Fitoterapia 82 (2011) 1237–1240

Contents lists available at SciVerse ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

A new biphenyl and antimicrobial activity of extracts and compounds from *Clusia burlemarxii*

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ARTICLE INFO

Article history:
Received 19 January 2011
Accepted in revised form 16 August 2011
Available online 27 August 2011

Keywords:
Clusiaceae
Clusia burlemarxii
Biphenyl
Antimicrobial activity

ABSTRACT

Phytochemical investigation on *Clusia burlemarxii* (Clusiaceae) led to isolation and identification of nine compounds. Were isolated from leaves 3-O-α-L- rhamnopyranosylquercetin, 3-O-α-L-rhamnopyranosylkaempferol, 4-hydroxy-5,5-dimethyldihydrofuran-2-one, 2*Z*-δ-tocotrienoloic acid and friedelin and were isolated from trunk betulinic acid, protocatechuic acid, lyoniresinol, and a new biphenyl 2,2-dimethyl-3,5-dihydroxy-7-(4-hydroxyphenyl)chromane. The structures were determined by ^{1}H, ^{13}C-NMR, DEPT, HMBC, HMQC, HRESIMS. The Minimal Inhibitory Concentration against *Streptococcus mutans*, *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, *Salmonella choleraesuis*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Cladosporium cladosporioides* was also determined. Extracts and compounds showed significant activity against tested Gram-positive bacteria, none activity against tested Gram-negative bacteria and fungi.

1. Introduction

Clusiaceae is a tropical and temperate family of around 43 genera and 1610 species. In Brazil, there are 21 genera and about 180 species with wide occurrence [1]. Many plants belonging to this family have been used by traditional medicine in different regions of the world [2–5] whose extracts and pure compounds have shown antibacterial [6], antifungic [7,8], antiviral [9] e antitumoral activities [10–15].

The genus *Clusia* is constituted of about 200 species and its occurrence is restricted to tropical and subtropical regions of South and Central America [1]. The species of this genus are known as producers of polyprenilated benzophenones and triterpenes [16–18]. Biphenyls [15,18,19], quinones [20] and tocotrienols [18,21] are rarely found. Lignans were never found in the genus; in the family only two, syringaresinol from roots of *Vismia guaramirangae* [22] and kaerophyllin from *Montrouziera sphaeroidea* [23] were previously described.

Clusia burlemarxii is a wild shrub with distribution restricted to campo rupestre areas (rocky fields) of Chapada Diamantina, Bahia, Brazil. Phytochemical investigation on this species led to isolation and identification of nine compounds: 3-O-α-L-rhamnopyranosylquercetin, 3-O-α-L-rhamnopyranosylkaempferol, 4-hydroxy-5,5-dimethyldihydrofuran-2-one, 2*Z*-δ-tocotrienoloic acid, and friedelin were isolated from leaves, and betulinic acid, protocatechuic acid, lyoniresinol and a new biphenyl 2,2-dimethyl-3,5-dihydroxy-7-(4-hydroxyphenyl)chromane were isolated from trunk. As a part of our search for new source of antimicrobial compounds, in vitro preliminary tests were performed to determine the inhibitory activity of extracts and pure compounds against *Streptococcus mutans*, *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, *Salmonella choleraesuis*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Cladosporium cladosporioides*.

2. Experimental

2.1. General procedures

Optical rotations were measured on a Perkin-Elmer model 341 digital polarimeter. NMR spectra were recorded on a VARIAN GEMINI 2000 spectrometer operating at 300 MHz.

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doi:10.1016/j.fitote.2011.08.012

HRESIMS was obtained in positive mode in a UtrO-TOF Bruker Daltonics (Billa Rica, USA). CC was performed using silica gel (230–400 mesh). TLC was carried out by using silica gel plates prepared with $GF_{254+366}$ (Merck); spots were visualized by UV light or iodine vapor.

2.2. Plant material

C. burlemarxii was collected in campo rupestre areas (rocky fields) in Mucugê, Bahia, northeastern region of Brazil. The voucher specimen (ALCB-61584) is stored at Alexandre Leal Costa herbarium of the Biology Institute, UFBA.

2.3. Extraction and isolation

The dried and powdered leaves (1.6 kg) were extracted by three times maceration with 95% ethanol at room temperature. The combined extract was concentrated under reduced pressure at 40 °C and then it was dissolved in a mixture of ethanol and water (8:2). This solution was successively extracted three times with hexane yielding 2.54 g, and three times with EtOAc yielding 3.52 g. The dried and powdered trunk (3.9 kg) was extracted by maceration three times with methanol at room temperature. The concentrated methanol extract was partitioned with hexane yielding to 3.63 g and with EtOAc yielding 12.61 g.

The ethyl acetate extract (3.52 g) of leaves was subjected to a CC over silica gel using as eluent mixtures of chloroform and methanol of increasing polarity. Eighteen fractions were obtained. From fraction 1, by recrystallization in methanol, friedelin (8 mg) was isolated [24]. The second fraction (120 mg) was subjected to CC on silica gel using as eluent mixtures of ethyl acetate and methanol of increasing polarity providing 4-hydroxy-5,5-dimethyldihydrofuran-2-one [25] (10 mg) and 2*Z*-δ-tocotrienoloic acid (5 mg) [18]. Combined fractions 14–17 (350 mg) were subjected to a CC on silica gel using as eluent mixtures of chloroform and methanol of increasing polarity yielding 3-O-α-L- rhamnopyranosylquercetin (20 mg) and 3-O-α-L-rhamnopyranosylkaempferol (30 mg) [26].

The ethyl acetate extract (12.61 g) from the trunk was subjected to filtration on a CC over silica gel using as eluent mixtures of dichloromethane and ethyl acetate gradient of increasing polarity. Twenty one fractions were obtained. The fractions CBM1-13 to CBM1-15 were grouped (330 mg) and then fractionated on a CC of silica gel using as eluting system mixtures of dichloromethane and ethyl acetate yielding 53 fractions. From fractions CBM2-36 to CBM2-40 the protocatechuic acid was isolated (20 mg). The fractions CBM2-42 to CBM2-46 were grouped (60 mg) and submitted to a TLC over silica gel eluted with chloroform:methanol (85:15) yielding (+)-lyoniresinol (20 mg) [27]. The fractions CBM1-16 to CBM1-21 after being grouped (9 g) were subjected to liquid–liquid extractions, three times with dichloromethane and then three times with ethyl acetate. The material extracted with dichloromethane (2 g) was subjected to a column of silica gel eluted with mixtures of dichloromethane and acetone in increasing polarity gradient leading to two substances, betulinic acid [28] (30 mg) and a new biphenyl 2,2-dimethyl-3,5-dihydroxy-7-(4-hydroxyphenyl)chromane, 1 (12 mg).

(−)-2,2-dimethyl-3,5-dihydroxy-7-(4-hydroxyphenyl) chromane (1): Yellow greenish semi-solid; $[\alpha]_D^{25}$-103,45 (*c* 0.29, CH_3OH). RMN ^1H e ^{13}C, see Table 1. HREIMS: *m/z* 309.1153 $[M+Na]^+$.

2.4. Antimicrobial tests

The determination of minimum inhibitory concentration (MIC) of extracts and isolated compounds was performed by using the successive microdillution test in 96 wells plate according to the methodology of Bicalho et al. (2003) [29]. Nutrient broth and malt were used as culture media for bacteria and fungi, respectively. The stock solutions of samples were prepared by dissolving 2 mg of pure substance or 8 mg of extract in 5 ml of dimethylsulfoxide in water (20% v/v). Chloramphenicol and ciclopirox olamine (Loprox) were used as positive control for bacteria and fungi, respectively, in the same concentration of stock solution (2000 μg ml^{-1} for extract and 400 μg ml^{-1} for pure substances). The concentrations of samples and positive controls ranged from 500 μg ml^{-1} to 3.90 μg ml^{-1} for the extracts and from 100 μg ml^{-1} to 0.78 μg ml^{-1} for the pure substances. The incubation period was 24 h for bacteria and 72 h for fungi. MIC was determined through the emergence of turbidity in the wells. Nine microbial strains were used to access the antimicrobial properties of the test sample: the bacteria *S. mutans* (ATCC 5175), *S. aureus* (ATCC 6538), *B. subtilis* (ATCC 6633), *M. luteus* (ATCC 10240), *E. coli* (ATCC 94863), *S. choleraesuis* (ATCC 14028), *P. aeruginosa* (clinical isolate) and the fungi *Aspergillus ninger* (ATCC 16 404) and *C. cladosporioides* (IMI 178 517). All tests were performed in triplicate. Extracts were considered active when there was inhibition at concentrations below or equal to 500 μg ml^{-1}. The substances were considered active when there was inhibition at concentrations below or equal to 100 μg ml^{-1}.

3. Results and discussion

The structures of compounds were determined using ^1H and ^{13}NMR, DEPT, HMQC, HMBC and MS and by comparison

Table 1
^1H NMR (300 MHz) and ^{13}C NMR (75 MHz) data for compound 1 in $(CD_3)_2CO$ (δ in ppm and *J* in Hz).

No.	δ_H	δ_C
1	–	140.9
2	6.47 (1H, d, *J* = 1.7)	106.8
3	–	155.2
4	–	107.2
5	–	156.8
6	6.62 (1H, d, *J* = 1.7)	105.2
7	–	133.1
8, 12	7.39 (2H, d, *J* = 8.7)	128.4
9, 11	6.87 (2H, d, *J* = 8.7)	116.3
10	–	157.8
2′	–	77.4
3′	3.78 (1H, dd, *J* = 8.1 5.7)	69.9
4′	2.54 (1H, dd, *J* = 17.1 8.1)	27.1
	2.94 (1H, dd, *J* = 17.1 5.7)	
5′	1.23 (3H, s)	20.2
6′	1.35 (3H, s)	26.1
OH-5	8.60 (1H, bs)	–
OH-10	8.60 (1H, bs)	–
OH-3′	4.27 (1H, bs)	–

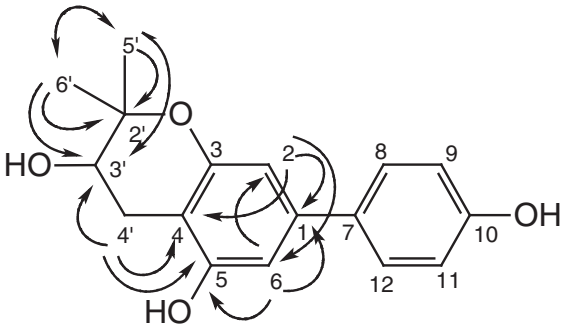

Fig. 1. Structure of compound 1 showing selected HMBC correlations.

of data with literature. From leaves, five compounds were obtained where friedelin, 3-O-α-L- rhamnopyranosylquercetin and 3-O-α-L-rhamnopyranosylkaempferol are commonly found in plants. The 4-hydroxy-5,5-dimethyldihydrofuran-2-one is very rare in plants and this is the first report to the family Clusiaceae. The 2*Z*-δ-tocotrienoloic acid is also rarely found in the family where the E isomer is the most common. From trunk, four compounds were isolated where betulinic acid and protocatechuic acid are common in plants. However, lignans are very rare in the family where the (+)-lyoniresinol is the third lignan isolated in Clusiaceae. Biphenyls have some occurrence related in the family and this is the first report for the compound 2,2-dimethyl-3,5-dihydroxy-7-(4-hydroxyphenyl) chromane.

The ^{1}H NMR spectra of 1 showed two doublets of two hydrogen at δ 6.47 (1.7 Hz) and δ 6.62 (1.7 Hz) suggesting the presence of a 1,3,4,5-tetrasubstituted aromatic ring. Additionally, it showed two doublets of two hydrogen each at δ 6.87 (8.7 Hz) and δ 7.39 (8.7 Hz) suggesting the presence of another aromatic ring with 1,4 substitution pattern. Two singlets at δ 1.23 and δ 1.35 for three hydrogen each were assigned to a *gem*-dimethyl group. A double doublet at δ 3.78 (8.1 and 5.7 Hz) suggested the presence of a methyne oxygenated group attached to a methylene group. Two double doublets at δ 2.54 (17.1 and 8.1 Hz) and δ 2.94 (17.1 and 5.7 Hz), confirmed the presence of an α-carbinolic methylene group. The ^{13}C NMR data indicated six non-hydrogenated aromatic carbons including three oxygenated at δ 157.8, δ 156.8 and δ 155.2 besides signals of four aromatic hydrogenated carbons, where two of them, those at δ 128.4 and δ 116.3, corresponded to two CH each. In the aliphatic region of spectrum signals were observed at δ 77.4 (C), δ 69.9 (C), δ 27.1 (CH_2), δ 26.1 (CH_3), and δ 20,2 (CH_3) compatible with a 2,2-dimethylpyran group with a hydroxyl at C-3. The *J* values observed for H-3′ (Table 1) indicated that the pyran ring assumes a half-chair conformation in which H-3′ is *pseudo*-axial.

DEPT and ^{13}C NMR data analysis indicated the presence of two methyl, one methylene, seven methyne and seven non-hydrogenated carbons. Based on these data and in the pseudo molecular ion peak at *m/z* 309.1153 $[M+Na]^{+}$ the molecular formula, $C_{17}H_{18}O_4$ was deduced to compound 1.

The HMBC data (Fig. 1) indicated that compound 1 is a biphenyl with a 3′-hydroxi-2′,2′-dimethylpyran moiety located at C-3 and C-4 and with two additional hydroxyl groups at C-5 and C-10. The correlations of H-2 with C-3 and C-7, the correlations of H-6 with C-5 and C-7, the correlations of H-9 with C-7 and C-10 and the correlations of H-4′ with C-3 and C-4 determined unequivocally the localization of substituent groups in the biphenyl skeleton. A similar compound presenting in C-5 a methoxyl group instead of a hydroxyl was isolated from roots of *Garcinia linii* [30].

Biphenyls are substances found mainly in plants of Rosaceae [31–33] in which they are identified as phytoalexins. In *Clusia* they are rare and in literature the isolation of five from *C. paralicola* [15,19] and one from *C. melchiorii* [18] was reported. Compound 1 was probably biosynthesized through the action of a biphenyl synthase in an intermediary produced by the condensation of 4-hydroxybenzoyl-CoA, derived from 4-coumaroyl-CoA, with three acetyl units, derived from malonyl-CoA [34]. The enzyme catalyzes the cyclization of the intermediary via an intramolecular aldol condensation followed by a CO_2 elimination producing the 3,4,10-trihydroxybiphenyl. A prenyltransferase promoted a prenylation of C-4 with subsequent epoxidation of double bond and cyclization to produce compound 1 (Fig. 2).

The antimicrobial activities of substances and extracts were assayed against four Gram-positive bacteria (*S. aureus, S. mutans, B. subtilis* and *M. luteus*), three Gram-negative bacteria (*E. coli, S. choleraesuis* and *P. aeruginosa*) and two fungi (*A. niger* and *C. cladosporioides*).

The MIC obtained in the initial assessment of *C. burlemarxii* extracts demonstrated a very promising activity from the leaves ethanol extract against *B. subtilis* (31.25 μg ml^{-1}) and *S. aureus* (62.50 μg ml^{-1}) and from the trunk methanol extract against *B. subtilis* (62.50 μg ml^{-1}), *S. mutans* (62.50 μg ml^{-1}) and *M. luteus* (31.25 μg ml^{-1}).

The antimicrobial activity of the isolated compounds showed that compound 1 exhibited significant activity against all tested Gram positive bacteria which was stronger against *M. luteus* (25 μg ml^{-1}) and *S. aureus* (50 μg ml^{-1}) and weaker

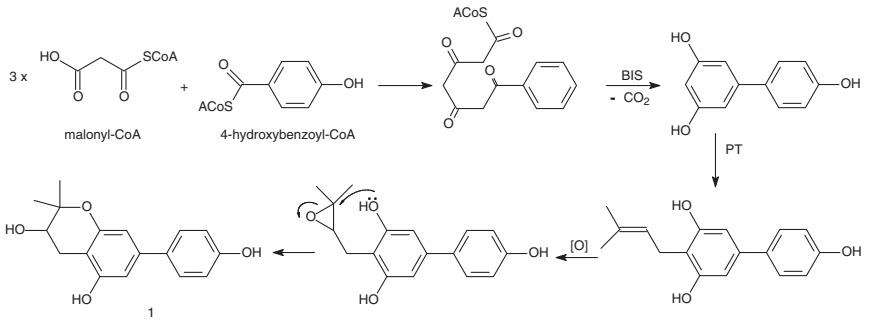

Fig. 2. Proposed biosynthesis of compound 1. BIS, biphenyl synthase; PT, prenyltransferase.

against *B. subtilis* (100 μg ml^{-1}) and *S. mutans* (100 μg ml^{-1}). Lyoniresinol and 3-O-α-L-rhamnopyranosylkaempferol were more selective showing strong activity only against *S. aureus* (25 μg ml^{-1}) whereas 3-O-α-L- rhamnopyranosylquercetin exhibited moderate activity only against *B. subtilis* (50 μg ml^{-1}) and was weaker against *S. aureus* (100 μg ml^{-1}). The other compounds showed no activity against the microorganisms tested. Neither extracts nor compounds showed activity against tested Gram negative bacteria and fungi.

Acknowledgments

The authors would like to thank Dr. Norberto P. Lopes from FCFRP-USP for the HRESIMS and Dr. Walmir da Silva Garcez from UFMS for α_D data. This work was supported by the FINEP, CAPES, CNPq, and FAPESB.

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