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A new tropane alkaloid and other constituents of *Erythroxylum rimosum* (Erythroxylaceae)

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ABSTRACT

A new tropane alkaloid, named the 7β-acetoxy-3β,6β-dibenzoyloxytropane (**1**), was isolated from a methanol extract of *Erythroxylum rimosum* O.E. Schulz leaves. Other known compounds were detected, including quercetin, kaempferol-3-O-α-L-arabinofuranoside, (+)-catechin, epicatechin, quercetin-3-O-α-arabinofuranoside, quercetin-3-O-α-arabinopyranoside, quercetin-3-O-β-arabinopyranoside, quercetin-3-β-glucopyranoside, kaempferol, quercetin-3-O-β-galactopyranoside, β-sitosterol, α-amyrin, β-amyrin, and the ester derivatives of these two amyrins. Compound **1** exhibited weak inhibition of acetylcholinesterase. Structural identification was performed using IR, ESIHRMS and one- and two-dimensional NMR data analyses and confirmed by comparison with literature data.

1. Introduction

The *Erythroxylum* genus is the largest member of the Erythroxylaceae family, corresponding to 97% of its species, and members of this genus are found in the tropic and subtropical regions of South America with significant biodiversity. Brazil is considered the main center of biodiversity of *Erythroxylum* species due to the presence of 116 out of a total of 187 species recorded in tropical America (Plowman and Hensold, 2004). In the northeastern region, especially in the Bahia State, the largest concentration of these species is found in "Restinga" (sandy soil of along the coast) and in the Atlantic forest. Some species of this genus have been utilized in traditional medicine for various therapeutic purposes, including as diuretic, tonic, stimulant, anti-diarrheal, an inhalant for asthma, as a remedy against intestinal parasites, fever, amenorrhea, bleeding, kidney disorder, influenza, sinusitis, and stomach derangement. In addition, it is used to fight fatigue and hunger (Hegnauer, 1981; Rahman et al., 1998; Plowman, 1984; Al-Said et al., 1986; Chaves et al., 1988). Whereas cocaine is found in only a few species of this genus, tropanic alkaloids and diterpenoids are quite common (David et al., 2007). To date, there are no chemical or biological studies dealing with *E. rimosum*. This study describes the isolation, structural characterization and acetyl and butylcholinesterase inhibition activity of a new tropane alkaloid **1**. In addition, the identification of flavonoids (**2a**–**2h**) and triterpenoids (**3** and **4**) (Fig. 1) from *E. rimosum* O. E. Schulz leaves is also presented.

2. Results and discussion

The molecular formula $C_{24}H_{25}NO_6$ of compound **1** was deduced by the molecular ion observed at *m/z* 424.1774 (calc. 424.1760) in the ESIHRMS. The IR spectrum showed bands in the 1600–1450 cm^{-1} region that correspond to the C=C aromatic ring, and intense absorptions at 1718 cm^{-1}, 1272 cm^{-1} and 1112 cm^{-1}, which were assigned to the conjugate acyl group and C–O stretching vibrations of the esters. The ^1H NMR spectrum exhibited a single signal at δ 2.63 for the methyl group attached to the nitrogen (Table 1) and the typical tri-oxygenated tropane alkaloid signals at δ 5.81, 5.91 and 5.36. The ^1H NMR spectrum also showed signals integrating to 10 hydrogens attached to the aromatic carbons, which are attributed to the two aromatic acid residues, and the characteristic signal of the methyl of acetyl group. These structural characteristics observed by all the ^1H NMR data were

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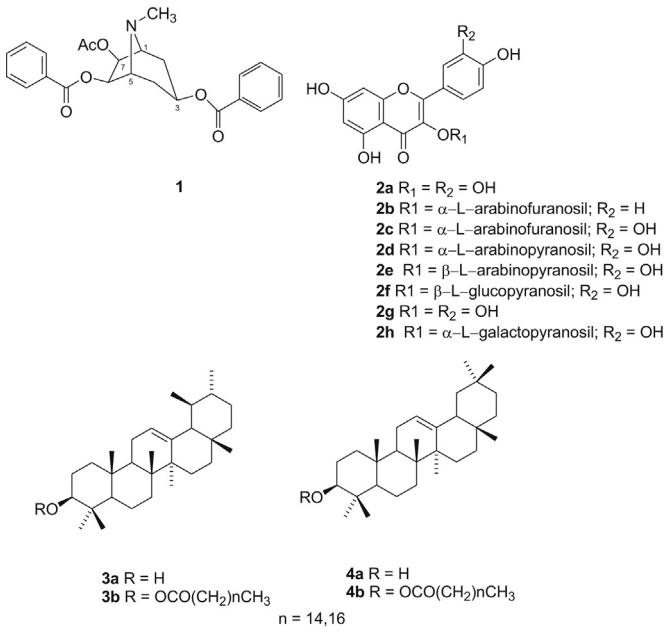

Fig. 1. Chemical structures of the *E. rimosum* isolates.

confirmed by ^{13}C NMR spectra (including DEPT experiments) through of the characteristic resonances of the aromatic groups, sp^3 carbons as well as the N–CH_3 and acetyl groups. In the ^{13}C NMR spectra, the typical signals of trioxygenated tropane alkaloid were corroborated by the oxymethine carbons at δ 67.1, 77.2, 77.6 as well as the characteristic chemical shift of N–CH_3 at δ 38.2, among others (Table 1). Therefore, based on information obtained from the ^1H and ^{13}C NMR spectra, it was possible to characterize two benzoyloxy and one acetyloxy as substituents of the tropane moiety. The assignment of all hydrogenated carbons was made possible by evaluating the heteronuclear correlations observed in the *g*HMQC experiment. The location of each substituent in the tropane skeleton was assigned by correlations observed in the *g*HMBC spectrum and it was corroboreted by *g*COSY, *g*TOCSY, NOESY-1D and HOMODEC experiments. The *g*HMBC spectrum allowed for the identification of each benzoyl unit, from the correlations between the *ortho* hydrogens of each of these units and the respective signals for the carbonyl carbons (Fig. 2). Thus, a correlation was observed between the hydrogens at δ 7.99 and 8.10 and the acyl carbons at δ 165.9 and 165.6, respectively. The location of these benzoyl units at the C-3 and C-6 positions in the tropane skeleton was established from the correlations between the hydrogens at δ 5.91 and 5.36 and the acyl carbons at δ 165.9 and δ 165.6, respectively. Likewise, the methyl hydrogens of the acetyl group at δ 1.91 showed correlations with the signal at δ 170.1, which is located at C-7 due the correlations observed between the signal at δ 5.81 (H-7) and 170.1. The assignment of signals at δ 5.81 and 5.91 to H-7 and H-6 of the tropane skeleton, respectively, was also verified by the HOMODEC analysis. The strong irradiation of only one of the two nuclei results in the loss of coupling (decoupling) in the other nucleus, indicating the proximity of the two signals. Additional structural characterization of compound **1** focused on the stereochemical orientation of the three substituents in relation to the nitrogen bridge. The relative stereochemistry was determined by 1D NOESY. The NOE observed between N-CH_3 and H-2'/H-6' made it possible to precisely determine the β-stereochemistry, i.e., the *exo* arrangement of the C-3 benzoyl. This method also enabled the assignment of the methyl N-CH_3 at the pseudo-axial position. The β-orientation of the other benzoyl group at C-6 was confirmed by the NOE observed for both H-6 and H-7 and H-2″/H-6″ of this benzoyl unit. The NOE of N-CH_3 hydrogens and H-2_{exo}, H-4_{exo} and H-2' of C-3 benzoyl group indicate this ring possesses a boat conformation and this statement agrees with the observed multiplicity and coupling constant of H-3 (Fig. 3). Finally, the correlations observed between hydrogens at δ 1.9 and both hydrogens at δ 5.91 and 5.81 clearly define the bicyclic conformation. Thus, the spectroscopic data obtained were compared to literature values, allowing for the identification of substance **1** as the alkaloid 7β-acetoxy-3β,6β-dibenzoyloxytropane, a new tropane alkaloid.

Table 1
^1H and ^{13}C NMR data and correlations observed in the gCOSY, gTOCSY-1D, NOESY-1D and HOMODEC spectra for compound **1** ($CDCl_3$, δ, *J* in Hz, 500 and 125 MHz).

Position	^1H	^{13}C	gCOSY	gTOCSY-1D	NOESY-1D	HOMODEC
1	3.33[a] (*sl*)	64.7[a]	H-2_{exo}	H-6, H-2_{exo}, H-2_{endo}	N-CH_3	H-2_{exo}
7	5.81 (d, *J* = 6.5)	77.2		H-6	H-2″/H-6″, H-2_{endo}, H-4_{endo}	H-6
6	5.91 (d, *J* = 6.5)	77.6			H-2″/H-6″	H-7
4_{endo}	1.90 (*m*)	30.4			H-2″/H-6″	
4_{exo}	2.33[b] (*m*)	[a]				
5	3.42[a] (*s*)	64.8[a]	H-4_{exo}	H-3, H-2_{exo}, H-4_{endo}	N-CH_3	H-4_{exo}
3	5.36 (*t*, *J* = 5.0)	67.1	H-2_{exo}			
2_{endo}	1.90 (*m*)	30.5			H-7, H-6	
2_{exo}	2.32[b] (*m*)			H-3, H-5, H-1, H-2_{endo}		
N-Me	2.63 (*s*)	38.2			H-2′/H-6′, H-5, H-1, H-4_{exo}, CH_3CO	
Benzoyl C-3						
1'		129.8[a]				
2′ and 6′	7.99 (*d*, *J* = 8.2)	129.7	H-3′			
3′ and 5′	7.39 (*t*, *J* = 8.0)	128.4				
4′	7.50 (*t*, *J* = 8.0)	133.1				
C=O		165.9				
Benzoyl C-6						
1″		130.0[a]				
2″ and 6″	8.10 (*d*, *J* = 8.5)	129.6	H-3″			
3″ and 5″	7.47 (*t*, *J* = 7.7)	128.7				
4″	7.52 (*t*, *J* = 8.5)	133.3				
C=O		165.6				
CH_3CO	1.91 (*s*)	20.8; 170.1				

[a] Assignments may be reversed; *s* = singlet, *sl* = broad singlet, *d* = doublet, *t* = triplet, *m* = multiplet.
[b] Assignments may be reversed; *s* = singlet, *sl* = broad singlet, *d* = doublet, *t* = triplet, *m* = multiplet.

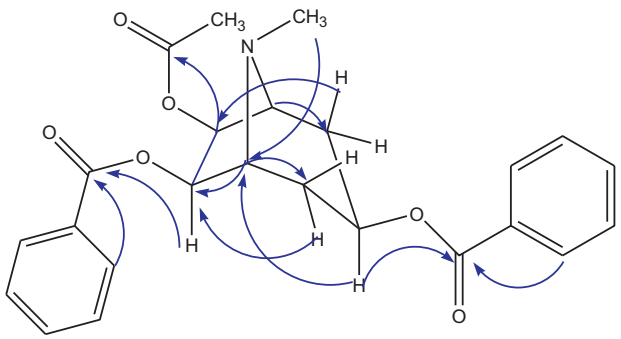

Fig. 2. Main correlations observed in the gHMBC contour map for **1**.

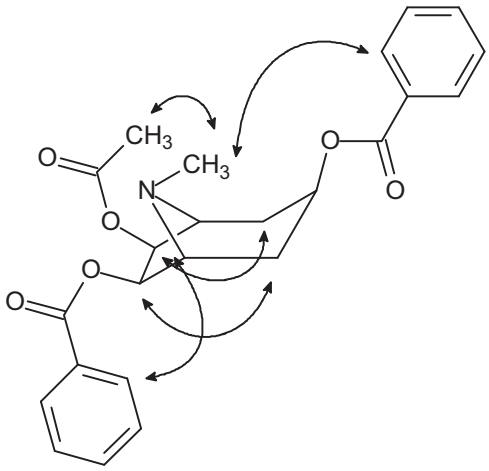

Fig. 3. Correlations observed in the NOESY-1D spectra for **1**.

The identification of quercetin (**2a**) (Meira et al., 2008), kaempferol-3-O-α-L-arabinofuranoside (**2b**) (Kim et al., 1994), catechin and epicatechin (Harborne, 1996), quercetin-3-O-α-arabinofuranoside (**2c**) (Ossipov et al., 1995), quercetin-3-O-α-arabinopyranoside (**2d**), quercetin-3-O-β-arabinopyranoside (**2e**) (Rodrigues et al., 2011), quercetin-3-β-glucopyranoside (**2f**) (Barreiros et al., 2005), kaempferol (**2g**) (Harborne, 1996), quercetin-3-O-β-galactopyranoside (**2h**) (Santos et al., 2005), α-amyrin and β-amyrin esters (Chávez et al., 1996), α-amyrin, β-amyrin (Barreiros et al., 2005), and β-sitosterol (Meira et al., 2008) were accomplished via direct comparison of the obtained spectra with the literature data.

The effect of **1** on the AChE (acetylcholinesterase) and BuChE (butylcholinesterase) activities were monitored using different concentrations of these compound. The results of these experiments reveal that compound **1** inhibited the activity of AChE and BuChE enzymes with IC_{50} values of 4.67×10^3 μmol L^{-1} and 2.71×10^4 μmol L^{-1}, respectively, which were greater than the physostigmine standard (IC_{50} = 141.51 and 271.17 μmol/L, respectively).

The chemical composition of *E. rimosum* is in agreement with that of other members of its genus. Flavonoid 3-glycoside derivatives are chemotaxonomic markers for the *Erythroxylum* species (Inigo and Pomilio, 1985). In addition, a new tropane alkaloid, 2β-acetoxy-3β, 6α-dibenzoyloxytropane (**1**) was isolated, which is also the taxonomic marker of family that is typically found in *Erythroxylum* leaves. Tropane and pyrrolidine alkaloids are biosynthesized from *N*-methyl-Δ^1-pyrrolinium cation, previously formed by *N*-methylprutescine/putrescine. The bicyclic structure of the tropane skeleton (tropinone) is achieved by repeat Mannich-like reactions with acetyl-CoA. Action of two tropinone redutases (TRI and TRII) leads tropine (C3 α-OH) and pseudotropine (C3-β-OH), respectively. The pseudotropine pathway is responsible in forming polihydroxylated tropane alkaloids (Dräger, 2006).

3. Experimental

3.1. General experimental procedures

The ^1H (300 and 500 MHz) and ^{13}C (125 MHz and 75 MHz) NMR spectra of uni-and bi-dimensional of compounds **1–16** were acquired on Varian INOVA 500 (11.7 T) and Varian INOVA 300 (7.05 T) spectrometers, using traditional pulse sequences for the homonuclear and heteronuclear correlation experiments. Optical rotations were performed using a PerkinElmer model 343 Polarimeter. The IR spectra were recorded on a Shimadzu model IRAffinity-1 spectrophotometer using a film and KBr pellets. The mass spectra were obtained on a Bruker Daltonics UltrO-TOFq (ESI-TOFMS) using direct injection and spray-electron detection in the positive mode. A Fisons TM Platform II spectrometer by direct injection in the positive or negative detection mode through "ESI" was also utilized. Silica gel TLC plates (Merck) stained with iodine (Dragendorff, Liebermann–Burchard) and exposed to UV light (254/366 nm) were used to monitor the chromatographic purification process.

3.2. Plant material

The leaves of *Erythroxylum rimosum* were collected from the city of Palmeiras, (Bahia State, BA) and identified by Prof. Maria Iracema Bezerra Loyola. A voucher was deposited at Herbarium of "Universidade Estadual de Feira de Santana" (HUEFS) under number 127631.

3.3. Extraction and isolation

The dried and ground up leaves of *E. rimosum* (835 g) were extracted with MeOH. The resulting extract was suspended in H_2O and partitioned between hexane, $CHCl_3$ and EtOAc to obtain hexane (10.44 g), $CHCl_3$ (5.05 g) and EtOAc (11.4 g) phases. The $CHCl_3$ phase was subjected to CC over Si gel 60 and eluted with mixtures of hexane:EtOAc in crescenting polarity order. The fractions eluted with hexane:EtOAc (2:8 and 1:9) were grouped and subsequently purified by CC (Si gel; $CHCl_3$:MeOH). The fractions eluted with $CHCl_3$:MeOH (99:1) were purified using a Sephadex LH-20 in MeOH, allowing for the isolation of 2β-acetoxy-3β,6α-dibenzoyloxytropane (11.3 mg).

The EtOAc phase was purified by CC using $CHCl_3$:CH_3OH in the order of decreasing polarity to afford ten 50 mL fractions. Fractions 4 and 10 consisted of quercetin (84.1 mg) and quercetin-3-*O*-β-galactopyranoside (102.3 mg), respectively. The remaining fractions were subjected to successive fractionations and recrystallization. Fractions 5, 8 and 9 were purified by Sephadex LH-20 using a combination of CH_2Cl_2:CH_3OH 1:1 and 0:1, yielding kaempferol-3-O-α-L-arabinofuranoside (178.1 mg), quercetin-3-β-glucopyranoside (14.5 mg) and kaempferol (3.0 mg), respectively. Fraction 6 was purified by CC (SiO_2; $CHCl_3$:CH_3OH in order of decreasing polarity) producing catechin and epicatechin (366.1 mg). Fraction 7 was purified by recrystallization from methanol, yielding quercetin-3-O-α-arabinofuranoside (304.8 mg), quercetin-3-O-α-arabinopyranoside and quercetin-3-O-β-arabinopyranoside (95.1 mg).

The hexane phase was purified by CC using hexane:EtOAc in order crescenting of polarity to afford eight fractions. Fractions 4, 5 and 6 were purified by CC (SiO2; hexane:EtOAc 95:05, 9:1 and 7:3) and generated a mixture of α-amyrin and β-amyrin esters

(351.8 mg), α-amyrin and β-amyrin (185.5 mg), and β-sitosterol (87.4 mg).

7β-Acetoxy-3β,6β-dibenzoyloxytropane (**1**): yellow oil; $[\alpha]_D^{25} = -21^\circ$ (MeOH, $c = 0.01$); ESIHRMS m/z 424.1774 [M+ H] (requires 424.1760), IR (film) ν_{max} 2930 (C–H), 2849 (C–H), 1718 (C=O), 1600–1450 (C=C), 1272–1112 (C–O); ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 1.

3.4. Biological activity

Compound **1** was evaluated by TLC, which showed positive spots in the presence of a bioautographic TLC test for evaluating the anticholinesterase activity (Marston et al., 2002). The in vitro inhibition of AChE and BuChE was determined by spectrophotometry using the colorimetric method (Ellman et al., 1961). Briefly, 15 μL of acetylcholine iodide or butylcholine iodide, and 62 μL of DTNB (3 mM) were incubated with 5 μL of pure compound (4–500 μmol L^{-1}), physostigmine (positive control), or buffer (blank) for 15 min in a 96-well microplate. The reaction was initiated by the addition of 12 μL of the enzyme (0.22 U/mL). The change in the absorbance was recorded at 405 nm using a microplate reader (Biotek EL 800). DTNB, AChE and the substrate were dissolved in 0.1 M sodium phosphate buffer (pH 7.4). All samples were analyzed in triplicate.

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