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Original Article

Zornioside, a dihydrochalcone *C*-glycoside, and other compounds from *Zornia brasiliensis*

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ABSTRACT

The secondary metabolites of the aerial parts of *Zornia brasiliensis* Vogel, Fabaceae, and the biological activity of one of these secondary metabolites were characterized in this study. A phytochemical investigation was performed using chromatographic techniques including analytical and preparative reverse-phase HPLC column sequences, which resulted in the isolation of fourteen compounds: one previously undescribed *C*-glycosylated dihydrochalcone (zornioside), one cyclitol (D-pinitol), one glycosylated megastigmane (roseoside) and eleven phenolic compounds: 7-methoxyflavanone, 7,4′-dimethoxyisoflavone, medicarpin, 2′-4′-dihydroxychalcone, ononin, isoorientin-3′-*O*-methyl ether, isovitexin, glycosylated (*Z*)-*O*-coumaric acid, glycosylated (*E*)-*O*-coumaric acid, dihydromelilotoside, and isoorientin. The structures of the isolated compounds were determined based on 1D and 2D-NMR, HRESIMS, IR and CD spectroscopic analyses. The cytotoxic activity of zornoside was assessed against tumor cell lines (MCF-7, HCC1954, T-47D, 4T1, HL60), and a non-tumor cell line (RAW264.7) using MTT assay. The compound zornioside was selectively cytotoxic for HL60 leukemia cells (IC_{50}: 37.26 μM).

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Introduction

The genus *Zornia*, Fabaceae, has about eighty species distributed in the world (Sciamarelli and Tozzi, 1996), 36 species are in the Brazilian territory, among which, fifteen species are endemic (Perez, 2009). Some specimens of *Zornia* have already been studied for their pharmacological activity: relaxing on smooth muscle, cytotoxic activity, anticonvulsant activity, antioxidant potential, anti-inflammatory and antibacterial and antitumor (Rojas et al., 1999; Brahmachari et al., 2009; Arunkumar et al., 2012; Belcavello et al., 2012; Greetha et al., 2012; Arunkumar et al., 2014). Previous phytochemical studies of the genus *Zornia* demonstrated the presence of several isoflavones (Ren et al., 2012; Leuner et al., 2013).

The species *Zornia brasiliensis* Vogel is commonly known as “urinária”, “urinana” and “carrapicho” in Brazil, and it is typically used as a diuretic and for the treatment of venereal diseases (Agra et al., 2007). *Z. brasiliensis* is distributed in the North, Northeast, Midwest, and Southeastern regions from Brazil associated to the Amazonian, Caatinga, Cerrado, and Atlantic Rain Forest phytogeographical domains (BFG, 2015), but mainly in the Brazilian Northeast (Mohlenbrock, 1961), and Venezuela (Missouri Botanical Garden, 2017). Three flavones have been isolated from the aerial parts of this species, and one of these, 7-methoxyflavone, demonstrated antinociceptive effects (Silva et al., 2013). A previous study of *Z. brasiliensis* revealed the chemical composition and antitumor activity of the essential oil of its leaves (Costa et al., 2015). In this article, we report the results of the phytochemical study of the crude ethanolic extract of *Z. brasiliensis*. Fourteen compounds were isolated and identified, all reported for the first time in this species. Among these compounds, a dihydrochalcone, zornioside (**1**), was isolated and identified for the first time. The other known compounds were identified by comparing their spectroscopic data with data reported in the literature.

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Materials and methods

General experimental procedures

For semi-preparative HPLC, a SHIMADZU 10AVP Series system was used with a SPD-M10AVP detector, two LC-6AD pumps and a Rheodyne injector with a SCL-10A VP interface. A Shimadzu Shim-Pack C18 (250 mm × 10 mm and 5 μm particle size) column was used. Analytical chromatographic analyses were performed using a Shimadzu Prominence HPLC system equipped with a LC-20AT binary solvent pump, an SIL-20A autoinjector, a DGU-20A degassing system, a SPD-M20A diode array detector and a CBM-20A system controller. The column used was a Phenomenex Gemini® C18 (250 mm × 4.6 mm ID filled with 5-μm particles) with a SecurityGuard Gemini® C18 (4 mm × 3.0 mm ID filled with 5-μm particles) pre-column. Preparative chromatographic analyses were performed using a Shimadzu HPLC system equipped with a LC-6AD binary solvent pump, a Rheodyne injector, a SPD-M10A diode array detector and a SCL-10A system controller. The column used was an ACE C18 (250 mm × 21.2 mm and 5 μm particle size). The organic solvents used were acetonitrile and methanol, both HPLC grade (TEDIA®, Brazil), and ultrapure water obtained with a Milli-Q purification system (Millipore®). The NMR analyses were performed on a 500 MHz Varian NMR spectrometer operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR, on a 200 MHz Varian Mercury NMR spectrometer operating at 200 MHz for ^{1}H NMR and 50 MHz for ^{13}C NMR and on a Bruker Ascend 400 spectrometer operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Deuterated chloroform ($CDCl_3$), deuterated acetone (CD_3COCD_3), deuterated methanol (CD_3OD) and deuterated dimethyl sulfoxide (DMSO-d_6) (all from Cambridge Isotope Laboratories) containing TMS as an internal standard were used.

To obtain mass spectra, low and high resolution Bruker mass spectrometers, model Ion Trap-amaZonX and model micrOTOF II, respectively, were used in electrospray ionization positive (ESI^+) or negative (ESI^-) modes. Samples were diluted in a water:acetonitrile or water:methanol solution (Milli-Q Millipore®/Tedia®, Brazil). For CD assays, a Jasco J-815 UV-Visible absorbance spectropolarimeter was used. Infrared analyses were obtained in the 4000–400 cm^{-1} region using a Shimadzu IRPrestige-21 spectrophotometer with 1 mg of sample in potassium bromide (KBr) tablets and frequency measured in cm^{-1}.

Plant material

The plant material of *Zornia brasiliensis* Vogel, Fabaceae (aerial parts) was collected in the municipality of Serra Branca (07°29′46″S and 36°44′36″W, altitude: reaching 712 m elevation), Paraíba state, Brazil, in March 2016. Collection authorization: N. 53894-1, granted by the Instituto Chico Mendes de Conservação da Biodiversidade through the Sistema de Autorização e Informação em Biodiversidade. This material was identified by the botanist Dr. José Iranildo Miranda de Melo from the Universidade Estadual da Paraíba. An exsiccata is deposited at the Herbarium Arruda Câmara, Campus I of the, Universidade Estadual da Paraíba, under code 1862.

Extraction and isolation

The aerial parts of *Z. brasiliensis* (5 kg) were extracted by maceration with 95% ethanol at room temperature to obtain the crude ethanol extract (CEE). An aliquot of the CCE was subjected to a liquid vacuum chromatography with silica deactivated using as eluent the solvents: hexane, dichloromethane, ethyl acetate, ethyl acetate–methanol (9:1, v/v) and ethyl acetate–methanol (1:1, v/v). After fractionation, the extractive solutions resulting from this process were concentrated in rotary evaporator, obtaining the respective fractions (Silva et al., 2013).

The dichloromethane fraction (10 g) was subjected to column chromatography (CC) using silica gel (ART 7734, MERCK, 0.060–0.200 mm and 70–230 mesh ASTM) as the stationary phase and hexane, ethyl acetate and methanol, either pure or in binary mixtures, as eluents in increasing order of polarity (collected a total of thirty fractions with 100 ml each). Fr 13 (hexane:ethyl acetate: 9:1, v/v) was subjected to preparative HPLC using an isocratic system of water and acetonitrile (55:45), thus obtaining substance **2** (R_T 45 min, 30 mg). Fr 25 and 26 (hexane:ethyl acetate: 8:2, v/v) were pooled and subjected to preparative HPLC under the same conditions, obtaining substance **3** (R_T 36.6 min, 35 mg).

The ethyl acetate fraction (10 g) was subjected to CC under conditions analogous to those described previously for the dichloromethane fraction (collected a total of fifty fractions with 100 ml each). Fractions 25–29 (hexane:ethyl acetate: 8.5:1.5, v/v) were subjected to CC using the same conditions indicated for the dichloromethane fraction, yielding 59 fractions of 50 ml each, and fractions 11–16 (hexane:ethyl acetate: 95:5, v/v) were pooled on a preparative plate and eluted in a hexane-ethyl acetate (80:20, v/v) system five times. The presence of three stains with different retention factors was observed under ultraviolet light. The stain with the highest retention factor was separated from the other stains and filtered in an ethyl acetate–methanol (80:20, v/v) system using a porous funnel. The fraction obtained from the filtration was subjected to semi-preparative HPLC using an isocratic system of water and acetonitrile (38:62, v/v), thus obtaining substances **4** (R_T 17.3 min, 31 mg) and **5** (R_T 22.6 min, 27 mg).

An aliquot of the ethyl acetate–methanol (9:1, v/v) fraction (3 g) was subjected to CC using Sephadex® LH-20 (GE Healthcare) as stationary phase and pure methanol as a mobile phase (yielding 29 fractions). Fractions 9–16 were subjected to a new CC using Sephadex® LH-20 (GE Healthcare) and methanol as the mobile phase. Thus, 9 were obtained, while fractions Fr 2–7 were submitted to a third CC under the same conditions previously described. From this column, 19 fractions were obtained, and fraction 8 generated substance **6** (21.0 mg).

An aliquot of the ethyl acetate–methanol fraction (1:1, v/v) (10 g) was subjected to CC under conditions similar to those described previously for the dichloromethane fraction (collected a total of 21 fractions with 250 ml each). Fractions 8–9 were subjected to preparative HPLC using an isocratic system consisting of water (0.1% and formic acid) and methanol (65:35, v/v) to obtain substances **1** (R_T 31.6 min, 22.8 mg), **7** (R_T 57.1 min, 33.8 mg), **8** (R_T 51 min, 17.3 mg), **9** (R_T 13.8 min, 16 mg), **10** (R_T 28.5 min, 12.6 mg), **11** (R_T 21.8 min, 13.7 mg) and **12** (R_T 20.6 min, 19.1 mg). The fraction 14 was subjected to CC using Sephadex® LH-20 (GE Healthcare) and pure metanol (yielding twenty fractions) the fraction 12 obtained from this column resulted in substance **13** (18.5 mg), and the fraction 20 resulted in substance **14** (16 mg).

(3S)-1-{2′,4′-dihydroxy-3′-[(2″R,3″S,4″S,5″R,6″S)-3″,4″,5″-trihydroxy-6″-(hydroxymethyl)oxan-2″-yl]phenyl}-3-hydroxy-3-phenylpropan-1-one (**1**): yellow oil; IR_{max} 1613, 3434 cm^{-1}; ^{1}H and ^{13}C NMR data (500 MHz and 125 MHz, respectively, in DMSO-d_6), see Table 1; HRESIMS *m/z* 421.1508 $[M+H]^+$ (calcd. for $C_{21}H_{25}O_9$, 421.1493).

Electronic circular dichroism (ECD) calculation

All geometric optimizations and conformational searches were performed using Spartan'16 for Windows (Wavefunction, Irvine, CA, USA). The geometry of the chemical structure of the compound was initially optimized with a Merck Molecular Force Field (MMFF) force field (Halgren, 1996) and a new geometric optimization was then performed based on the semi-empirical method, Austin Model

Table 1
^{1}H- and ^{13}C-NMR spectroscopic data for compound **1**.

No.	δ_C, type	δ_H (*J*, Hz)	HMBC
1	145.2, C	–	–
2	125.9, CH	7.42, m	4, β
3	128.1, CH	7.33, m	1, 5
4	127.0, CH	7.24, m	2
5	128.1, CH	7.33, m	1, 2
6	125.1, CH	7.42, m	4, β
1′	112.5, C	–	–
2′	132.8, CH	7.77, d (8.8)	6′, 4′, C=O
3′	108.1, CH	6.40, d (8.8)	4′, 5′, 1′
4′	164.2, C	–	–
5′	112.7, C	–	–
6′	164.1, C	–	–
α	47.1, CH_2	3.09, dd (4.4, 14.8)3.32, m	β, C=O 1
β	70.2, CH	5.12, dd (4.4, 8.8)	α, 1 2
C=O	202.8, C	–	–
1″	73.2, CH	4.61, d (10.0)	5′, 2″6′, 4′, 5″, 3″
2″	70.5, CH	4.01, t (8.8)	1″, 3″
3″	79.0, CH	3.19, m	–
4″	69.7, CH	3.19, m	2″
5″	81.5, CH	3.19, m	–
6″	61.4, CH_2	3.66, dd (1.2, 11.2)	–

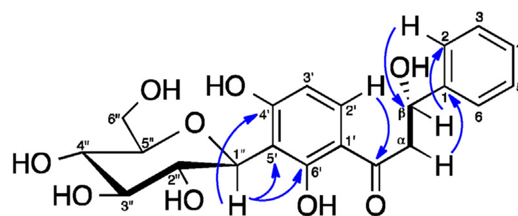

Fig. 1. HMBC correlations (→) and the interaction ^{1}H–^{1}H COSY (dark bond) for **1**.

1 (AM1) (Dewar et al., 1985). A systematic search method was used which analyzed conformers and selected the conformers with the lowest minimum energy using AM1 and a Monte-Carlo algorithm (Metropolis and Ulam, 1949). After that, the lowest minimum energies were selected and optimized based on a vibrational mode calculation using DFT (density functional theory) (Becke, 1988). DFT calculations were performed using Spartan 16 (Wavefunction, Irvine, CA, USA; Vereecken et al., 1998). Each structure was examined at the B3LYP/6-311G* level and the lowest energy structures were selected for the calculations. The global minimum on the potential energy surface was used for the determination of each geometry. The calculated ECD spectra were obtained by density functional theory (DFT) and time-dependent DFT(TD-DFT) using Gaussian 09 and analyzed using GUIs GaussSum v 3.0 (O'Boyle et al., 2008).

Cytotoxicity assay

MTT cell viability assay (Mosmann, 1983) was performed to study the cytotoxic effect of zornoside. MCF-7 (breast adenocarcinoma), HCC1954 (mammary gland carcinoma), T-47D (mammary gland carcinoma), 4T1 (mammary gland carcinoma), HL60 (promyelocytic leukemia), RAW264.7 (macrophage) cells were cultured in a RPMI 1640 or DMEM medium supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in humidified air with 5% CO_2 at 37 °C. Briefly, cells were seeded in 96-well plates (100 μl of 2×10^5 cells/ml) and zornoside (0.39–50 μg/ml) dissolved in DMSO:Medium (1:99 v/v; 100 μl) was added to each well and incubated for 72 h. DMSO 1% was used as negative control. MTT was added at a final concentration of 0.5 mg/ml, incubated for 4 h, and then solubilized with 100 μl of DMSO. Formazan production was measured at 570 nm in a plate spectrophotometer.

Results and discussion

*Structural elucidation and identification of the isolated compounds (**1**–**14**)*

Compounds **1**–**14** were isolated from the ethanolic extract of the aerial parts of *Z. brasiliensis* by column chromatography followed by preparative and semi-preparative HPLC. Known substances were determined by comparing their spectroscopic data with those in the literature. The known compounds were identified as 7-methoxyflavanone (**2**) (Kostrzewa-Susłow and Janeczko, 2012), 7,4′-dimethoxysoflavone (**3**) (Veitch et al., 2003), medicarpin (**4**) (Baill et al., 2000), 2′-4′-dihydroxychalcone (**5**) (Mikell and Khan, 2012), ononin (**6**) (Fedoreyev et al., 2008), isoorientin-3′-*O*-methyl ether (**7**) (Delazar et al., 2006), isovitexin (**8**) (Peng et al., 2005), glycosylated (*Z*)-*O*-coumaric acid (**9**) (Canuto et al., 2010), glycosylated (*E*)-*O*-coumaric acid (**10**) (Canuto et al., 2010), dihydromelilotoside (**11**) (Taskova et al., 2005; Wu et al., 2002), roseoside (**12**) (Shen and Terazawa, 2001; Rao, 2017), D-pinitol (**13**) (Yu et al., 2005), and isoorientin (**14**) (Huang et al., 2015).

Compound **1** showed a peak at *m/z* 421.1508 $[M-H]^+$, as determined by high-resolution electrospray ionization mass spectrometry (HRESIMS), that is compatible with the molecular formula $C_{21}H_{24}O_9$ (calcd. 421.1493). The ^{1}H NMR and Heteronuclear Single-Quantum Coherence (HSQC) spectra showed signals of two aromatic rings, one tetra-substituted [δ_H 7.77 (1H, d, *J* = 8.8 Hz), 6.40 (1H, d, *J* = 8.8 Hz)] and another monosubstituted [δ_H 7.42 (2H, m), 7.33 (2H, m), 7.24 (1H, m)], and one carbinolic hydrogen [δ_H 5.12 (1H, dd, *J* = 4.4, 8.8 Hz), δ_C 70.2(C-β)] (Table 1). In addition to two non-equivalent methylene hydrogens [δ_H 3.09 (1H, dd, *J* 4.4, 14.8 Hz), 3.32 (1H, m), δ_C 47.1(C-α)], the ^{1}H–^{1}H COSY (Fig. 1) showed a correlation between the carbinolic and methylenic hydrogens, revealing that these are vicinal, and a Heteronuclear Multiple-Bond Correlation (HMBC) experiment revealed a correlation between these hydrogens and the carbonyl [δ_C 202.8 (C=O)]; however, the only correlation identified was between the carbinolic hydrogen and C-2 (δ_C 125.9). Therefore, this β is the carbonyl (δ_C 202.8), whereas the methylenic hydrogens have a correlation with C-1 (δ_C 145.2], and α is thus the carbonyl (δ_C 202.8).

The carbons at [δ_C 108.1 (C-3′), 112.5 (C-1′), 112.7 (C-5′), 132.8 (C-2′), 164.1 (C-6′), 164.2 (C-4′)] confirmed the presence of a tetra-substituted aromatic ring in **1**. In HMBC, another correlation was observed between H-2′ [δ_H 7.77 (1H, d, *J* = 8.8), δ_C 132.8] and the carbonyl (δ_C 202.8) confirming that this C=O is bonded to the tetra-substituted aromatic ring. The four carbon signals [δ_C 145.2 (C-1), 125.9 (C-2), 127.0 (C-4) and 128.1 (C-5)], attributed to the existence of six carbons, confirm the presence of a monosubstituted aromatic ring. HMBC showed a correlation of H-2/6 [δ_H 7.42 (2H, m), δ_C 125.9(C-2/6)] with C-β (δ_C 70.2), confirming the binding of the monosubstituted ring to C-β. The presence of the signal [δ_H 4.16 (1H, d, *J* = 10.0 Hz)] associated with other signals from aliphatic carbinolic hydrogens [δ_H 4.01 (1H, t, *J* = 8.8 Hz), 3.66 (dd, 2H, *J* = 1.2. 11.2 Hz), 3.19–3.10 (3H, m)] revealed the presence of an osidic unit in **1**; however, the absence of the hydrogen and of the anomeric carbon shows that this unit is a C-glycoside. HSQC showed the existence of six carbons correlated with these hydrogens, and the value of the H-1″ coupling constant (*J* = 10.0 Hz) confirms that it is β-glucose. HMBC showed a correlation between H-1″ [δ_H 4.16 (1H, d, *J* = 10.0 Hz), δ_C 73.6] and [δ_C 112.7 (C-5′)], [δ_C 164.2 (C-4′)], [δ_C 164.1 (C-6′)], there by confirming the insertion of β-glucose at the 5′-position through a C—C bond.

Electronic circular dichroism (ECD) has been used for decades successfully as a powerful tool for the absolute configuration assignment of natural products with various chromophores

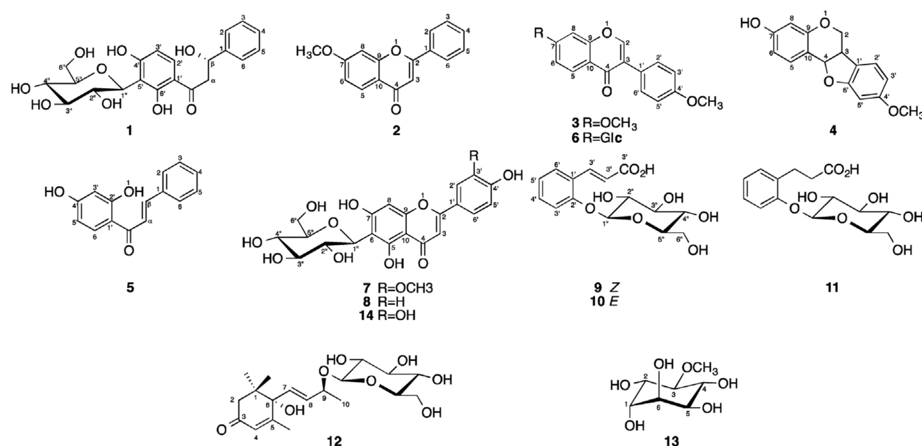

Table 2
Cytotoxic activity against tumor and non-tumor cells of zornoside.

Cell lines	[a]IC_{50} (μM)	IC_{50} (μM)Doxorubicin
MCF-7	76.26 (57.38–101.35)	1.9
T47D	76.09 (53.14–89.76)	1.43
4T1	93.42 (73.90–109.26)	2.0
HCC 1954	81.88 (68.57–97.74)	1.43
HL-60	37.26 (27.28–43.00)	0.3
RAW 264.7	106.92 (96.52–118.78)	2.8

[a] Data are presented as IC_{50} values and 95% confidence intervals obtained by non-linear regression for all cell lines from two independent experiments. Exposure time: 72 h. MCF-7, breast adenocarcinoma; HCC1954, mammary gland carcinoma; T-47D, mammary gland carcinoma; 4T1, mammary gland carcinoma; HL-60, promyelocytic leukemia; RAW264.7, macrophage.

(Slade et al., 2005). The calculated ECD can be used to validate the empirical rules deduced previously, but it is also widely used to aid to determine the absolute configuration of a chiral molecule, comparing the calculated and experimental ECD spectra, the similarity of the spectra is crucial for the absolute configuration assignment. The development of the computational technologies has allowed use some quantum chemical calculations to simulate the ECD spectra (Li et al., 2010). The TD-DFT (Time Dependent Density Functional Theory) has been widely used to aid to determine the absolute configuration of several secondary metabolites (Gan et al., 2009; Kamel et al., 2009; Li et al., 2010). The TD-DFT method was successful in calculating ECD spectra of chiral flavonoids thanks to its feasibility (Ding et al., 2007; Ding et al., 2009).

The TD-DFT calculations of the ECD spectra of the compounds were compared with the experimental. This analysis can be done to determine the absolute configuration of diastereoisomers (Kwit et al., 2009). Then, were made comparations of simulated ECD spectra were performed for the compounds (β*S*)-zornioside and (β*R*)-zornioside. The simulated ECD spectra of the former show no peaks in any wavelength from 800 nm until 200 nm that corroborate with the experimental ECD spectra (Fig. 2) that is different from the simulated spectra of the configuration βR, these results allow to propose the configuration of the isolated dihydrochalcone. Therefore, this is the first report of this dihydrochalcone in the literature.

*Cytotoxic activity of zornioside (**1**)*

The results of the cytotoxic activity assay are shown in Table 2. The IC_{50} (drug concentration at which 50% of the cells are viable relative to the control) values, calculated from the dose–survival curves generated by the MTT assays performed after the zornoside treatment, ranged from 37.26 (27.28–43.00) μM, for HL-60 cells, to 93.42 (73.90–109.26) μM, for 4T1 cells. Then, as can be viewed, the compound was selectively cytotoxic for HL60 promyelocytic leukemia cells. On non-tumor cell line (macrophage RAW 264.7), zornoside showed weak cytotoxicity, IC_{50} value of 106.92 (96.52–118.78) μg/ml.

With the exception of compounds **3** and **6**, all other compounds are reported for the first time in the genus *Zornia*, the compound **1** being first described in the literature. Compound **1** demonstrated better cytotoxic activity against the HL-60 (37.26 μM) cell line. Thus, the study showed that specie *Z. brasiliensis* is a bioproducer of phenolic compounds and zornioside has cytotoxic potential.

Supplementary materials

Supplementary data (1D and 2D NMR, MS, CD and Fourier transform infrared (FTIR) spectroscopic data of compound **1** and 1D NMR spectroscopic data of compounds **2**–**14**).

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

YMN, LSA, RLL, ADSS and CVOC participated in phytochemical analysis. MSS and JFT participated in interpretation of the NMR spectra. JIMM participated in botanical identification. MTS participated in acquisition and interpretation of computational data. MVS, SSA and MAGF participated in acquisition and interpretation of biological assays.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgment

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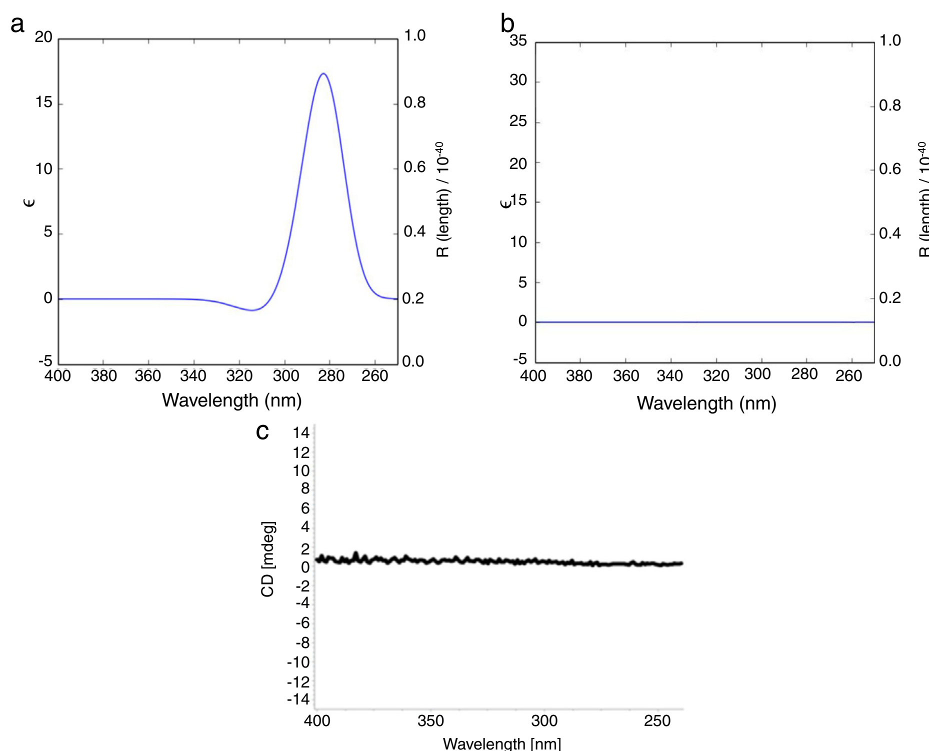

Fig. 2. Comparison of ECD spectra of (βR)-zornoside (a), (βS)-zornoside (b), simulated using Gaussian band shapes (σ = 0.4 eV), and to the experimental ECD spectrum (c).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.02.003.

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