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Artigo

PHENOLIC DERIVATIVES AND OTHER CHEMICAL COMPOUNDS FROM *Cochlospermum regium*

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This study describes the chemical investigation of the ethyl acetate fraction obtained from the hydroethanolic extract of the xylopodium of *Cochlospermum regium* (Mart. & Schr.) Pilger, which has been associated with antimicrobial activity. Phytochemical investigation produced seven phenol derivatives: ellagic acid, gallic acid, dihydrokaempferol, dihydrokaempferol-3-*O*-β-glucopyranoside, dihydrokaempferol-3-*O*-β-(6"-galloyl)-glucopyranoside, pinoresinol, and excelsin. It also contained two triacylbenzenes, known as cochlospermines A and B. The hydroethanolic extract and its fractions exhibited antimicrobial activity (0.1 mg/mL) against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Gallic acid showed activity against *S. aureus*. Dihydrokaempferol-3-*O*-β-(6''-galloyl)-glucopyranoside is reported here for the first time in the literature.

Keywords: *Cochlospermum*; antimicrobial activity; dihydrokaempferol-3-*O*-β-(6´´-galloyl)-glucopyranoside.

INTRODUCTION

Cochlospermum regium (Mart. & Schr.) Pilger is a shrub in the Cochlospermaceae family that is represented by a few species. It is common in the Brazilian cerrado where it is considered animal fodder, an ornamental plant and a medicinal plant. There are reports of *C. regium* (popularly known as "algodãozinho") use in folk medicine in the states of Goias, Mato Groso, Mato Grosso do Sul, Distrito Federal, and São Paulo. This species is also found in the state of Minas Gerais, where a description of its traditional use was recorded by Saint-Hilaire. Saint-Hilaire described the use of this plant by the population of Paracatu City, mainly for relieving internal pains and the healing of already formed abscesses. Other reports of the use of this species have been cited in regional books.[1]

A survey of medicinal plants requested from and/or indicated by herb sellers operating in Campo Grande City, Mato Grosso do Sul, Brazil was perfomed. Among the five most often used plants was the roots of *Cochlospermum regium*, which have been used for the treatment of various diseases related to inflammation (arthritis, rheumatism, and acne) and genitourinary infection. This preliminary inquiry led to our research to identify the compound responsible for the antimicrobial, analgesic and/or anti-inflammatory activity of *Cochlospermum regium*.[2] A preliminary phytochemical analysis with antimicrobial and analgesic assessments resulted in the confirmation that the extracts and root fractionations possessed antimicrobial activity.[3] Dihydrokaempferol 3-*O*-β-glucopyranoside (**5**)[3] was isolated from these materials, but this substance is devoid of antimicrobial activity.[3] However, it does possess antinociceptive activity.[4] Other pharmacological investigations performed with the root of *C. regium* corroborated the observations that have been reported in folk medicine, suggesting analgesic, antiedematogenic, antimicrobial, non-toxic acute, and subacute toxicological effects of this root.[5] Specifically, the antinociceptive effect of **5** was evaluated and its pharmacological mechanism of action was assessed using different routes of drug administration.[5]

Additional phytochemical studies have led to the isolation of acetophenone 1-hydroxytetradecanone-3, an ester of *p*-hydroxycinnamic acid, flavonoids, naringenin, and dihydrokaempferol from the roots of *C. regium*.[6]

Two other species of *Cochlospermum* are considered native to Brazil, *C. vitifolium* and *C. onirocense. C. insigne,* a relative of *C. regium*, is also mentioned in this work. *C. vitifolium* is cited as being native to the north and northeast regions of Brazil, whereas *C. onirocense* is located predominantly in the northern region of the country.[7]

The current work describes the phytochemical and antimicrobial results obtained from the hydroethanolic extracts from the root of *C. regium.* The purpose of this work was to confirm the pharmacological reasons for the plant's use in folk medicine.

EXPERIMENTAL

General

Thin layer chromatography (TLC) plates (60 GF254 silica gel, Merck) and microcrystalline cellulose (Merck) were used for analytical separations and chromatography. Silica-gel 60 (70-230 mesh, Aldrich) and Sephadex LH-20 were used (25-100 μm, Sigma) for column chromatography.

All NMR experiments were performed on a Bruker DPX-300 instrument (^{1}H 300 MHz, ^{13}C 75 MHz) using $CDCl_3$ as a solvent and tetramethylsilane (TMS) as an internal standard. The chemical shifts are reported in δ units and coupling constants (*J*) in Hz.

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The FTIR spectra were obtained on a Bomem, Hartmann & Braum spectrophotometer (Michelson MB series) using KBr pellets. Mass spectral data (HR-ESI-MS) for ellagic acid (**1**), dihydrokaempferol (**2**), ethyl gallate (**4**), dihydrokaempferol-3-*O*-β-glucopyranoside (**5**) and dihydrokaempferol-3-*O*-β-(6´´-galloyl)-glucopyranoside (**6**), were collected on a UltrOTOFq (Bruker Daltonics) equipped with an infusion pump set to a flow rate of 3000 μL/h. The instrument was equipped with an ESI source used in the negative ion mode. Trifluoroacetic acid/Na (Na-TFA, 10.0 μg/mL) and sodium formate (10 μM) solutions were used for internal and external calibrations, respectively.

A Shimadzu GC/MS QP-2010 instrument equipped with a DB-5-5MS (30 m x 0.25 mm x 0.25 μm) capillary column was used for obtaining the spectral data for the mixture of cochlospermines A (**7**) and B (**8**). The instrument was set at an electron impact of 70 eV and used He as the carrier gas (flow rate of 1.3 mL/min). The column temperature was 300 °C with an interface temperature of 280 °C. The total analysis time was 120 min.

Plant material, extraction, and isolation

The underground system (xylopodium) of *C. regium* was collected in Terenos (MS, Brazil, in May, 1992) and identified by G. Hatschbach. A voucher specimen was deposited in the CG/MS Herbarium (04375). The dried powder (100 g) was extracted with 70% ethanol by exhaustive maceration, as described in the literature.[3] After concentrating under reduced pressure, a brown amorphous residue was obtained (14.9 g). The residue was solubilized in methanol:water (9:1), which was submitted to a partition. The partitioning provided hexane (0.2 g), ethyl acetate (5.3 g), and butanol (1.8 g) fractions, as well as a marc. The ethyl acetate fraction (2 g) was chromatographed on a silica-gel column with a chloroform:methanol gradient as a solvent (92:5 to 25:75 v/v). The chromatography furnished 110 fractions of 30 mL each. From the fractions, ethyl gallate (**4**, artifact from fractions 16-18, 15 mg, 0.003%), dihydrokaempferol (**2**, 5 mg, f 29, 0.0013%), dihydrokaempferol-3-*O*-β-glucopyranoside (**5**, 23 mg, f 40, 0.005%), and gallic acid (**3**, 17 mg, f 98, 0.06%) were isolated.

The combined material from fractions 55-41 (1.0 g) was chromatographed on a Sephadex Gel LH-20 column using methanol as an eluent. This column furnished 95-10 mL sub-fractions. The sub-fractions 25-30 (100 mg), after being fractionated two more times on a Sephadex Gel LH-20 column, yielded 50 10 mL sub-fractions. From these sub-fractions **3** (10 mg), **5** (30 mg), and **6** (dihydrokaempferol-3-*O*-β-(6"-galloyl)-glucopyranoside, sub-fractions 28-32, 5 mg, 0.001% p/p) were isolated. Sub-fractions 46-54 furnished a yellow precipitate identified as **1** (ellagic acid, 15 mg). Sub-fractions 50-63 provided a mixture of excelsin (**9**) and pinoresinol (**10**) (7 mg).

Lastly, compounds **7** and **8** were obtained as a mixture. These compounds were isolated from the crystal precipitate that was formed from the hydroethanolic extract following filtration and storage in the refrigerator.

Antimicrobial activity

The antimicrobial activity of the extracts, fractions, and substances **1** and **3** were evaluated by an agar diffusion technique (using the middle Müller–Hinton) against standard strains of *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212, and *K. pneumoniae* ATCC 13883. Disks of sterile filter paper (6 mm in diameter and 0.9 mm thick) were impregnated with 10 μL of each sample solution (dissolved in DMSO) to obtain disks impregnated with 1 mg of the crude extract or the fractions or with 0.1 mg of the isolated compounds, **1** and **3**. The bacterial suspensions were prepared by diluting the inoculated samples in saline solution until a turbidity comparable to a standard scale of 0.5 McFarland units was reached. The turbidity measurements were performed on an ultra-violet spectrophotometer at an absorbance of 0.08 to 0.10, operated at 625 nm. This procedure provided solutions at a concentration of 1.10^8 UFC/mL.[8]

After the inoculation of the bacteria in the culture medium, three disks of each sample were deposited. The reading of the halos of bacterial growth inhibition were measured after 24 h of incubation at 37 °C, as recommended by the National Clinical and Laboratory Standards Institute.[8] The positive control was obtained with standard disks of gentamicin (10 μg, Cecon). The negative control was obtained with disks impregnated with 10 μL of DMSO. Each sample was observed in triplicate. The values of the halos of the bacterial growth inhibition (in mm) are expressed as the mean and standard deviation (mean ± SD) of six readings.

*Dihydrokaempferol-3-O-β-(6"-galloyl)-glucopyranoside (**6**)*

An R*f* value of 0.45 in chloroform:acetic acid:methanol:water (64:32:12:8) with blue fluorescence in UV light (256 nm) is produced with the NP /PEG reagents.[9] The ^{1}H NMR (300 MHz, CD_3OD) values for δ are 7.24 (2H, d, *J* = 8.5 Hz, H-2' and H-6'), 7.10 (2H, s, H-6"' and H-3"'), 6.73 (2H, d, *J* = 8.5 Hz, H-3' and H-5'), 5.93 (1H, d, *J* = 2Hz, H-6), 5.87 (1H, d, *J* = 2Hz, H-8), 5.24 (1H, d, *J* = 11Hz, H-2), 4.81 (1H, *J* = 11Hz, H-3), 4.36 (1H, d, *J* = 6Hz, H-6"a and H-6"b), and 3.08-3.43 (4H, m, sugar portion). The ^{13}C NMR (75 MHz, CD_3OD) values for δ are 64.4 (C-6"), 71.3 (C-4"), 74.5 (C-2"), 75.7 (C-3"), 76.70 (C-5"), 77.5 (C-3), 83.5 (C-2), 96.4 (C-6 and C-8), 102.1 (C-1"), 102.6 (C-10), 110.2 (C-6"'), 110.2 (C-6"' and C-2"'), 121.5 (C-1"'), 139.9 (C-4"'), 146.5 (C-3"' and C-5"'), 116.2 (C-5' and C-3'), 128.5 (C-1'), 130.4 (C-6' and C-3'), 130.7 (C-2'), 159.2 (c-4), 164.0 (C-9), 164.9 (C-5), 168.3 (C-7), 169.0 (C-7"'), and 195.8 (C-4);. HR-ESI-MS *m/z* 601.1235 [M-H] ($C_{28}H_{25}O_{15}^-$ 601.11194), and the fragmentation occurred at *m/z* 449.1156 $[C_{28}H_{25}O_{15}]^-$ and at *m/z* 271.0481 $[C_{21}H_{21}O_{11}]^-$.

RESULTS AND DISCUSSION

The ^{1}H and ^{13}C NMR spectrum of **2**[10] and **5**[3] revealed the presence of a set of signals that are characteristic of dihydrokaempferol and its 3-O-glycoside derivative, respectively. These substances have been previously described in the xylopodium of *C. regium*.[3] The spectrum of substance **2** showed typical signals similar to the signals from a ^{13}C spectrum derived from kaempferol, which was only substituted in ring B in the C-4'. This was evidenced by the presence of two signals, each corresponding to two carbons, at δ 115 and δ 129, in addition to the presence of signals at δ 83.3 and 72.3, corresponding to C-2 and C-3, respectively. The ^{1}H NMR resonances at δ 4.97 and 4.52 (d, *J* = 11 Hz) indicated a *trans*-configuration for the H-2 and H-3; thus, this corroborates the proposal of the increased prevalence of the dihydrokaempferol flavanone in nature.[3,11-13] Substance **5** was confirmed to be the 3-O-glycoside of **2** because the ^{1}H NMR spectrum showed signs of additional sugar residues between 3.0 and 4.0 ppm. In addition, the ^{13}C spectrum had resonances between 70 and 80 ppm, with additional signs of a CH_2OH group (δ 62.6 and 3.60 and 3.73 by gHSQC) and an anomeric carbon at δ 102.0.

Compound **6** was isolated as a pale yellow amorphous solid. Spectral data acquired by one- (^{1}H, ^{13}C, and DEPT) and two-dimensional (gHSQC and gHMBC) experiments showed a close resemblance between a part of the spectral data and the data obtained for compounds **2**, **3**[10] and **5**.[3]

Spectral data for **6** were consistent with a flavanone monoglucoside, similar to the spectral data for **5**. However, the data showed an additional set of signals characteristic of a galloyl ester moiety.

The esterification of the methylene carbinolic sugar (H-6") could be confirmed due to the displacement of the signals that appear downfield (H-6", brs, δ 4.36). This is in contrast to those observed for **5** (δ 3.60 and 3.73). In addition, the ^{1}H-^{13}C gHMBC contour correlation map corroborated with this proposal. The resonance of the carbinolic methylene hydrogens at δ H 4.36 (H-6") correlated with the resonance of the gallate portion of the acid carbon at δ C169.0 (C-7").

A molecular ion peak at *m/z* 601.1235 [M-H] was found in the HR-ESI-MS of substance **6**, suggesting the formula $C_{28}H_{25}O_{15}^-$. A fragmentation at *m/z* 449.1156 ($C_{21}H_{21}O_{11}^-$) suggests the loss of the galloyl portion. Another peak at *m/z* 271.0481 ($C_{15}H_{11}O_5^-$), coincides with the fragmentation pattern of a dihydrokaempferol moiety. Thus, substance **6** was identified as the dihydrokaempferol-3-*O*-β-(6´´-galloyl)-glucopyranoside.[3,11,12]

The set of signals and chemical shifts of the mixture containing substances **7** and **8**, both of which were acquired by RMN ^{1}H and ^{13}C and DEPT 135º, indicated the presence of a carbonyl group (δ 199.3), a quaternary carbon (δ 137.84), the methine carbon (δ 131.10) of an aromatic ring or conjugated alkene, methylene carbons (δ 38.9-22.67) and a methyl group (δ 14.10). The presence of a symplet at δ 8.66 was associated with only one methylene carbon signal (at δ 131.10), suggesting the presence of a conjugated olefin between the methylene carbon and the quaternary carbon (δ 137.84). The GC-MS analysis of this mixture allowed the separation of substances **7** and **8** with retention times of 81.87 (84.9% area) and 119.9 min (15.09% area), respectively. The respective peaks measured molecular ions of *m/z* 624 ($C_{42}H_{72}O_3$) and 652 ($C_{44}H_{76}O_3$). The set of spectral signals associated with the molecular mass and the pattern of the chemotaxonomy of *Cochlospermum* sp. allowed us to characterize substances **7** and **8** as the triacylbenzenes known as cochlospermines A and B.[10] The spectral data of substances **9** and **10** were compared and confirmed with those previously reported for excelsin and pinoresionol, both of which have been previously isolated from *C. vitifolium* (Figure 1).[10]

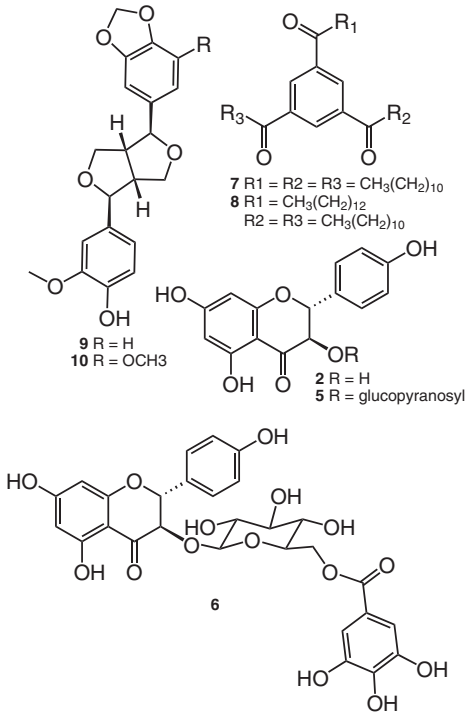

Figure 1. *Secondary metabolites isolated from the root of C. regium: dihydrokaempferol (**2**), dihydrokaempferol-3-O-β-glucopyranoside (**5**), dihydrokaempferol-3-O-β-(6´´-galloyl)-glucopyranoside (**6**), cochlospermines A (**7**) and B (**8**), excelsin (**9**), and pinoresinol (**10**)*

Compounds **1** and **3**, which represent the patterns of the ellagic and gallate chemical skeletons, were submitted to antimicrobial evaluation. A representative of the dihydrokaempferol carbon skeleton has been previously tested, and it did not show activity. The hydroethanolic extract and all the fractions obtained by partitioning were submitted for testing to determine their antimicrobial activity against *S aureus* and *P. aeruginosa.*[3] The inhibitory activity (measurement of halos of bacterial growth inhibition) of these extract and fractions was greater against *S. aureus.* Table 1 highlights the results obtained with the hydroethanolic extract (16.4 mm), the EtOAc extracts (15.0 mm) and the BuOH extracts (13.8 mm). Only substance **3**, which was isolated from the ethyl acetate fraction, showed a significant inhibitory effect (14.0 mm) against *S. aureus.* The inhibition of *P. aeruginosa* was modest, with halos between 7.0 and 9.5 mm in diameter. Specifically, the additive effects of the EtOAc fraction (9.5 mm) and the hydroethanolic extract (9.0 mm) as well as the less polar fractions were more active (Hex: 8.3 mm; $CHCl_3$: 8.0 mm). A similar study performed by Oliveira *et al.*,[3] reported that the inhibitory effect of the root of *C. regium* against the wild strains of both *Staphyloccocus aureus* and *Escherichia coli* were similar for the hydroethanolic extract and the EtOAc fraction and higher than that of the BuOH fraction. These samples were obtained from patients in the University Hospital of the Federal University of Mato Grosso do Sul – UFMS. Our results corroborate the results obtained by these authors in regard to the analysis against *S. aureus.* The antimicrobial activity observed in the hydroethanolic extract and in the ethyl acetate fraction may be related to the presence of gallic acid.[14,15] Its presence in the alcoholic extract of *Cesalpinea mimosoides*, followed by its isolation as a single active compound, confirms the antimicrobial effect observed in this species.[14]

Table 1. Antimicrobial activity of the extract, fractions, and substances from the fractions obtained from the root of *C. regium* against the microorganisms *S. aureus* and *P. aeruginosa*

Samples	Inhibition halo (mm)	
	S. aureus	*P. aeruginosa*
hydroethanolic extract	16.4 ± 1.3	9.0 ± 1.0
Hex fraction	9.8 ± 0.4	8.3 ± 0.5
$CHCl_3$ fraction	10.0 ± 0.4	8.0 ± 1.2
EtOAc fraction	15.0 ± 0.7	9.5 ± 0.5
BuOH fraction	13.8 ± 1.9	7.7 ± 0.8
marc	12.8 ± 1.3	7.0 ± 0.0
1	-	-
3	14.0 ± 0.7	0.00
Gentamicin	22.0 ± 0.0	22.0 ± 2.8
DMSO	0.0	0.0

- no activity

CONCLUSION

The phytochemical investigation of the ethyl acetate fraction obtained from the hydroethanolic extract of *C. regium* provided seven phenolic derivatives (ellagic acid, gallic acid, dihydrokaempferol, dihydrokaempferol-3-*O*-β-glucopyranoside, and dihydrokaempferol-3-*O*-β-(6´´-galloyl)-glucopyranoside, pinoresinol, and excelsin) and two triacylbenzenes, Cochlospermine A and B. With the exception of dihydrokaempferol and its glucoside, this is the first finding showing that these substances are contained in this species. This study also describes the presence

of dihydrokaempferol-3-*O*-β-(6´´-galloyl)-glucopyranoside for the first time. These results provide information concerning gender and corroborate the pattern of substances found in *Cochlospermum*, which are characterized by the presence of flavonoids, triacylbenzenes, and gallic acid derivatives.

The popular use of this species led us to investigate and identify the specific pharmacological agents responsible for the biological activity of *C. regium* to justify its use in medical infections and inflammation.[2] The ethyl acetate fraction from the hydroethanolic extract contained the most active component. In previous studies, compound **5** had been isolated from this fraction and was found to lack an antimicrobial effect.[3] However, **5** showed antinociceptive activity in previous studies.[5] Prior to our study, no constituent with antimicrobial activity had been isolated from this species. However, the presence of gallic acid, **3**, provides sufficient support to justify the popular use of this species in treating infection.

SUPPLEMENTARY MATERIAL

Available at http://quimicanova.sbq.org.br, in PDF file, with free access.

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REFERENCES

1. Souza V. C.; Lorenzi, H.; *Botânica Sistemática: guia ilustrado para identificação das famílias de fanerógamas nativas e exóticas no Brasil baseado em APG II,* 2ª ed., Nova Odessa: São Paulo, 2008; Silberbauer-Gottsberger, I.; *Oreades* 1981/82, p. 15-30; Brandão, M. G. L.; *Plantas Úteis de Minas Gerais, na Obra dos Naturalistas,* 1ª ed., Código: Belo Horizonte, 2010.
2. Nunes, G. P.; Silva, M. F.; Resende, U. M.; Siqueira, J. M. de; *Rev. Bras. Farmacogn.* **2003**, *13*, 83; Sólon, S.; Brandão, L. F. G.; Siqueira, J. M. de; *Rev. Elet. Farm.* **2009**, *6*, 1.
3. Lima, D. P. de; Castro, M. S. A.; Mello, J. C. P.; Siqueira, J. M. de; Kassab, N. M.; *Fitoterapia* **1995**, *66*, 545; Oliveira, C. C.; Siqueira, J. M. de; Souza, K. C. B. de; Resende, U. M.; *Fitoterapia* **1996**, *67*, 176.
4. Castro, M. S. A.; *Tese de Doutorado***,** Universidade Federal de São Paulo, Brasil, 2000.
5. Toledo, M. I.; Siqueira, J. M. de; Araújo, L. C. L.; Oga, S.; *Phytother. Res.* **2000**, *14*, 359.
6. Ritto, J. L. A.; Oliveira, F. de; Carvalho, J. E. de; Dias, P. C.; *Lecta* **1996**, *14*, 27.
7. http://www.tropicos.org/NameSearch.aspx; http://floradobrasil.jbrj.gov.br, accessed in February 2011.
8. National Committee of Clinical Laboratory Standards; *Performance standards for antimicrobial disk susceptibility test. Ninth International Supplement. M100-S9,* NCCLS: Wayne, 1999.
9. Wagner, H.; Bladt, S.; *Plant Drug Analysis - A Thin Layer Chromatography Atlas,* 2nd ed., Springer: New York, 1996.
10. Almeida, S. C. X.; Lemos, T. L. G.; Silveira, E. R.; Pessoa, O. D. L.; *Quim. Nova* **2005**, *28*, 57.
11. Sólon, S.; Lopes, L.; Sousa, P. T.; Schmeda-Hirschmann, G.; *J. Ethnoph.* **2000**, *72*, 173.
12. Islam M. T.; Tahara, S.; *Phytochemistry* **2000**, *54*, 901; Ruangrungsi, N.; Tappayuthpijarn, P.; Tantivatana, P.; *J. Nat. Prod.* **1981**, *44*, 541; Agrawal, P. K.; Thakur, R. S.; Bansal, M. C.; *Carbon-13 NMR of Flavonoids*, Elsevier Science Publisher ed.: New York, 1989.
13. Baderschneider, B.; Winterhalter, P.; *J. Agric. Food. Chem.* **2001**, *49*, 2788.
14. Chanwitheesuk, A.; Teerawutgulrag, A.; Kilburn, J. D.; Rakariyatham, N.; *Food Chem.* **2007**, *100*, 1044.
15. Scalbert, A.; *Phytochemistry* **1991**, *30*, 3875.