Phytochemistry Letters 13 (2015) 267–274

Contents lists available at ScienceDirect

Phytochemistry Letters

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

Phytochemical analysis of the hot tea infusion of *Hedyosmum brasiliense*

Solomon K.S. Amoah[a], Eirini Kouloura[b], Livia Macedo Dutra[d], Andersson Barison[d], Letícia M. Wildner[c], Maria L. Bazzo[c], Maria Halabalaki[b], Leandros A. Skaltsounis[b], Maique W. Biavatti[a,*]

[a] Programa de Pós-Graduação em Farmácia, Universidade Federal de Santa Catarina – UFSC, Florianópolis, SC, Brazil
[b] Laboratory of Pharmacognosy & Natural Products Chemistry, Faculty of Pharmacy, University of Athens, Panepistimioupoli, Zografou, 15771 Athens, Greece
[c] Laboratório de Biologia Molecular e Micobactérias, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil
[d] NMR Center, Universidade Federal do Paraná–UFPR, Curitiba, PR, Brazil

ARTICLE INFO

Article history:
Received 13 April 2015
Received in revised form 23 June 2015
Accepted 6 July 2015
Available online xxx

Keywords:
Hedyosmum brasiliense
Sesquiterpene lactones
Eudesmanolide
Neolignans
Rosmarinic acid
CPC

ABSTRACT

The leaves of the neotropical dioecious shrub *Hedyosmum brasiliense* (HB) are employed popularly as a sedative, aphrodisiac and as a substitute for green tea.

The aim of this work was to study the composition of the hot tea infusion from the fresh leaves of HB, comparing its androecious or gynoecious parts, including the isolation and identification of new secondary metabolites. The characterization of the HB tea infusion was achieved by means of UHPLC–ESI (±)-HRMS and the isolation of its secondary metabolites was done through FCPC and MPLC. Both *female* and *male* plant extracts presented similar chemical profiles, with rosmarinic acid (RA) as the main compound. The FCPC analysis led to a one-step isolation of RA 97% pure. A new sesquiterpene lactone (1-α-acetoxyeudesma-3,7(11)–dien–8,12–olide) is reported herein for the first time. The additional compounds isolated and characterized by NMR and LC–MS are isorinic acid, a glycosylated flavonol, two neolignans reported for the first time for HB and three other sesquiterpene lactones which were previously isolated from the ethanol extract. Neither the extract nor its major constituent (RA) exhibited *in-vitro* antimycobacterial activity.

1. Introduction

Hedyosmum brasiliense Miq., Chloranthaceae (HB) is an aromatic and dioecious neotropical shrub endemic to Brazil (Souza and Lorenzi, 2008). It is employed in folk medicine irrespective of its androecious or gynoecious parts, in the form of tea or brewed in white wine as a sedative, anti-inflammatory, diuretic, aphrodisiac and also for the treatment of migraines and diseases of the ovary as well as a substitute for green tea (Di Stasi et al., 1988; Reitz, 1965). Reported pharmacological or biological information indicate that the ethanol extract, the essential oil and sesquiterpene lactones from *H. brasiliense* possess antinociceptive (Trentin et al., 1999), antimicrobial (Kirchner et al., 2010) and neurochemical properties (Goncalves et al., 2012; Tolardo et al., 2010). Recently, some sesquiterpene lactones and phenolic compounds from fractions of the ethanol extract have been isolated (Amoah et al., 2013). However, the composition of the aqueous infusion of this species has not been studied and no study has been done to ascertain whether there are differences in the chemical profiles of its androecious and gynoecious plants.

The objective of this work was to find out the secondary metabolites composition of the hot tea infusion from the fresh leaves of *H. brasiliense* using a UHPLC–ESI(±)-HRMS platform for the comparison of the female and male plant of HB. Moreover, the isolation of its secondary metabolites was performed using orthogonal techniques, such as centrifugal partition chromatography (CPC) and medium pressure liquid chromatography (MPLC), with an enrichment step using adsorption resin. This study reports the isolation and identification of nine secondary metabolites from HB, one of them is a new sesquiterpene lactone, together with known phenolic acids, flavonoids and neolignans. Considering the previous reported antimicrobial activity of HB (Kirchner et al., 2010) the antimycobacterial activity of the aqueous extract and its main compound was also investigated in this work.

* Corresponding author at: Laboratório de Farmacognosia, CIF/CCS/UFSC Campus Universitário/Trindade–88,040-900 Florianópolis, SC, Brazil. Tel.: +55 48 3721 3493.
E-mail address: maique.biavatti@ufsc.br (M.W. Biavatti).

http://dx.doi.org/10.1016/j.phytol.2015.07.013
1874-3900/

2. Experimental

2.1. General procedures (instruments and solvents)

All nuclear magnetic resonance (NMR) experiments were performed on an Avance III 600 spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 600 and 150 MHz for ^{1}H and ^{13}C, respectively, equipped with a 5 mm BBI probe. 1 and 2D NMR spectra were recorded using deuterated chloroform ($CDCl_3$) or methanol (CD_3OD). Chemical shifts (δ) are expressed in ppm with reference to the solvent signals (δ_H 7.26/δ_C 77.0) and (δ_H 3.34/δ_C 49.86), respectively, and coupling constants in Hz. The 2D NMR experiments (COSY, HSQC, HMBC, and NOESY) were performed using standard Bruker microprograms. Mass spectrometry analyses were performed on an LTQ-Orbitrap Discovery instrument (Thermo Finnigan, San Jose, CA, USA) coupled or not to an Accela LC system consisting of pump and autosampler. Xcalibur software version 2.0.7 was used for the acquisition and the processing of data.

Fast centrifugal partition chromatography (FCPC) was performed using a CPC Kromaton with a 1000 mL column and a Laboratory Alliance pump with a pressure safety limit of 50 bar. A manual sample injection valve was used to introduce the samples into the column, with the rotation adjusted at 600 rpm and the flow rate held at 20 mL/min. MPLC separation was carried out using a Sepacore MPLC system (Büchi C-650 pump) with a maximum applied pressure of 10 bar. Chromatographic separations were performed on a Buchi PP Cartridge with dimensions 12 mm × 150 mm filled with normal phase silica gel (Merck 0.02–0.04 mm).

Solvents used for centrifugal partition chromatography (CPC), medium pressure liquid chromatography (MPLC) and preparative thin layer chromatography were of analytical grade (Fisher Scientific). Ultra gradient grade solvents (MeOH, ACN) for HPLC separations were purchased from Carlo Erba, and deionized water with a resistivity of 18MΩcm was used. Solvents of LC–MS grade were purchased from Fluka/Riedel-de Haën (Switzerland).

2.2. Plant material and extraction

H. brasiliense leaves from androecious and the gynoecious plants were separately collected at the municipal area of Antonio Carlos in the Santa Catarina State, Brazil in December 2011. The plant was identified by Dr. Ademir Reis and compared with the voucher specimen (#2031) deposited at the Lyman Bradford Smith Herbarium (UNIVALI, Itajaí—Santa Catarina).

The fresh harvested leaves of the androecious and the gynoecious plant (2 kg each) were infused with hot deionized water (10 L

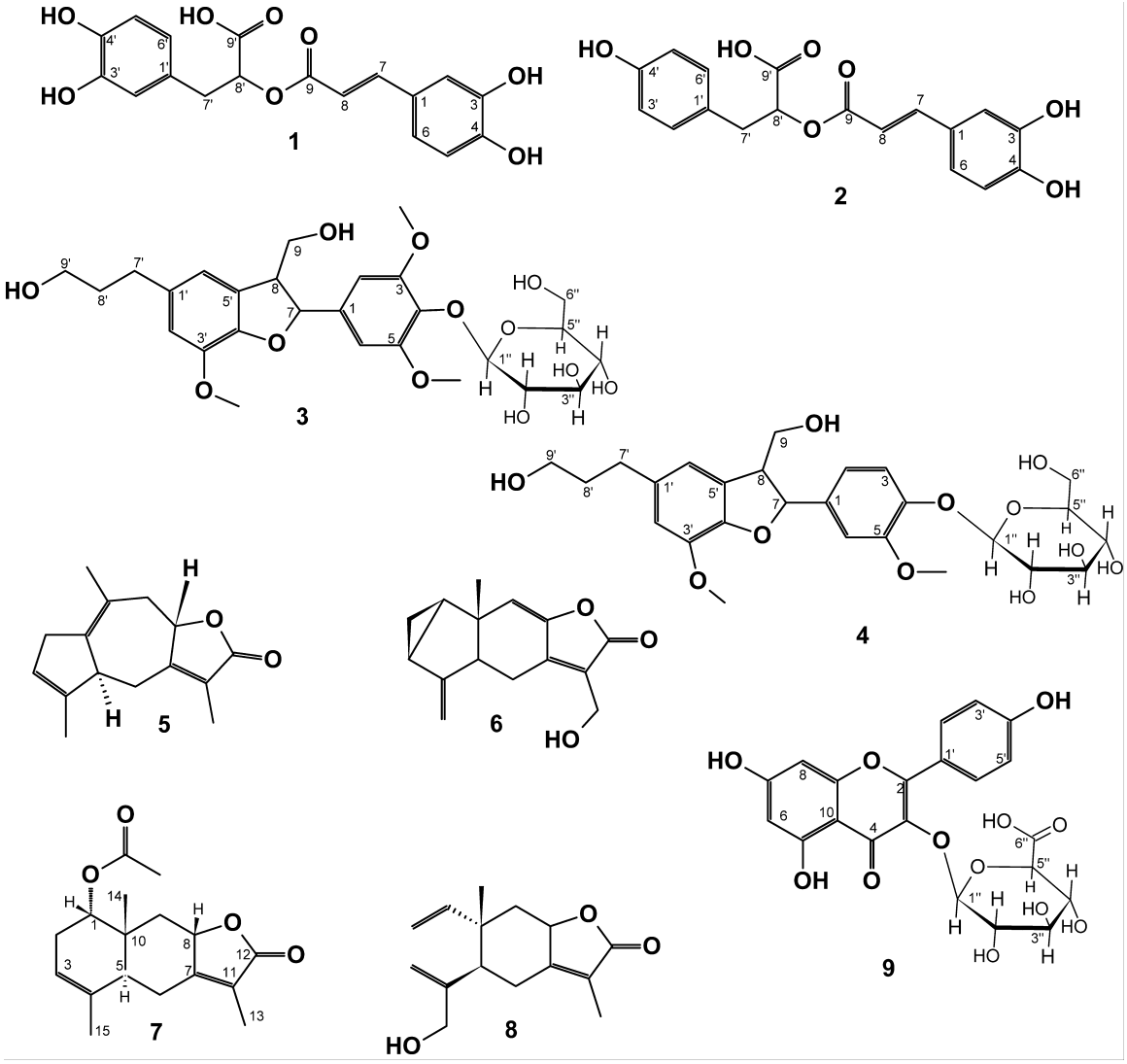

Fig. 1. Compounds from *Hedyosmum brasiliense*.

each) (≈100 °C) for 30 min. The resulting extracts were frozen and freeze-dried to yield 31 and 28 g of the *male* crude aqueous extract (MCE) and *female* crude aqueous extract (FCE), respectively.

2.3. Sample preparation

All samples (extracts, fractions and isolated compounds) were diluted in methanol. For LC–MS analysis, solutions of 500 μg/mL for the extracts and of 10 μg/mL for the isolated compounds were used. For Semi preparative HPLC, fractions were diluted to obtain a solution of 20 mg/mL.

2.4. LC–MS analysis

A Hypersil Gold (100 × 2.1 mm, 3 μm) column was used for the analysis and a flow rate of 0.5 mL/min, with a mobile phase of water −0.1% formic acid (solvent A) and methanol (solvent B) was applied according to the following stepwise gradient program (v/v): 0 min 1% B linear, 3 min 1% B, 8 min 60% B, 22 min 100% B, 25 min 100% B, 27 min 1% B, 30 min 1% B. The ionization was performed using an ESI source in the positive and negative modes. ESI–MS parameters were as follows in positive mode: Source voltage 4.5 kV, capillary voltage 25 V, tube lens voltage 85 V, capillary temperature 300 °C, Sheath gas 37, auxiliary gas flow (N_2) 10 and in negative mode: Source voltage 3.5 kV, capillary voltage −17 V, tube lens voltage −65 V, capillary temperature 300 °C, Sheath gas 40, auxiliary gas flow (N_2) 10. Full scans were acquired in profile mode at 30,000 (FWHM). Resolution and detection were performed considering a mass range of *m/z* 100–1000. For direct infusion experiments, a syringe pump (500 μL syringe, Hamilton Bonaduz AG, Bodanuz, Switzerland) was coupled with the LTQ-Orbitrap mass spectrometer. For these experiments, the ESI operation parameters and the acquisition parameters were identical to the aforementioned apart from the nitrogen flow which was adjusted to 12 and 6 arb units as sheath and auxiliary gas, respectively. The flow rate of the injection was set at 5 μL/min.

2.5. LC-DAD analysis

The chromatographic profiling of all extracts, fractions, and purity determination of isolated compounds was performed by LC-DAD. An ultrafast liquid chromatography (Prominence UFLC Shimadzu, Japan) apparatus connected to a SPD-20A UFLC UV–vis detector was applied using a Zorbax Eclipse XDB-C18 column (2.1 mm × 150 mm, 3,5 um). The mobile phase was composed of H_2O–MeOH (0.1% of formic acid in H_2O 80:20 → 20:80) gradient at a flow-rate of 0.2 mL/min and was detected at 254 nm, 280 nm and 330 nm.

2.6. Isolation of compounds

In order to obtain an enriched fraction of secondary metabolites Fig. 1, adsorption on Amberlite FPX66 resin (Dow Chemicals, 5 kg) was used, activated in a separatory funnel with methanol. Subsequently, the methanol was removed and the resin was washed thoroughly with deionized water (4L). 5 g of the FCE was dissolved in 700 mL of water and poured into the separating funnel containing the resin, allowed to stand for an hour with intermittent stirring with a rod. The extract was then removed and the resin washed with water to remove excess sugars (discarded). Subsequently, elution was done with methanol to obtain a methanol fraction (FCE-M). This process was repeated four times such that 20 g of the FCE were treated. The combined methanol extracts were dried under reduced pressure to afford 7.5 g of FCE-M.

The fractionation of the FCE-M was performed by FCPC using a two-phase solvent system consisted of *n*-butanol-ethyl acetate–water (4:1:5). In details, for the preparation of the two-phase solvent system, each solvent was added to a separatory funnel and the mixture was equilibrated at room temperature. The two phases were separated and the stationary phase consisting of the lower phase (polar) was pumped into the column and then the upper (mobile) phase was pumped in the descending mode until equilibration of the biphasic solvent system. FCE-M (5 g) was injected into the column diluted in 50 mL of the lower phase and the separation yielded 20 fractions: A1–A13 in ascending mode and A14–A20 in the descending mode.

CPC fractions A4–A6 afforded rosmarinic acid (RA) (1.7 g) (**1**) 97% pure**.** Fractions A9, A10 and A18 were combined (246 mg) due to their similarity and subjected to gel filtration on Sephadex (LH-20) with methanol as the mobile phase. This led to the subfractions A23–T23. Subfraction E23 (27.9 mg) was subjected to normal phase preparative TLC eluted with dichloromethane-ethyl acetate (9:1) which led to compound **3** (3.0 mg), identified as the neolignan (7*S*, 8*R*)-5-methoxydihydrodehydrodiconiferyl

Table 1
NMR data (600 MHz, $CDCl_3$) for **7**.

Position	δ ^{1}H (mult., J Hz)	δ ^{13}C	HMBC	COSY
1	4.72_{eq} m	74.5	C-2, C-3, C-5, C-10, and COMe	H-2_{peq},
2	2.46_{peq} m	29.0		H-2_{pax}, H-1
	2.11_{pax} m			
3	5.34 m	119.0		H-2_{peq}, H-2_{pax} and H-15
4		132.8		
5	2.29 m	41.2		H-6_{ax} and H-6_{aq}
6	2.18_{ax} ddm (13.8, 13.6)	24.8	C-5, C-7 and C-11	H-6_{eq} and H-5
	3.01_{eq} dd (13.8; 3.8)		C-5, C-7, C-8, C-10, and C-11	H-6_{ax} and H-5
7		161.4		
8	4.92_{ax} m	78.4	7	H-9_{ax}, H-9_{eq}
9	2.08_{eq} m	41.0	C-1, C-8, C-10, and C-14	H-8 and H-9_{ax}
	1.33_{ax} dd (11.9, 11.8)		C-1, C-5, C-7, C-8, C-10, and C-14	H-8 and H-9_{eq}
10		37.2		
11		120.9		
12		174.5		
13	1.87 s	8.3	C-7, C-11 and C-12	H-8
14	1.00 s	16.0	C-1, C-5, C-9, and C-10	
15	1.77 s	21.0	C-3, C-4 and C-5	H-3
COMe		170.4		
COMe	2.02 s	21.2	COMe	

NMR chemical shifts are given in ppm relative to TMS as an internal reference at δ 0.00. Long-range ^{1}H–^{13}C (HMBC) correlations were optimised for 8 Hz, and are from the stated hydrogen(s) to the indicated carbon.

alcohol-4-*O*-β-D-glucopyranoside. Subfractions K23–M23 (50.2 mg) were submitted to semiprep-HPLC using H_2O-MeOH (90:10 → 10:90) as gradient at a flow rate of 3 mL/min, from which **1** (3 mg) and **2** (4.2 mg, Isorinic acid) were obtained. Similarly, the CPC fraction A8 (134.5 mg) was also subjected to semiprep-HPLC and afforded **9** (1.5 mg) identified as the flavonoid kaempferol-3-*O*-β-D-glucuronide. CPC fraction A15 (30.6 mg) was submitted to MPLC starting with dichloromethane (50%) in hexane increasing to 100% dichloromethane. Subsequently, separation was continued by increasing polarity with ethyl acetate (EtOAc) until 100%. This led to the production of subfractions A25–K25 as well as A26–P26. There was a spontaneous crystallization of B25 affording **5** (11.8 mg) which was elucidated as the sesquiterpene lactone podoandin. Subfraction K25 (19 mg) was submitted to TLC-prep which led to the isolation of compounds **6** (4.9 mg) and **7** (1.3 mg) identified also as sesquiterpene lactones onoseriolide and 1-α-acetoxyeudesma-3,7(11)–dien–8,12–olide respectively, with compound **7** as a new eudesmanolide. Subfraction C26 (4.9 mg) was also subjected to TLC-prep which yielded **8** and was characterized as the sesquiterpene lactone 15-hydroxy-isogermafurenolide. CPC fraction A13 (15.7 mg) was subjected to Prep RP-TLC (H_2O–MeOH, 6:4) which afforded **4** identified as the neolignan (7*S*, 8*R*)-urolignoside (2 mg).

1-α-acetoxyeudesma-3,7(11)–dien–8,12–olide (**7**), (4a*S*,8*S*,8a-*R*,9a*S*)-3,5,8a-trimethyl-2-oxo-2,4,4a,7,8,8a,9,9a-octahydronaphtho[2,3-b]furan-8-yl acetate. HR-ESI–MS ($[M+H]^+$): *m/z* found: 291.1587, calculated = 291.159086. HR-ESI–MS ($[M+Na]^+$): 313.1405. Molecular formula $C_{17}H_{22}O_4$; ^1H and ^{13}C NMR data ($CDCl_3$): See Table 1 spectra available as supplementary information.

2.7. Antimycobacterial assay

The extracts and isolated compounds were screened for antimycobacterial activity in concentrations ranging from 100 μg/mL to 1.56 μg/mL against the reference strain *Mycobacterium tuberculosis* H37Rv. The resazurin microtiter assay plate was carried out as described by Palomino et al. (2002) with the following modifications. The method was performed in 7H9-S medium containing Middlebrooks broth (Himedia, India), 0.5% glycerol (Amresco, USA) and supplemented with OADC (oleic acid, albumin, dextrose and catalase) enrichment (Himedia, India). One hundred microliters of serial two fold dilutions of each extract or isolated compound were added in 96-well microplates, followed by 100 μL of the inoculum, wich was prepared in 7H9-S medium, adjusted to a McFarland tube no. 1 and diluted 1:20. Isoniazid (Sigma–Aldrich, USA) 1.0 μg/mL was used as positive control and sterility control without inoculation and a growth control containing no drug were also included. The plates were covered, sealed and incubated at 37 °C. After 7 days of incubation, 30 μL of 0.01% resazurin solution (Sigma, USA) was add to each well and the plates were re-incubated for 24 h. A color change from blue to pink indicates reduction of resazurin and therefore bacterial growth.

3. Results and discussion

3.1. Comparative LC–MS profiling of male and female HB extracts and its HR-MS analysis

Lokvam and Braddock (1999) have shown differences in the TLC profiles of resins from the male and female neotropical dioecious plant *Clusia grandiflora*. Considering that people use indiscriminately HB infusion as remedy made from both sexes, LC–MS analysis in the negative mode of separate plants were conducted. Similarities in their profiles were found (Fig 2). Both sexes showed rosmarinic acid (**1**) as a major peak with a molecular ion at 359.077 *m/z* $[M-H]^-$ and minor peaks at retention times of about 11.3, 11.6 and 13.3 min. The total ion chromatogram of both FCE and MCE extracts showed no significant differences. The profiling of both male and female extracts of HB by UHPLC–ESI–MS led to the detection of all described compounds in both extracts (Table 2). In particular, the ionization of all compounds was feasible in positive mode (Figs. 4 and 5).

Hydroxycinnamic acid derivatives, compounds **1** and **2**, were detected at 12.20 and 13.22 min respectively from their molecular ions $[M+H]^+$ in positive mode. Interestingly, the fragmentation of

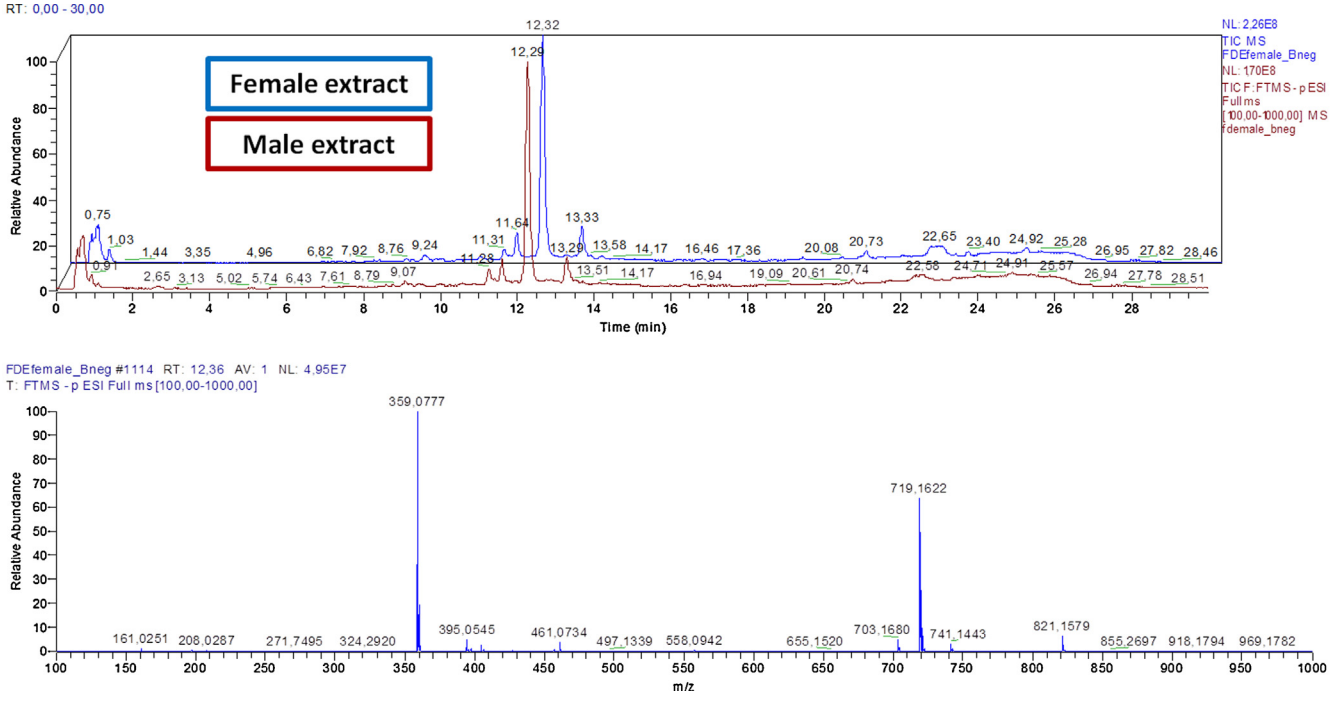

Fig. 2. Total Ion chromatogram of the FCE (Female crude extract) and MCE (Male crude extract).

both compounds led to the generation of caffeoyl ions at *m/z* 163.0369 corresponding to $C_9H_7O_3$. This finding is in accordance with studies reporting the presence of this typical ion under CID fragmentation in ESI(−) (Hossain et al., 2010).

The neolignans **3** and **4** ionized only in positive mode generating ions $[M+Na]^+$ (Table 2). Their detection at 11.21 and 10.86 min respectively, confirmed their relative polar nature. The fragmentation of the $[M+Na]^+$ ion resulted in the cleavage of the glycoside moiety leading to the loss of 162 Da from the structure. The sesquiterpene derivatives were observed between 15.5 and 18.1 min due to their nonpolar properties, generating both molecular $[M+H]^+$ and $[M+Na]^+$ ions. The fragmentation of podoandin (**5**) in ESI(+) revealed a major fragment ion at *m/z* 213.1277 corresponding to the loss of a H_2O and an ion at *m/z* 185.1327 corresponding to an additional loss of carboxyl group (−28 Da) suggesting the rupture of the lactone ring. In the case of onoseriolide (**6**), a fragment ion at *m/z* 199.0756 denotes the cleavage of a C_2H_6O moiety probably due to of the localization of the double bond at C8-C9 which probably hampers the elimination of the carboxylate group. Finally, compound (**7**) presented a fragment ion at *m/z* 231.1383 revealing the loss of an acetoxy group from the core structure.

3.2. Isolation and identification of compounds from female HB extract

Since the chemical profiles of the FCE and MCE extracts were similar, the FCE extract was chosen for fractionation with the FPX66 resin and subsequently subjected to FCPC. Macroporous resins have been useful for phytochemical analysis in natural products due to an extensive presence of sugar compounds in polar extracts (Ma et al., 2011; Papaspyridi et al., 2012; Zhang et al., 2008). The resin was used to separate carbohydrates from other secondary metabolites. The obtained fraction (FCE-M) which represents 37% of the crude aqueous extract was subjected to FCPC technique for separation and purification of the metabolites.

This orthogonal technique is highly efficient because of its lack of irreversible adsorption to a stationary phase and also by the complete recovery of sample making its use in combination with other techniques an interesting strategy to maximize the separation and isolation of metabolites (Simmler et al., 2014). The FCPC analysis of FCE-M with the biphasic solvent *n*-butanol-ethyl acetate–water (4:1:5), led to a one-step isolation of rosmarinic acid (RA) (**1**) in high purity (97%). When analyzed on the UFLC it was found to possess a purity index of 0.96 (Fig. 3). This is the first time that RA is reported for this species. RA was identified by extensive 1D and 2D NMR and comparison with published data (Ha et al., 2012). Previously, RA had been identified in *Chloranthus oficinalis* (Petersen et al., 2009) and *Sarcandra glabra* (Zhu et al., 2008). RA is known to possess many biological and pharmacological activities including anticancer and neurochemical properties as well as potent antioxidant properties (Krajcovicová and Melus, 2013). The presence of this compound as a major component in the hot infusion of *H. brasiliense* may be one of the reasons why it used to treat various affections in folk medicine (Reitz, 1965). *H. brasiliense* may be considered a source of RA for the food and cosmetic industries due to the high yield of this compound which is employed in food preservation and cosmetics. From the same FCE-M fraction subjected to FCPC, the following compounds were obtained:

Compound **2** was identified as isorinic acid (Satake et al., 1999), also known as caffeoyl-4′-hydroxy-phenyllactic acid, that is assumed to be one of the intermediates for the biosynthesis of **1** (Petersen et al., 2009). Compounds **3** and **4** were identified as neolignans (7*S*, 8*R*)-5-methoxydihydrodehydrodiconiferyl alcohol-4-*O*-β-D-glucopyranoside (Takara et al., 2003) and (7*S*, 8*R*)-urolignoside (Shen et al., 1998) respectively. These compounds are being described for the first time in the genus *Hedyosmum* although they have also been found in other Chloranthaceae species such as *Chloranthus japonicus* (Kuang et al., 2009) and *Sarcandra glabra* (Wu et al., 2012). Compounds **2–4** were identified through MS and NMR data comparing to those reported in the literature. Likewise compounds **5** and **6** were found to be the sesquiterpene lactones podoandin and onoseriolide respectively based on their MS and NMR data comparing to those reported in the literature (Kubo et al., 1992) (Trentin et al., 1999). Recently, a series of pharmacological assays have shown that the sesquiterpene lactone podoandin has antidepressant activities in mice (Goncalves et al., 2012; Tolardo et al., 2010).

The new sesquiterpene lactone **7** was obtained as a colourless gum with a molecular formula $C_{17}H_{22}O_4$, deduced from its NMR data and its HR-ESIMS showing the pseudo-molecular peak at *m/z* 291.1587 $[M+H]^+$ (calc. 291.159086) and *m/z* 313.1405 $[M+Na]^+$ and NMR data. The 1H NMR spectrum of **7** showed methyl singlets at δ 1.00 (H-14), 1.77 (H-15) and 1.87 (H-13) as well as a singlet at δ 2.02 characteristic of an acetate methyl group. The methyl hydrogens at δ 1.87 showed direct $^1H–^{13}C$ correlation with a carbon at δ 8.3 according to HSQC NMR experiment which is consistent with the presence of a neighboring carbonyl group. Moreover, these hydrogens showed long-range $^1H–^{13}C$ correlations with the carbons at δ 161.4 (C-7), 120.9 (C-11) and 174.5 (C-12), according to HMBC NMR experiment indicating the presence of a five-member α,β-unsaturated-γ-lactone. The hydrogens at δ 2.02 showed direct $^1H–^{13}C$ correlation with a carbon at δ 21.2, and a long-range $^1H–^{13}C$ correlation with an acetate carbonyl at δ

Table 2
HR-MS and Tandem data of (**1–9**) in ESI(+).

No	Rt	$[M+H]^+$				$[M+Na]^+$				RDB	Molecular formula
		m/z Experimental	Delta (ppm)	MS/MS	Loss	*m/z* Experimental	Delta (ppm)	MS/MS	Loss		
1	12.2	361.0909	2.51	163.0369	$C_9H_{10}O_5$					11	$C_{18}H_{16}O_8$
2	13.22	345.0964	2.97			367.0783	2.97			11	$C_{18}H_{16}O_7$
3	11.21					575.2081	2.97	413.157	$C_6H_{10}O_5$	10	$C_{27}H_{36}O_{12}$
4	10.86					545.1987	2.22			10	$C_{26}H_{34}O_{11}$
5	18.09	231.1374	2.23	213.1277 185.1327	H_2O CH_2O_2	253.1194	2.17			7	$C_{15}H_{18}O_2$
6	16.02	245.1169	1.51	227.1069 199.0756	H_2O C_2H_6O	267.0988	1.56			8	$C_{15}H_{16}O_3$
7	15.74	291.1587	1.36	273.1491 231.1383	H_2O $C_2H_4O_2$	313.1405	1.59			7	$C_{17}H_{22}O_4$
8	13.51	249.1480	2.17	231.1381 203.1431 185.1325	H_2O CH_2O_2 CH_4O_3	271.1299	2.08			6	$C_{15}H_{20}O_3$
9	12.25	463.0862	2.14	287.0554	$C_6H_8O_6$	485.0679	3.03			13	$C_{12}H_{18}O_{12}$

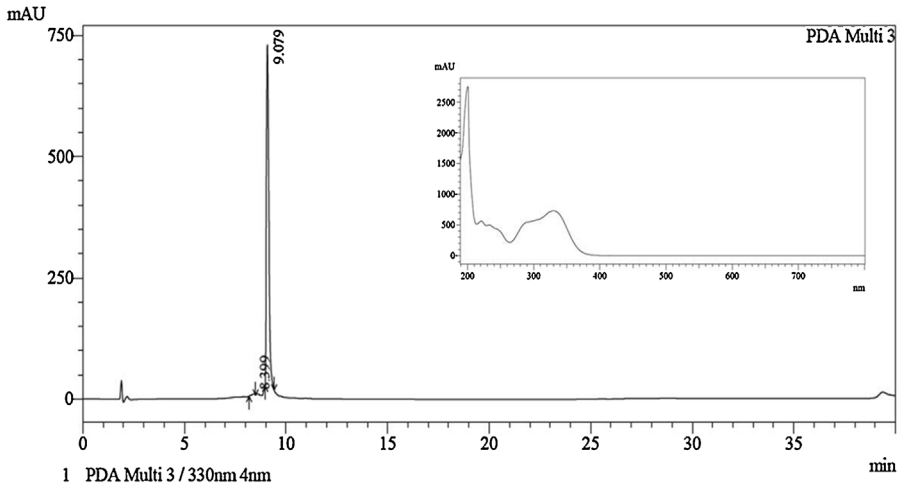

Fig. 3. Chromatogram and UV spectrum of rosmarinic acid (**1**).

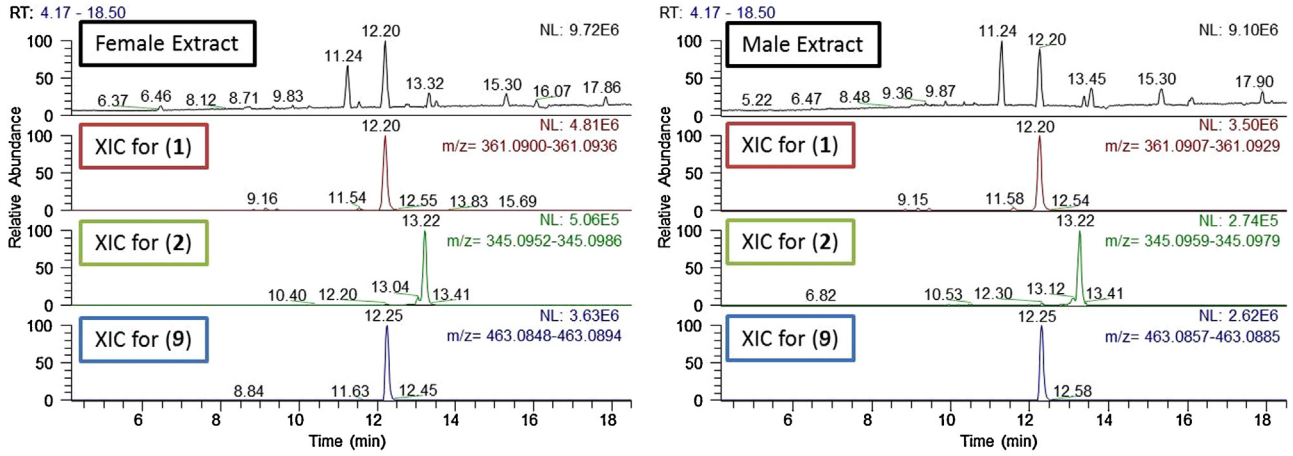

Fig. 4. Base peak chromatograms of the female and male HB extracts in ESI(+) and extracted ion chromatograms for compounds **1, 2, 9**.

170.4. The location of the acetyl group was established based on the long-range $^{1}H–^{13}C$ correlation observed for the hydrogen at δ 4.72 (H-1) with the carbonyl at δ 170.4; H-1 showed further HMBC correlations with carbons resonating at δ 37.2 (C-10), 41.2 (C-5) and 119.0 (C-3). The hydrogens of the methyl group at δ 1.00 showed direct $^{1}H–^{13}C$ correlation with the carbon at δ 16.0 (C-14) and $^{1}H–^{13}C$ long-range correlations with the carbons at δ 74.5 (C-1), 41.2 (C-5), 41.0 (C-9), and 37.2 (C-10). The hydrogens of the methyl group at δ 1.77 showed direct $^{1}H–^{13}C$ correlation with the carbon at δ 21.0 (C-15) and $^{1}H–^{13}C$ long-range correlations with the carbons

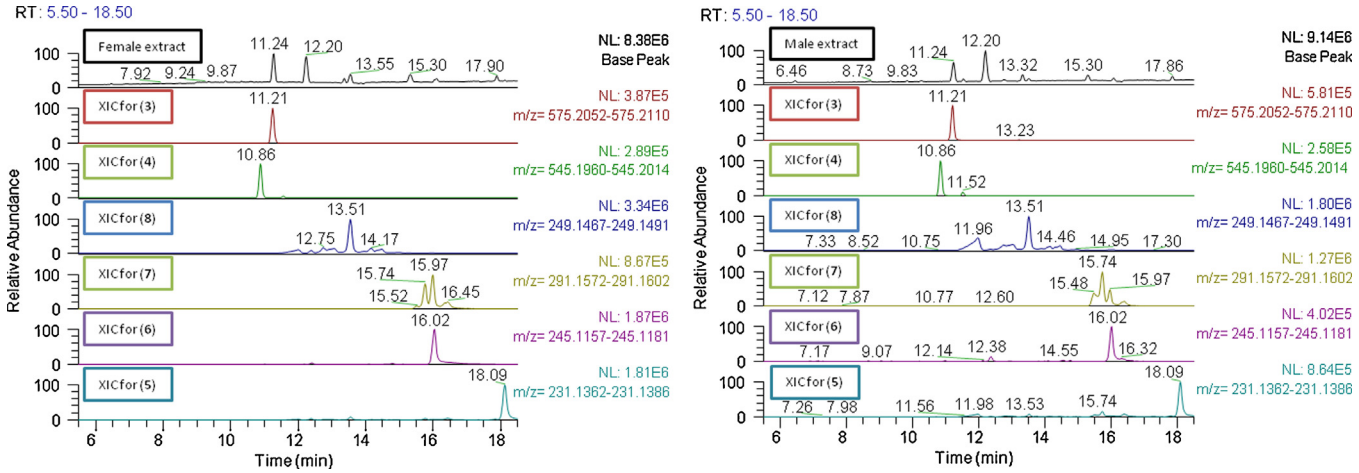

Fig. 5. Base peak chromatograms of the female and male HB extracts in ESI(+) and extracted ion chromatograms for compounds **3–8**.

at δ 41.2 (C-5), and with the olefin carbons at δ 132.8 (C-4) and 119.0 (C-3). According to the HSQC experiment, C-3 is bound to hydrogen at δ 5.34 (H-3). According to $^{1}H–^{1}H$ correlations from COSY NMR experiment, this hydrogen couples to the hydrogens at δ 2.46 (H-2peq) and 2.11 (H-2pax) which are directly connected to the carbon at δ 29.0 as observed in the HSQC NMR experiment. Further COSY correlations were revealed between the hydrogens at δ 2.46 (H-2peq) and 2.11 (H-2pax) and the deshielded hydrogen at δ 4.72 (H-1).

The methylene hydrogen signals at δ 2.08 (H-9eq) and 1.33 (H-9ax) gave a direct $^{1}H–^{13}C$ correlation with the carbon at δ 41.0, and showed HMBC correlations with the carbons at δ 161.4 (C-7) and 78.3 (C-8). Moreover, COSY correlations indicated a coupling between these hydrogens and the deshielded hydrogen at δ 4.92 (H-8ax). Additionally, the quaternary carbon at δ 161.4 (C-7) showed $^{1}H–^{13}C$ long-range correlations with the methylene hydrogens at δ 2.18 (H-6ax) and 3.01 (H-6eq) which in turn correlated with the carbon at δ 41.2 (C-5).

In order to determine the relative stereochemistry of the molecule, 1D NOE NMR experiments were performed. By irradiation of the methyl hydrogens H-14 a positive NOE was observed on H-1, H-8, H-2pax, H-6ax and also H-9eq, with no enhancement of the signal from the hydrogen H-5. The irradiation of H-1, caused a NOE enhancement on the signals of H-14, H-2pax. These data confirmed the *trans*-fused decalin system of the eudesmane skeleton (Ando et al., 1994), leding to the identification of compound **7** as the new eudesmane sesquiterpene lactone 1-α-acetoxyeudesma-3,7(11)–dien–8,12–olide and the overall analysis of 1D and 2D NMR data enabled complete and unequivocal ^{1}H and ^{13}C NMR chemical shifts assignments (Table 1). Previously, only two similar semisynthetic compounds have been reported, one that was acetylated (Zdero and Bohlmann, 1989) and the other derived from the germacranolide laurenobiolide (Haruhiko and Kenichi, 1976). This is the first eudesmanolide described in the genus *Hedyosmum*. Some eudesmane sesquiterpene lactones have been previously identified in *Chloranthus elatior* (Wang et al., 2012).

Differently from compound **7**, compound **8** presented typical NMR data of terminal methylenes of the elemene skeleton, turned it also to be a sesquiterpene lactone identified as 15-hydroxy-isogermafurenolide based on its NMR data in conjunction with those published in the literature (Amoah et al., 2013).

The structure of compound **9** was established as kaempferol-3-*O*-β-D-glucuronide (Granica et al., 2013). To the best of our knowledge this is the first time that this flavonoid glycoside is reported in *H. brasiliense*. This compound has been previously found in the *n*-butanol extract of *S. glabra* and shown to have activity against *Staphylococcus aureus* (Yuan et al., 2008). The isolation of other flavonoid glycosides like kaempferol-3-*O*-α-L-rhamnopyranosyl (1 → 6)-β-D-glucopyranoside and kaempferol-3-*O*-β-D-glucopyranoside have been reported for *H. bonplandianum* (Cardenas et al., 1993) and from other Chloranthaceae species (Cao et al., 2008).

3.3. Antimycobacterial assay

The FCE-M extract and its isolated major compound (**1**) did not show any significant activity against *M. tuberculosis*. However, extracts of *Thymbra spicata* var spicata, *Salvia tomentosa*,*Salvia fruticosa* and *Origanum onites* which have **1** as one of the major constituents have shown moderate-high activities against *M. tuberculosis*, suggesting minority compounds can be more active in these above mentioned plant extracts (Askun et al., 2009).

4. Conclusion

From the aqueous extract of *H. brasiliense* nine compounds were isolated and characterized, in which five are reported for the first time in HB and one of them is a new sesquiterpene lactone (1-α-acetoxyeudesma-3,7(11)–dien–8,12–olide), for the first time reported. Additionally, three sesquiterpene lactones previously isolated from the ethanol extract were also found in the aqueous hot infusion of *H. brasiliense*. The male and female plants of this dioecious plant possess the same profile of secondary metabolites.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to CAPES, CNPq, Finep, Fundação Araucária, UFPR and the CHEMBIOFIGHT PROJECT (code number 269301) for financial support and fellowships and the Centro de Biologia Molecular (CEBIME) from UFSC for LC- DAD analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2015.07.013.

References

Amoah, S.K.S., de Oliveira, F.L., da Cruz, A.C.H., de Souza, N.M., Campos, F.R., Barison, A., Biavatti, M.W., 2013. Sesquiterpene lactones from the leaves of *Hedyosmum brasiliense* (Chloranthaceae). Phytochemistry 87, 126–132.

Ando, M., Arai, K., Kikuchi, K., Isogai, K., 1994. Synthetic studies of sesquiterpenes with a cis-fused decalin system, 4. Synthesis of (+)-5βH-eudesma-3, 11-diene, (−)-5βH-eudesmane-4β, 11-diol, and (+)-5βH-eudesmane-4α 11-diol, and structure revision of a natural eudesmane-4, 11-diol isolated from Pluchea arguta. J. Nat. Prod. 57, 1189–1199.

Askun, T., Tumen, G., Satil, F., Ates, M., 2009. In vitro activity of methanol extracts of plants used as spices against *Mycobacterium tuberculosis* and other bacteria. Food Chem. 116, 289–294.

Cao, C.M., Peng, Y., Shi, Q.W., Xiao, P.G., 2008. Chemical constituents and bioactivities of plants of Chloranthaceae. Chem. Biodivers. 5, 219–238.

Cardenas, L.C., Rodriguez, J., Villaverde, M.C., Riguera, R., Cadena, R., Otero, J.A., 1993. The analgesic activity of *Hedyosmum bonplandianum*: flavonoid glycosides. Planta Med. 59, 26–27.

Di Stasi, L.C., Costa, M., Mendacolli, S.L.J., Kirizawa, M., Gomes, C., Trolin, G., 1988. Screening in mice of some medicinal plants used for analgesic purposes in the state of Sao Paulo. J. Ethnopharm. 24, 205–211.

Goncalves, A.E., Burger, C., Amoah, S.K., Tolardo, R., Biavatti, M.W., de Souza, M.M., 2012. The antidepressant-like effect of *Hedyosmum brasiliense* and its sesquiterpene lactone, podoandin in mice: evidence for the involvement of adrenergic, dopaminergic and serotonergic systems. Eur. J. Pharmacol. 674, 307–314.

Granica, S., Czerwińska, M.E., Żyżyńska-Granica, B., Kiss, A.K., 2013. Antioxidant and anti-inflammatory flavonol glucuronides from *Polygonum aviculare* L. Fitoterapia 91, 180–188.

Ha, T.J., Lee, J.H., Lee, M.-H., Lee, B.W., Kwon, H.S., Park, C.-H., Shim, K.-B., Kim, H.-T., Baek, I.-Y., Jang, D.S., 2012. Isolation and identification of phenolic compounds from the seeds of *Perilla frutescens* (L.) and their inhibitory activities against α-glucosidase and aldose reductase. Food Chem. 135, 1397–1403.

Haruhiko, T., Kenichi, T., 1976. Sesquiterpenes of lauraceae plants. IV. Germacranolides from *Laurus nobilis* L. Chem. Pharm. Bull. 24, 667–671.

Hossain, M.B., Rai, D.K., Brunton, N.P., Martin-Diana, A.B., Barry-Ryan, C., 2010. Characterization of phenolic composition in lamiaceae spices by LC-ESI–MS/MS. J. Agric. Food Chem. 58, 10576–10581.

Kirchner, K., Wisniewski, A., Cruz, A.B., Biavatti, M.W., Netz, D.J.A., 2010. Chemical composition and antimicrobial activity of *Hedyosmum brasiliense* Miq., Chloranthaceae, essential oil. Rev. Bras. Farmacogn. 20, 692–699.

Krajcovicová, Z., Melus, V., 2013. Bioactivity and potential health benefits of Rosmarinic acid. Univ. Rev. 7, 8–14.

Kuang, H.-X., Xia, Y.-G., Yang, B.-Y., Wang, Q.-H., Lü, S.-W., 2009. Lignan constituents from *Chloranthus japonicus* Sieb. Arch. Pharm. Res. 32, 329–334.

Kubo, I., Ying, B.P., Castillo, M., Brinen, L.S., Clardy, J., 1992. Podoandin, a molluscicidal sesquiterpene lactone from *Podocarpus andina*. Phytochemistry 31, 1545–1548.

Lokvam, J., Braddock, J.F., 1999. Anti-bacterial function in the sexually dimorphic pollinator rewards of *Clusia grandiflora* (Clusiaceae). Oecologia 119, 534–540.

Ma, C.H., Liu, T.T., Yang, L., Zu, Y.G., Yang, F.J., Zhao, C.J., Zhang, L., Zhang, Z.H., 2011. Preparation of high purity biphenyl cyclooctene lignans from *Schisandra* extract by ion exchange resin catalytic transformation combined with macroporous resin separation. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879, 3444–3451.

Palomino, J.-C., Martin, A., Camacho, M., Guerra, H., Swings, J., Portaels, F., 2002. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 46, 2720–2722.

Papaspyridi, L.M., Aligiannis, N., Topakas, E., Christakopoulos, P., Skaltsounis, A.L., Fokialakis, N., 2012. Submerged fermentation of the edible mushroom *Pleurotus ostreatus* in a batch stirred tank bioreactor as a promising alternative for the effective production of bioactive metabolites. Molecules 17, 2714–2724.

Petersen, M., Abdullah, Y., Benner, J., Eberle, D., Gehlen, K., Huecherig, S., Janiak, V., Kim, K.H., Sander, M., Weitzel, C., Wolters, S., 2009. Evolution of rosmarinic acid biosynthesis. Phytochemistry 70, 1663–1679.

Reitz, R., 1965. Clorantaceas,–Flora Ilustrada Catarinense. Herbário Barbosa Rodrigues. Itajai 4–10.

Satake, T., Kamiya, K., Saiki, Y., Hama, T., Fujimoto, Y., Kitanaka, S., Kimura, Y., Uzawa, J., Endang, H., Umar, M., 1999. Studies on the constituents of fruits of *Helicteres isora* L. Chem. Pharm. Bull 47, 1444–1447.

Shen, Y.C., Hsieh, P.W., Kuo, Y.H., 1998. Neolignan glucosides from *Jasminum urophyllum*. Phytochemistry 48, 719–723.

Simmler, C., Nikolić, D., Lankin, D.C., Yu, Y., Friesen, J.B., van Breemen, R.B., Lecomte, A., Le, Que'mener, Audo, G.G., Pauli, G.F., 2014. Orthogonal analysis underscores the relevance of primary and secondary metabolites in licorice. J. Nat. Prod. 77, 1806–1816.

Souza, V.C., Lorenzi, H., 2008. Botânica sistemática: guia ilustrado para identificação das famílias de Fanerógamas nativas e exóticas no Brasil em APG II. Instituto Plantarum de Estudos da Flora, Nova Odessa.

Takara, K., Matsui, D., Wada, K., Ichiba, T., Chinen, I., Nakasone, Y., 2003. New phenolic compounds from Kokuto, non-centrifuged cane sugar. Biosci. Biotechnol. Biochem. 67, 376–379.

Tolardo, R., Zetterman, L., Bitencourtt, D.R., Mora, T.C., de Oliveira, F.L., Biavatti, M. W., Amoah, S.K.S., Burger, C., de Souza, M.M., 2010. Evaluation of behavioral and pharmacological effects of *Hedyosmum brasiliense* and isolated sesquiterpene lactones in rodents. J. Ethnopharmacol. 128, 63–70.

Trentin, A.P., Santos, A.R.S., Guedes, A., Pizzolatti, M.G., Yunes, R.A., Calixto, J.B., 1999. Antinociception caused by the extract of *Hedyosmum brasiliense* and its active principle, the sesquiterpene lactone 13-hydroxy-8,9- dehydroshizukanolide. Planta Med. 65, 517–521.

Wang, F., Zhou, D.-S., Wei, G.-Z., Ren, F.-C., Liu, J.-K., 2012. Chlorantholides A–F, eudesmane-type sesquiterpene lactones from *Chloranthus elatior*. Phytochemistry 77, 312–317.

Wu, H., Hu, X., Zhang, X., Chen, S., Yang, J., Xu, X., 2012. Benzyl 2-β-Glucopyranosyloxybenzoate, a new phenolic acid glycoside from *Sarcandra glabra*. Molecules 17, 5212–5218.

Yuan, K., Zhu, J.X., Si, J.P., Cai, H.K., Ding, X.D., Pan, Y.J., 2008. Studies on chemical constituents and antibacterial activity from n-butanol extract of *Sarcandra glabra*. Zhongguo Zhongyao Zazhi 33, 1843–1846.

Zdero, C., Bohlmann, F., 1989. Eremophilanolides, eudesmanolides, guaianolides and other constituents from *Ondetia linearis*. Phytochemistry 28, 1653–1660.

Zhang, L., Yang, L., Niu, H., Zu, Y., 2008. Separation of vindoline, catharanthine and vinblastine from *Catharanthus roseus* (L.) G. Don with macroporous adsorption resin. Huagong Xuebao 59, 607–614.

Zhu, L.P., Li, Y., Yang, J.Z., Zuo, L., Zhang, D.M., 2008. Studies on chemical constituents of *Sarcandra glabra*. Zhongguo Zhongyao Zazhi 33, 155–157.