-	
7	31.4	2.62(m), 1.54	H-8	H-15	
11	27.4	1.58, 1.49	-	H-16a	
12	40,5	1.67, 1.38	-	H-14, 3H-17	
15	30.5	1.17, 1.69	H-14	2H-16	
16	28.2	2.75 (d, 11.3), 1.42	-	H-8, 2H-15	H-5
18	71.9	3.66 (dd, 12.0; 3.0); 3.32 (dd, 12.0; 3.0)	-	H-3, H-5, 3H-19	
CH_3					
17	30.3	1.31 (s)	-	-	
19	18.9	0.87 (s)	-	2H-3, H-5, H-18a	2H-18; 3H-20
20	17.7	1.02 (s)	-	2H-1, H-5	H-8, 3H-17, 3H-19

*Number of hydrogens bound to carbon atoms deduced by comparative analysis of {^{1}H}- and DEPT-^{13}C NMR spectra. Chemical shifts and coupling constants (*J*) were obtained of 1D ^{1}H NMR spectrum. ^{1}H-^{1}H-COSY and ^{1}H-^{1}H-NOESY experiments were also used to these assignments. Superimposed ^{1}H signals are described without multiplicity and chemical shifts deduced also using the HSQC and HMBC spectra.

Table 2. The DPPH free radical scavenging activity of *Stemodia maritima* extracts, stemodin (**3**) and crenatoside (**7**)

Sample	Concentration (mg/mL)			IC_{50} (mg/mL)
	1.0	0.1	0.005	
	% Antioxidant			
Trolox	99. 9	99.8	86.5	0.0026
Vit C	99.8	92.8	19.3	0.0430
3	-	-	-	-
7	99.9	99.8	93.7	0.0022

and are uncorrected. IR spectra were recorded using a Perkin Elmer 1000 FT-IR spectrophotometer. Optical rotation was measured on a Perkin Elmer 341 polarimeter. High resolution electrospray ionization mass spectroscopy (ESI-MS), was performed in positive mode on a LCMS-IT-TOF SHIMADZU micromass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C); chemical shifts are given in ppm relative to residual C_5D_5N (8.74, 7.58, 7.22 and 150.4, 135.9, 123.9 ppm). Silica Gel 60 (Merck, 230 - 400 mesh) was used for analytical TLC. Silica gel 60 (Merck, 60 F_{254}, 0.2 mm) was used for column chromatography. The HPLC pump employed consists of a ternary LC-20AT SHIMADZU and a detector (UV SPD-M20A). Separations were performed on a Phenomenex RP 18 (250 x 10 mm, 5 μm) column.

All compounds were visualized with TLC by spraying with vanillin/perchloric acid/EtOH followed by heating.

Table 3. Values of the minimal inhibitory concentration (MIC) (μg/mL) of stemodin (**3**) and stemodinoside B (**4**)

Bacteria	3	4
Sthaplyloccocus aureus (ATCC 12692)	512	≥1024
Sthaphyloccocus aureus (ATCC 6538)	ND	512
Sthaphyloccocus aureus (ATCC 358)	512	ND
Sthaphyloccocus aureus (ATCC 12624)	ND	≥1024
Pseudomonas aeruginosa (ATCC 15442)	512	512
Bacillus cereus (ATCC 33018)	512	≥1024
Escherichia coli (ATCC 27)	512	≥1024
Escherichia coli (ATCC 25922)	≥1024	512
Aeromonas carveae (ATCC 15468)	ND	≥1024
Klebsiela pneumonia (ATCC 10031)	ND	256
Shiguella flexneri (ATCC 12022)	ND	512
Vibrio choloreae (ATCC 15748)	ND	≥1024
Listeria monocytogenes (ATCC 7644)	ND	256

ND – not determined.

Plant material

Leaves and roots of *Stemodia maritima* L. were collected during the flowering stage in Fleixeiras-CE, (Northeastern Brazil). A voucher specimen (#38483) was deposited at the Herbarium Prisco Bezerra from the Universidade Federal do Ceará - Brazil.

Extraction and isolation

A portion of the air-dried, powdered leaves (830.0 g), were extracted with 95% EtOH at room temperature. The EtOH-dried extract was partitioned with MeOH/H_2O 4:1 and subjected to liquid-liquid re-partition with hexane, CH_2Cl_2 and EtOAc. After drying with Na_2SO_4 and the careful removal of CH_2Cl_2 in vacuo, the CH_2Cl_2 phase was subjected to silica gel column chromatography (Merck 60-120 60 Mesh) using a hexane, CH_2Cl_2 and EtOAc solvent system in gradient (1:0:0; 1:1:0; 0:1:0; 0:1:1; 0:0:1 *v/v*); five fractions were obtained (Fr **A**-**E**). The fraction eluted with hexane:CH_2Cl_2 (1:1:0, Fr-**B**, 384.4 mg) was further separated chromatographically over a Sephadex LH-20 column eluted with MeOH to yield the compound **1**[7] (16.0 mg). The fraction eluted with CH_2Cl_2 (0:1:0, Fr-**C**, 681.1 mg) was further separated using silica gel CC with increasing amounts of EtOAc in hexane as the eluent. The fourth fraction (hexane-AcOEt 4:1, 123.0 mg) furnished a solid residue, which was recrystallized under acetone to give the new natural compound **2** (12.9 mg). The fraction eluted with CH_2Cl_2:EtOAc (0:1:1, Fr-**D**, 758.4 mg) furnished a solid residue, which gave the compound **3**[8] (128.2 mg) upon recrystallization under acetone.

After the extraction of essential oils by hydrodistillation, the aqueous extract of a part of the fresh leaves (1,000 g) of *S. maritima* was submitted to liquid-liquid separation with EtOAc. After drying over anhydrous sodium sulfate and concentration under reduced pressure, the EtOAc fraction (2.0 g) was separated chromatographically using silica gel with an EtOAc-MeOH solvent system in gradient (1:0; 9:1; 8.5:1.5; 7.7:2.5; 1:1 *v/v*). Fractions eluted with EtOAc:MeOH 9:1 were subjected to column chromatography over silica gel 60 and successively eluted by EtOAc:MeOH (1:0; 9.5:0.5; 9:1; 8:2). The fractions eluted with EtOAc:MeOH (1:0) yielded the compound **4**[5] (34.6 mg), while the fractions eluted with EtOAc:MeOH (9.5:0.5) gave the compound **5**[9] (6.2 mg).

The fresh roots (649.0 g) of *S. maritima* were exhaustively extracted with ethanol at room temperature to obtain an extract (57.3 g) that was then subjected to liquid-liquid separation with ethyl acetate. The organic fraction (4.0 g) was separated chromatographically over silica gel with CH_2Cl_2:EtOAc (1:0; 1:1; 0:1) to afford three subfractions F1-F3. From the combined fractions, F2 eluted with an 4:1 mixture of CH_2Cl_2:EtOAc; the obtained residue was submitted to exclusion chromatography on a Sephadex(TM) LH-20 (Amersham Biosciences, Sweden) using an eluent mixture of MeOH to yield the crude fraction A. This mixture was further purified by HPLC on the reversed-phase material RP-18 (solvent MeOH-H_2O 40:60) to yield the compounds **6**[10] (28.0 mg), **7**[9] (8.9 mg), and **8**[11] (13.0 mg).

Antioxidant activity

The antioxidant activities of stemodin (**3**) and crenatoside (**7**) were measured using the radical scavenging effect of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. One milliliter of a 60-μM DPPH ethanol solution was added to sample solutions of different concentrations and allowed to react at r.t. After 30 min, the absorbance values were measured at 517 nm, and the antioxidant activity was determined.[19] Each test was performed in triplicate. Data were evaluated through regression analysis. From the regression line, the IC_{50} values were determined as the percentage of scavenged free radical DPPH for 50% activity. The results were also presented as the percentage of scavenged free radical DPPH.

Antibacterial activity

Strains: *Escherichia coli* (ATCC 25922 and EC 27), *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538, ATCC 12692, SA358 and ATCC 12624), *Bacillus cereus* (ATCC 33018), *Escherichia coli* (ATCC 25922), *Escherichia coli* (ATCC 27), *Aeromonas carveae* (ATCC 15468), *Klebsiela pneumonia* (ATCC 10031), *Shiguella flexneri* (ATCC 12022), *Vibrio choloreae* (ATCC 15748), and *Listeria monocytogenes* (ATCC 7644) were used as positive controls. Clinical and methicillin-resistant *Staphylococcus aureus* (MRSA) were obtained from the Laboratório de Pesquisa de Produtos Naturais - URCA. All strains were stocked at room temperature in heart infusion agar slants (HIA, Difco). Prior to assay, the cells were grown overnight at 37 ºC in brain heart infusion (BHI, Difco).

Minimum inhibitory concentration

MIC values were determined in a microtitre assay[20] by inoculation of 100 μL of each strain suspended in two-fold concentrated (final concentration 10^5 colony-forming units/mL) BHI in a 96-well microtitre tray; two-fold serial dilutions were conducted by adding 100 μL of each natural product solution.

The final concentrations of the products ranged from 1024 to 8 μg/mL. The MICs were recorded as the lowest concentration for growth inhibition. All plates were incubated aerobically for 24 h at 37 ºC. All antimicrobial assays were performed twice, and the results were expressed as the average of the two repetitions.

SUPPLEMENTARY MATERIAL

Available at http://quimicanova.sbq.org.br, in pdf format with free access.

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