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Original Article

Chromenes from leaves of *Calea pinnatifida* and evaluation of their leishmanicidal activity

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ABSTRACT

Calea pinnatifida (R. Br.) Less., Asteraceae, is popularly known as “quebra-tudo”, “cipó-cruz” or “aruca”. This species is used in the folk medicine for the treatment of stomach pain, giardiasis and amoebiasis. The aim of this study was to isolate and identify chromenes from leaves of *C. pinnatifida* and evaluate their leishmanicidal activity. A fraction from leaves of *C. pinnatifida* was analyzed for their chemical constituents, resulting in the isolation and characterization of four known chromenes: 6-acetyl-7-hydroxy-2,2-dimethylchromene (**1**), 6-acetyl-7-methoxy-2,2-dimethylchromene (**2**), 6-(1-hydroxyethyl)-7-methoxy-2,2-dimethylchromene (**3**) and 6-(1-ethoxyethyl)-7-methoxy-2,2-dimethylchromene (**4**). Structure identification of isolated compounds involved analysis of spectral data of 1D and 2D-NMR. The isolated compounds are here reported for the first time in *C. pinnatifida*, and the chromenes **1** and **3** show a moderate leishmanicidal activity.

Introduction

Calea L. is a large genus of the Asteraceae family (tribe Heliantheae, subtribe Melampodiinae), containing approximately 125 species distributed essentially in tropical and subtropical zones of the Americas (Roque and Carvalho, 2011), with the greatest number of species being recorded in Brazil (Mondin and Bringel Jr., 2010). This genus has been reported in the literature to possess various biological properties, such as anti-inflammatory (Gomes and Gil, 2011), antiplasmodial (Kohler et al., 2002), antileishmanial (Wu et al., 2011), acaricidal (Ribeiro et al., 2011), antifungal (Flach et al., 2002), antidiabetic (Ramos et al., 1992), antimicrobial (Do Nascimento et al., 2004), antihypertensive (Guerrero et al., 2002), and cytotoxic activities (Nakagawa et al., 2005).

Calea pinnatifida (R. Br.) Less. is popularly known as “aruca”, “cipó-cruz” or “quebra-tudo” (Mors et al., 2000). This species is used in the folk medicine for treating digestive disorders, giardiasis and amoebiasis (Malhado Filho, 1947; Prusk and Urbatsch, 1988; Mors et al., 2000). Previous phytochemical investigations of the petroleum ether and ethyl acetate extracts from aerial parts of this plant and its essential oil revealed the presence of fatty esters, phenolic acids, sterols, monoterpenes, one polyacetylene, and one sesquiterpene lactone (Ferreira et al., 1980a,b; Kato et al., 1994).

Chromenes (benzopyrans) represent a class of secondary metabolites that have generated great attention because of their interesting biological and pharmacological properties (Ribeiro et al., 2011; Thomas and Zachariah, 2013). Several studies have demonstrated the insecticidal, antibacterial, fungicidal and cytotoxic activities of these substances (Bandara et al., 1992; Burkhardt et al., 1994; Iqbal et al., 2004; Chen et al., 2005). Furthermore, some compounds of this class of natural products have been described to have notable antiprotozoal effect (Alizadeh et al., 2008; Batista Jr. et al., 2008; Harel et al., 2011).

Herein, we report the isolation and the structure determination of four known chromenes, named as 6-acetyl-7-hydroxy-2,2-dimethylchromene (**1**), 6-acetyl-7-methoxy-2,2-dimethylchromene (**2**), 6-(1-hydroxyethyl)-7-methoxy-2,2-dimethylchromene (**3**) and 6-(1-ethoxyethyl)-7-methoxy-2,2-dimethylchromene (**4**). In addition, the isolated compounds were selected for leishmanicidal assays based on previously reported activity of related structurally compounds in other trypanosomatid protozoa (Harel et al., 2011).

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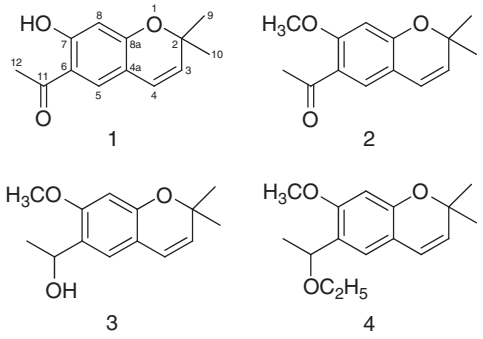

Materials and methods

General experimental procedures

Melting point was determined using an MQAPF-301 melting point apparatus. Optical rotation was measured in the solvent $CHCl_3$ on a Jasco P-2000. The ^1H and ^{13}C NMR spectra was obtained using a high resolution Bruker AVANCE-400 and Ascend 600 spectrometers, frequency of 400 and 600 MHz for ^1H, and 100 and 150 MHz for ^{13}C, respectively. NMR spectroscopic data were acquired in $CDCl_3$, TMS was used as internal standard, chemical shifts (δ) were given in ppm, and coupling constants (J) in Hz. 2D NMR experiments (HSQC, HMBC) were also performed using Bruker AVANCE-400 and Ascend 600 spectrometers.

Plant material

The leaves from *Calea pinnatifida* (R. Br.) Less., Asteraceae, were collected in September 2012, at the "Costa da Lagoa", Florianópolis, Santa Catarina, Brazil. Plant identification was performed by Dr. John F. Pruski, New York Botanical Garden, and a voucher specimen (MO-2383318 number) is deposited in Missouri Botanical Garden Herbarium (MO), St. Louis, Missouri, USA.

Extraction and isolation

Fresh leaves from *C. pinnatifida* (800 g) were extracted by maceration for 15 days at room temperature (*ca.* 25 °C) with ethanol 92%. After evaporation of the solvent under reduced pressure, 12 g of the ethanol extract of *C. pinnatifida* were obtained. The ethanol extract was re-dissolved in H_2O and fractionated with solvents of increasing polarity. The partitioning of this extract was performed with *n*-hexane, dichloromethane and ethyl acetate, respectively, yielding *n*-hexane (4.5 g), dichloromethane (0.5 g) and ethyl acetate (1.5 g) fractions, as well as a residual aqueous fraction (5.5 g). Initially, an aliquot of the hexane fraction (2.0 g) of the extract was subjected to column chromatography with silica gel 60. Elution was carried out using a solvent gradient of *n*-hexane:acetone in increasing polarities (100:0, 98:2, 95:5, 90:10, 70:30, 50:50, 0:100, respectively), obtaining some sub-fractions rich in chromenes (subfraction 1: 35 mg, sub-fraction 2: 20 mg, subfraction 3: 15 mg). Subsequently, these sub-fractions were purified by preparative TLC (*n*-hexane:acetone 85: 15). Sub-fraction 1 afforded 8.0 mg of **1** and 4.0 mg of **4**, sub-fraction 2 yielded 18.0 mg of **2**, and sub-fraction 3 afforded 5.0 mg of **3**.

6-Acetyl-7-hydroxy-2,2-dimethylchromene (**1**)

Yellow needles; mp: 78–80 °C; ^1H NMR ($CDCl_3$, 400 MHz): δ 7.31 (br s, 1H, H-5), 6.33 (br s, 1 H, H-8), 6.28 (d, 1H, *J* 9.9 Hz, H-4), 5.58 (d, 1H, *J* 9.9 Hz, H-3), 2.54 (s, 3H, H-12), 1.44 (s, 3H, H-9), 1.44 (s, 3H, H-10); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 77.9 (C-2), 128.9 (C-3), 121.0 (C-4), 113.5 (C-4a), 128.5 (C-5), 113.8 (C-6), 165.2 (C-7), 104.5 (C-8), 160.4 (C-8a), 28.6 (C-9), 28.6 (C-10), 202.3 (C-11), 26.1 (C-12).

6-Acetyl-7-methoxy-2,2-dimethylchromene (**2**)

Yellow oil; ^1H NMR ($CDCl_3$, 400 MHz): δ 7.54 (s, 1H, H-5), 6.38 (s, 1 H, H-8), 6.30 (d, 1H, *J* 9.8 Hz, H-4), 5.53 (d, 1H, *J* 9.8 Hz, H-3), 3.88 (s, 3H, H-13), 2.56 (s, 3H, H-12), 1.44 (s, 3H, H-9), 1.44 (s, 3H, H-10); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 77.7 (C-2), 128.4 (C-3), 121.4 (C-4), 114.0 (C-4a), 129.1 (C-5), 120.8 (C-6), 161.2 (C-7), 99.7 (C-8), 158.5 (C-8a), 28.4 (C-9), 28.4 (C-10), 197.6 (C-11), 31.9 (C-12), 55.6 (C-13).

6-(1-Hydroxyethyl)-7-methoxy-2,2-dimethylchromene (**3**)

Green gum; $[\alpha]_D^{20}$ = +45.49 (c = 0.2133 g ml^{-1}; $CHCl_3$; *ca.* 20 °C); ^1H NMR ($CDCl_3$, 400 MHz): δ 6.94 (d, 1H, *J* 0.3 Hz, H-5), 6.37 (s, 1 H, H-8), 6.27 (dd, 1H, *J* 9.7 0.3 Hz, H-4), 5.47 (d, 1H, *J* 9.7 Hz, H-3), 5.02 (q, 1H, *J* 6.5 Hz, H-11), 3.82 (s, 3H, H-13), 1.48 (d, 3H, *J* 6.5 Hz, H-12), 1.42 (s, 3H, H-9), 1.42 (s, 3H, H-10); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 76.5 (C-2), 127.9 (C-3), 121.9 (C-4), 113.7 (C-4a), 123.9 (C-5), 125.6 (C-6), 157.5 (C-7), 99.9 (C-8), 153.2 (C-8a), 28.2 (C-9), 28.2 (C-10), 65.8 (C-11), 22.9 (C-12), 55.6 (C-13).

6-(1-Ethoxyethyl)-7-methoxy-2,2-dimethylchromene (**4**)

Green oil; ^1H NMR ($CDCl_3$, 600 MHz): δ 7.00 (s, 1H, H-5), 6.34 (s, 1 H, H-8), 6.29 (d, 1H, *J* 9.7 Hz, H-4), 5.45 (d, 1H, *J* 9.7 Hz, H-3), 4.74 (q, 1H, *J* 6.4 Hz, H-11), 3.77 (s, 3H, H-13), 3.39 (dq, 1H, *J* 9.4 7.0 Hz, H-1a′), 3.37 (dq, 1H, *J* 9.4 7.0, 1b′), 1.43 (s, 3H, H-9), 1.43 (s, 3H, H-10), 1.35 (d, 3H, *J* 6.4 Hz, H-12), 1.18 (dd, 3H, *J* 7.0 7.0 Hz, H-2′); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 76.3 (C-2), 127.5 (C-3), 122.2 (C-4), 113.9 (C-4a), 123.9 (C-5), 124.6 (C-6), 157.6 (C-7), 99.3 (C-8), 152.8 (C-8a), 28.2 (C-9), 28.2 (C-10), 70.9 (C-11), 22.8 (C-12), 55.5 (C-13), 63.8 (C-1′), 15.5 (C-2′).

Leishmanicidal screening

Human macrophage cell line THP-1 (ATCC TIB202) was grown in RPMI-1640 without phenol red (Sigma-Aldrich, CO. St. Louis, MO, USA) supplemented with 10% FBS (Life Technologies, USA), 12.5 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml) and Glutamax (2 mM), at 37 °C in a 5% CO_2 incubator. *L. amazonensis* MHOM/BR/77/LTB0016 promastigotes, expressing β-galactosidase, were grown at 26 °C in Schneider's insect medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 5% heat inactivated fetal bovine serum FBS and 2% of human urine.

For the leishmanicidal screening against intracellular *L. amazonensis* amastigotes, THP-1 cells (3.0×10^4 per well) were cultivated in 96 well plates in RPMI-1640 medium supplemented as described above and treated with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) for 72 h at 37 °C in a 5% CO_2, to allow THP-1 cells differentiation into non-dividing macrophages (Schwende et al., 1996).

Four days culture promastigotes (4.0×10^6 parasites/ml) were washed with phosphate buffered saline, pH 7.4 (PBS) an incubated in RPMI-1640 supplemented with 10% human AB+ serum heat-inactivated for 1 h at 34 °C to parasite opsonization. THP-1 cells were incubated with a parasite/cell ratio of 10:1 for 3 h at 34 °C and 5% CO_2. After this period non-adherent parasite were removed by one wash with PBS and infected cells were incubated with 180 μl of full supplemented RPMI-1640 medium for another 24 h to allow the transformation of promastigotes into intracellular amastigotes.

Compounds **1–4** were solubilized in dimethyl sulfoxide (DMSO) and serially diluted (50–0.8 μg ml^{-1}). Infected cell layer were

treated by addition of 20 μl of each sample, in triplicate, followed by incubation for 48 h at 34 °C at 5% CO_2. After treatment, cells were carefully washed with PBS and incubated for 16 h 37 °C with 250 μl of chlorophenolred-ß-D-galactopyranoside (Sigma–Aldrich Co., St. Louis, MO, USA) (CPRG) at 100 μM and Nonidet P-40 (Amresco Inc, Solon, Ohio, USA) (NP-40) 0.1%. Optical density was read at 570/630 nm in an Infinite M200 TECAN, Austria. Amphotericin B (Bristol-Myers, Squibb) was used as positive control and DMSO 1% as negative control.

Results and discussion

The hexane fraction of the leaves from *C. pinnatifida* was phytochemically studied by column chromatography on silica gel and the chromene-rich fractions obtained were further purified by preparative TLC to isolate compounds **1**–**4**. Their chemical structures were established by physical data and 1D and 2D-NMR spectroscopy, and their spectral data were in agreement with published data (Steelink and Marshall, 1979; Fang et al., 1988; Zhai et al., 2010; Harel et al., 2011).

Compounds **1**–**4** showed closely related NMR data. The 1D and 2D-NMR spectral data of these compounds are consistent with those of a chromene skeleton. Thus, compounds **1, 2, 3** and **4** were identified as 6-acetyl-7-hydroxy-2,2-dimethylchromene (eupatoriochromene) (Harel et al., 2011), 6-acetyl-7-methoxy-2,2-dimethylchromene (methyleupatoriochromene) (Zhai et al., 2010), 6-(1-hydroxyethyl)-7-methoxy-2,2-dimethylchromene (encecalinol) (Fang et al., 1988) and 6-(1-ethoxyethyl)-7-methoxy-2,2-dimethylchromene (ethyl encecalol) (Steelink and Marshall, 1979), respectively. There are no spectroscopic or optical evidences to define the absolute stereochemistry of C-11 from compounds **3** and **4**. Optical rotation of encecalinol (**3**) was determined as +45.49 (see details above).

Compound **4** ($C_{16}H_{22}O_3$) was isolated as a green oil. Its ^{1}H NMR spectrum showed the presence of two singlets at δ 7.00 (s, 1H, H-5) and δ 6.34 (s, 1H, H-8), indicating the presence of a 1,2,4,5-tetrasubstituted benzene ring. These signals associated with a pair of AM doublets at δ 6.29 (d, 1H, *J* 9.7 Hz, H-4) and δ 5.45 (d, 1H, *J* 9.7 Hz, H-3), typical of a *cis*-olefin, suggested a benzopyran moiety that together with the presence of a singlet at δ 1.43 (s, 6H, H-9 and H-10), indicated a chromene skeleton. Furthermore, it was also possible to observe a singlet at δ 3.77 (s, 3H, H-13), typical of a methoxy group; a doublet at δ 1.35 (d, 3H, *J* 6.4 Hz, H-12) and a quartet at δ 4.74 (q, 1H, *J* 6.4 Hz, H-11), corresponding to a carbinol hydrogen. This compound also exhibited two doublet of quartets at δ 3.39 (dq, 1H, *J* 9.4 7.0 Hz, H-1a′) and 3.37 (dq, 1H, *J* 9.4 7.0, 1b′) coupled to a doublet of doublets at δ 1.18 (dd, 3H, *J* 7.0 7.0 Hz, H-2′), corresponding to an ethyl group attached to an oxygenated carbon δ C-11. This is the first report of the isolation of the ethyl encecalol in the genus *Calea*.

The ^{13}C NMR assignments were aided by heteronuclear shift correlation experiments such as HSQC and HMBC. The HSQC and HMBC spectra demonstrated the presence of 16 carbon signals, including two sp^2 aromatic carbons (δ_C 99.3, 123.9), two aromatic quaternary carbons (δ_C 113.9, 124.6), two oxygenated aromatic quaternary carbons (δ_C 152.8, 157.6), two olefinic carbons (δ_C 122.2, 127.5), three sp^3 oxygenated carbons (δ_C 55.5, 63.8, 70.9), one sp^3 oxygenated quaternary carbons (δ_C 76.3) and four methyl groups (δ_C 15.5, 22.8, 28.2, 28.2).

Eupatoriochromene has been previously isolated from *Calea* species, such as *C. serrata* (Steinbeck et al., 1997), *C. hispida* (Bohlmann et al., 1982b), *C. oxylepis* (Bohlmann et al., 1982a), *C. rotundifolia* (Bohlmann et al., 1981a) and *C. peckii* (Castro et al., 1989). Moreover, this compound has been tested on *Trypanosoma* (Harel et al., 2013) and insect larvae (Klocke et al., 1985).

Table 1
Leishmanicidal activity of isolated compounds from *Calea pinnatifida*.

Material tested 50 μg ml^{-1}	% Growth inhibition *L. amazonensis* amastigotes
Compound **1**	39.3 (±7.8)
Compound **2**	No activity
Compound **3**	32.3 (±5.7)
Compound **4**	No activity
Negative control (DMSO 1%)	0
Positive control (amphotericin B 2 μM)	86.6 (±5.0)

Methyleupatoriochromene was earlier isolated from *C. oxylepis* (Bohlmann et al., 1982a), *C. teucrifolia* (Bohlmann et al., 1981b), *C. rotundifolia* (Bohlmann et al., 1981a) and *C. morii* (Bohlmann et al., 1981b) and tested against some protozoan parasites (Harel et al., 2013) and insect species (Klocke et al., 1985).

Encecalinol was previously obtained from *C. morii* (Bohlmann et al., 1981b) and was evaluated against protozoan parasites (Harel et al., 2013), pathogenic bacteria (Rios et al., 2003), insect larvae (Klocke et al., 1985) and dermatophytes fungi (Aguilar-Guadarrama et al., 2009).

Ethyl encecalol has been described as an artifact produced during ethanol extraction of species *Encelia farinosa* Gray (Steelink and Marshall, 1979). In general, ethoxylated derivatives are unlikely from the biogenetic point of view. However, this particular compound was also isolated from the *n*-hexane extract of *Ageratum conyzoides*, whose separation and purification steps did not involve any ethanol in the isolation procedure (González et al., 1991). With the aim to confirm if the compound **4** is or not an artifact produced during the extraction with ethanol, a small portion of the plant material was extracted using MeOH and subjected to the same partition procedure. A comparison performed by TLC demonstrated that the ethyl encecalol was not identified in the hexane fraction obtained from MeOH extract, therefore suggesting that this compound may be really an artifact of extraction with ethanol.

The leishmanicidal screening data of the chomenes **1–4** against the amastigote forms of *Leishmania amazonensis* are shown in Table 1. Compounds **2** and **4** did not displayed leishmanicidal activity, while **1** and **3** exhibited moderate effect against *Leishmania* intracellular amastigotes. At a concentration of 50 μg ml^{-1} compounds **1** and **3** inhibited the parasite intracellular growth by 39.3% and 32.3%, respectively. Amphotericin B was used as positive control, producing inhibition of 86.6% at concentration 2 μM, and the negative control (DMSO 1%) did not show any inhibition. Comparing the structure of the four chromenes was found that the polar chromenes (**1** and **3**) were more bioactive than the non-polar chromenes (**2** and **4**), indicating that the polarity may play an important role in the leishmanicidal effect of these compounds.

Natural and synthetic chromenes have been reported to exhibit interesting antiprotozoal effect. A molecular docking analysis was carried out to evaluate potential *Leishmania* protein targets of antiprotozoal plant-derived chromenes and other phenolic compounds (Ogungbea et al., 2014). In this study, a chromene exhibited selective docking to *Leishmania major* N-myristoyltransferase, showing that chromenes may be promising as antiparasitic based on *in-silico* analysis. In other study, it was evaluated the leishmanicidal activity of twelve synthetic chromenes against *in vitro L. major* promastigotes, and some compounds exhibited important leishmanicidal effect (% growth inhibition>70%), showing the potential biological of this chemical class (Alizadeh et al., 2008).

Conclusions

The phytochemical investigation of the fresh leaves of *C. pinnatifida* afforded four known chromenes: 6-acetyl-7-hydroxy-2,2-dimethylchromene (**1**), 6-acetyl-7-methoxy-2,

2-dimethylchromene (**2**), 6-(1-hydroxyethyl)-7-methoxy-2,2-dimethylchromene (**3**) and 6-(1-ethoxyethyl)-7-methoxy-2,2-dimethylchromene (**4**), which are being reported in this species for the first time. Regarding the leishmanicidal activity, compounds **2** and **3** demonstrated moderate leishmanicidal effect.

Authors' contribution

TCL (PhD student) contributed in all steps of this study. DTMC and MS contributed to the biological studies. RJS contributed to isolation and purification of the compounds. AB and ADCS contributed to spectroscopic analysis. MS contributed to critical reading of the manuscript. MWB supervised the laboratory work and contributed to design of the study and critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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