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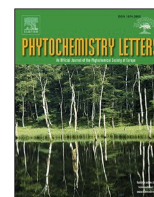

A new tropane alkaloid and other metabolites from *Erythroxylum macrocalyx* (Erythroxylaceae) and their antiproliferative activities

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A B S T R A C T

A new tropane alkaloid 7β-acetoxy-6β-benzoyloxy-3α-hydroxytropane (**1**) was isolated from the twigs of *Erythroxylum macrocalyx* Mart. (Erythroxylaceae), along with the known substances: 6β,7β-dibenzoyloxy-3α-hydroxytropane (**2**), 6β,7β-dihydroxy-3α-(phenylacetoxy)tropane (**3**), 3α-benzoyloxy-6β,7β-dihydroxytropane (**4**), 6β-benzoyloxy-3α-(4-hydroxy-3,5-dimetoxybenzoyloxy)tropane (**5**), ombuin-3-rutinoside-5-glucoside (**6**), lupeol (**7**), taraxerol (**8**) and lupenone (**9**). Compounds **1** and **2** were also isolated from the leaves. The structures were established by analyses of 1D- and 2D-NMR and MS data, as well as by comparison with literature data for known compounds. The structure of **2** was also supported by X-ray crystallography analyses. The compounds were evaluated *in vitro* for their antibacterial and antiproliferative activities. Compound **5** showed high antiproliferative activity on liver hepatocellular carcinoma cells (HepG2) with IC_{50} value of 3.66 μg mL^{-1} (8.29 μmol L^{-1}), but no cytotoxic effect ($IC_{50} > 25$ μg mL^{-1}) on human lymphoblast cell line. This study reveals the potential use of **5** as prototype for the synthesis of new antiproliferative agents.

1. Introduction

The genus *Erythroxylum* P. Browne is the major representative of the Erythroxylaceae family, comprising around 240 species widespread in neotropical regions (Araújo et al., 2015). In Brazil, 129 species were identified, of which 84 are endemic, including *E. macrocalyx* Mart. (Flora do Brasil, 2020).

Erythroxylum species are chemically characterized mainly by the production of tropane alkaloids, flavonoids and terpenoids (Chávez et al., 1996; Barreiros et al., 2005; González-Guevara et al., 2006; Oliveira et al., 2010; Nascimento et al., 2012; Albuquerque et al., 2014). These metabolites confer to *Erythroxylum* several pharmacological activities, including antibacterial (Albuquerque et al., 2014), antimicrobial, antiproliferative, proaptotic (Aguiar et al., 2012), hepatoprotective (Rodeiro et al., 2008; Syed and Namdeo, 2013), snake antivenom (Oliveira et al., 2016), antihypertensive (Lucas-Filho et al., 2010), multidrug resistance inhibitory activity (Silva et al., 2001), cytotoxic (Elias et al., 2016), among others (Restrepo et al., 2019).

There are few studies describing the anticancer activity of chemical constituents from *Erythroxylum* species, despite the diversity of biological properties related to this genus. The tropane alkaloid catuabine B, isolated from *E. caatingae*, showed very weak inhibition on human lung carcinoma cell line (NCI-H292) (Oliveira et al., 2011). Tropane alkaloids aromatic esters called pervilleines, isolated from different *Erythroxylum* species, have potential to be used as adjuvant in cancer therapy, to enhance the performance of chemotherapeutic drugs, such as

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vinblastine, since it has been shown to reverse multidrug-resistance tumor cells during the course of treatment (Silva et al., 2001; Mi et al., 2002; Chávez et al., 2002; Chin et al., 2006; Kinghorn et al., 2011; Beutler et al., 2012). In addition, a tropane alkaloid aliphatic ester and alkaloid-enriched fractions from *E. pugens* showed low cytotoxicity against four tumoral cell lines (Pereira et al., 2018). Two flavanols from *E. catuaba* (cinchonains **1a** e **1b**) were also cytotoxic against mouse lymphocytic leukemia (L1210) cells (Satoh et al., 2000). Finally, the diterpene 14-*O*-methylryanodanol from *E. passerinum* demonstrated a dose-dependent antiproliferative effect on glioblastoma-derived human (GL-15) cell line (Menezes-Filho et al., 2014).

Antibacterial activities of *Erythroxylum* species are also reported in the literature. The extract of leaves from *E. pulchrum* inhibited the growth of ten Gram-positive and Gram-negative bacterial strains, including *Staphylococcus aureus* ATCC 25,925 (MIC = 64 μg mL^{-1}) (Albuquerque et al., 2014). Extracts of five South African *Erythroxylum* species were tested against four bacterial strains and the methanol extracts of *E. delagoense* and *E. pictum* showed the highest activity (MIC = 250 μg mL^{-1}) against *Klebsiella pneumoniae* ATCC 13,883 (De Wet, 2011). Fractions of the stem bark ethanol extract from *E. suberosum* were assessed against five bacterial strains; the ethyl acetate and hydromethanol fractions showed antibacterial activity (MIC = 250 μg mL^{-1}) against *S. aureus* ATCC 25,923 (Violante et al., 2012). Bark extracts from *E. catuaba* ameliorate the infection caused by *Escherichia coli* and *S. aureus* in mice (Manabe et al., 1992). However, most studies reported only results for crude extracts or their fractions, and experiments with isolated compounds from *Erythroxylum* species are scarce.

In line with our ongoing research program focused on the discovery of natural products and their derivatives as new cytotoxic (Teixeira et al., 2007; Arantes et al., 2009; Rodriguez-Hernández et al., 2016; Santos et al., 2017; Nain-Perez et al., 2017), and antibacterial agents (Montanari et al., 2011; Nascimento et al., 2011; Santos et al., 2013), this work describes the first phytochemical investigation of the twigs and leaves from *E. macrocalyx* in the search for new antiproliferative and antibacterial compounds.

2. Results and discussion

2.1. Extraction and isolation

Twigs and leaves of *E. macrocalyx*, collected in Jequie municipality, Bahia state (Brazil), were dried, powedered and extracted with ethanol. This procedure resulted in solid residues, representing 6.5 % and 5.3 % of the mass of the extracted materials, respectively. The ethanol extracts were chromatographed on silica gel columns and some groups of fractions were further subjected to further chromatographic processes. These procedures resulted in the isolation, from the twigs, of the tropane alkaloids 7β-acetoxy-6β-benzoyloxy-3α-hydroxytropane (**1**), 6β,7β-dibenzoyloxy-3α-hydroxytropane (**2**) 6β,7β-dihydroxy-3α-(phenylacetoxy)tropane (**3**), 3α-benzoyloxy-6β,7β-dihydroxytropane (**4**), 6β-benzoyloxy-3α-(4-hydroxy-3,5-dimetoxybenzoyloxy)tropane (**5**), besides ombuin-3-rutinoside-5-glucoside (**6**), lupeol (**7**), taraxerol (**8**) and lupenone (**9**). Compounds **1** and **2** were also isolated from the leaves, whose crude extract revealed the presence of large amount of crystals. Structural formulas of the compounds (Fig. 1) were established by interpretation of their NMR (1D and 2D) and HRMS data and comparison with data from the literature.

2.2. Identification of Compounds 1 and 2

Compound **1** was isolated as a viscous pale yellow oil. Its molecular formula was assigned as $C_{17}H_{21}NO_5$ based on the positive HR-ESIMS that showed a peak with *m/z* 320.1494 $[M+H]^+$, accounting for eight degrees of unsaturation. Its ^{1}H NMR spectrum (Fig. S1 and Table 1) showed signals corresponding to a typical 3,6,7-*O*-tri-substituted tropane moiety, with chemical shifts at δ_H 4.15 (1H, t, *J* =4.7 Hz, H-3), 5.97 (1H, d, *J* =6.4 Hz, H-6$_{endo}$) and 5.86 (1H, d, *J* =6.4 Hz, H-7$_{endo}$). Signals of the other hydrogen atoms of the tropane ring are listed in Table 1.

The assignment of the signals due to H-6 and H-7 were confirmed from the ^{15}N-^{1}H HMBC spectrum (Fig. S8), which showed a three bond correlation of the N atom with H-7 and H-6. The structure was also supported by ^{1}H-^{1}H COSY experiment, that showed the correlations between H-1 and H-2$_{exo}$/H-2$_{endo}$, and between H-5 and H-4$_{exo}$/H-4$_{endo}$, confirming the absence of substitutions at positions 2 and 4. The presence of a hydroxyl group at C-3 was deduced from the molecular mass and typical chemical shift of H-3 (Chávez et al., 2002; Zanolari et al., 2003). The correlation of C-3 (δ_C 64.1) with H-2$_{endo}$ and H-4$_{endo}$, as well as, with H-1 and H-5, also confirmed the position of the hydroxyl group at C-3.

The presence of benzoate and acetate moieties in **1** was determined by the chemical shifts of hydrogen and carbon atoms from the monosubstituted phenyl ring and methyl group (acetate), and confirmed by the presence of acyl peaks (Table 1) at δ_C 166.2 (C-7′) and δ_C 170.5 (C-1″) in ^{13}C NMR spectra (Ribeiro et al., 2013).

The substitution pattern of the tropane moeity of **1** at positions 3, 6 and 7 was proposed from correlations observed in HMBC and ^{1}H-^{1}H COSY spectra (Table 1 and Fig. 2). The correlations observed in the HMBC contour map of the peak corresponding to H-6 and carbonyl carbon (C-7′) of benzoyloxy group, as well as a correlation between H-7 and carbonyl carbon (C-1″) of the acetate group, are in aggreement with these substituents at C-6 and C-7 on the tropane ring.

The relative stereochemistry at C-3, C-6 and C-7 was initially established by the couplings observed for H-3, H-6 and H-7, and confirmed by NOE difference (NOEdiff) and 2D (^{1}H-^{1}H COSY and HMBC) NMR experiments (Table 1, Fig. 2).

The coupling between H-6 and H-7, and absence of coupling between

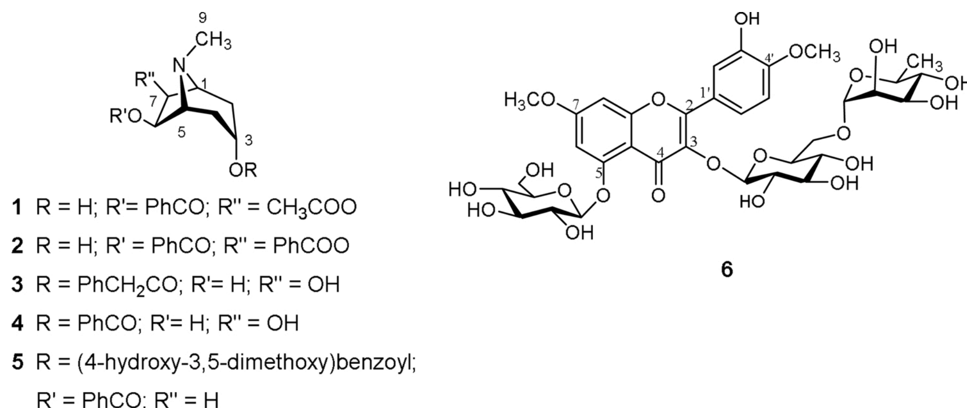

Fig. 1. Compounds (**1-6**) isolated from *E. macrocalyx*.

Table 1
^{1}H and ^{13}C NMR (1D and 2D) data* of compound **1**.

Position	δ_C	δ_H (*mult.*, *J* in Hz)	NOEdiff	HMBC	^{1}H-^{1}H COSY
1	65.1	3.26 (br *s*)		C-3; C-5; C-6; C-7	H-2_{exo}; H-2_{endo}
2_{endo}	32.9	1.76 (br *d*, 15.0)	H-3; H-2_{exo}; H-1; H-7	C-3; C-4	H-2_{exo}; H-1
2_{exo}	32.9	2.18 (*ddd*, 15.0;4.2;3.6)**		C-1; C-7	H-2_{endo}; H-1; H-3
3	64.1	4.15 (*t*, 4.7)	H-2_{exo}/H-4_{exo}; H-2_{endo}/H-4_{endo}	C-2/C-4; C-1/C-5	H-2_{exo}; H-4_{exo}
4_{endo}	32.9	1.78 (br *d*, 15.0)	H-3; H-4_{exo}; H-5; H-6	C-2; C-3	H-4_{exo}; H-5
4_{exo}	32.9	2.21 (*ddd*, 15.0;4.8;3.0)**		C-5; C-6	H-4_{endo}; H-5; H-3
5	65.1	3.36 (br *s*)		C-3; C-1; C-6; C-7	H-4_{exo}; H-4_{endo}
6	78.0	5.97 (*d*, 6.4)		C-4; C-1/C-5; C-7′	H-7
7	77.5	5.86 (*d*, 6.4)		C-2; C-1/C-5; C-1″	H-6
9	38.0	2.62 (*s*)	H-2_{exo}/H-4_{exo}; H-1; H-5; H-2'/H-6'; H-2''	C-1/C-5	—
1′	130.2	—			—
2′	129.7	8.03 (*dd*, 8.3; 1.3)		C-3′; C-4′; C-7′	H-3′
3′	128.5	7.43 (*dd*, 8.1;7.8)#		C-2′	H-2′; H-4′
4′	133.2	7.55 (*tt*, 7.4; 1.3)		C-2′/C-6′	H-3′/H-6′
5′	128.5	7.43 (*dd*, 8.1;7.8)#		C-6′	H-4′; H-6′
6′	129.7	8.03 (*dd*, 8.3; 1.3)		C-4′; C-5′; C-7′	H-5′
7′	166.2	—		—	—
1″	170.5	–		—	—
2''	21.1	1.97 (*s*)		C-1″	—

* Spectra obtained in $CDCl_3$, at 600 and 150 MHz, for ^{1}H and ^{13}C, respectiverly. **The coupling constants were calculated from homonuclear decoupling spectra (Supplementary data). #This signal resembles a triplet (see Fig. S1) due to the very close coupling constant values.

H-6 and H-5 and between H-7 and H-1 was verified in ^{1}H-^{1}H COSY contour map, indicating that H-6 and H-7 should be in the *endo* face, with a dihedral angle close to 90° with the neighboring hydrogens H-5 and H-1, respectively. A NOE observed between hydrogens of *N*-methyl group and H-2′/H-6′ of the benzoate group, and the CH_3CO group, are in aggreement with both ester substituents at the β-orientation (*exo*) at C-6 and C-7. A NOE was also observed between the *N*−CH_3 group with H-2 and H-4 (both *exo*). The unique correlations observed for H-3 in the ^{1}H-^{1}H COSY contour map (Table 1 and Fig. 2a) were with H-4_{exo}/H-2_{exo}, and therefore, the signal of H-3 was observed as a triplet (J =4.7 Hz). This multiplicity and coupling constant value are typical of a substituent at C-3 in α-orientation (Al-Said et al., 1989; Bringmann et al., 2000; Zanolari et al., 2003). Additionally, absence of NOE of H-3 (*exo*) with H-6 and H-7 (both *endo*), confirm the α-orientation of the hydroxyl group.

The signals of H-5 and H-1 are observed as broad singlets, however, it is possible to verify in the ^{1}H-^{1}H COSY contour map (Table 1, Fig. 2 and Fig. S4B) a correlation between H-5 and H-4 (both *exo* and *endo*), as well as of H-1 and H-2 (both *exo* and *endo*), indicating couplings between them. The signals of H-4_{exo} and H-2_{exo} have been observed as multiplet in ^{1}H NMR spectrum, due to the overlap of several lines. However, by homonuclear decoupling experiments (Fig. S5), accomplishing the selective irradiation at resonance frequencies of H-3, H-5 and H-1, it was possible to determine unambiguously the chemical shifts, multiplicity and coupling constants for H-4_{exo} and H-2_{exo}. The irradiation of H-3 resulted in a simplification of the signals of H-4_{exo} and H-2_{exo}, which were observed as double doublets. In the same way, irradiation of H-5 resulted in simplification of the signal due to H-4_{exo}, observed as double doublet. Since there is no coupling between H-5 and H-2_{exo}, the signal of H-2_{exo} remained unchanged, and its multiplicity was determined as double double doublet. Similarly, the irradiation of H-1 caused a change only in the signal of H-2_{exo}, that was simplified to a double doublet, while the signal of H-4_{exo} remained unchanged.

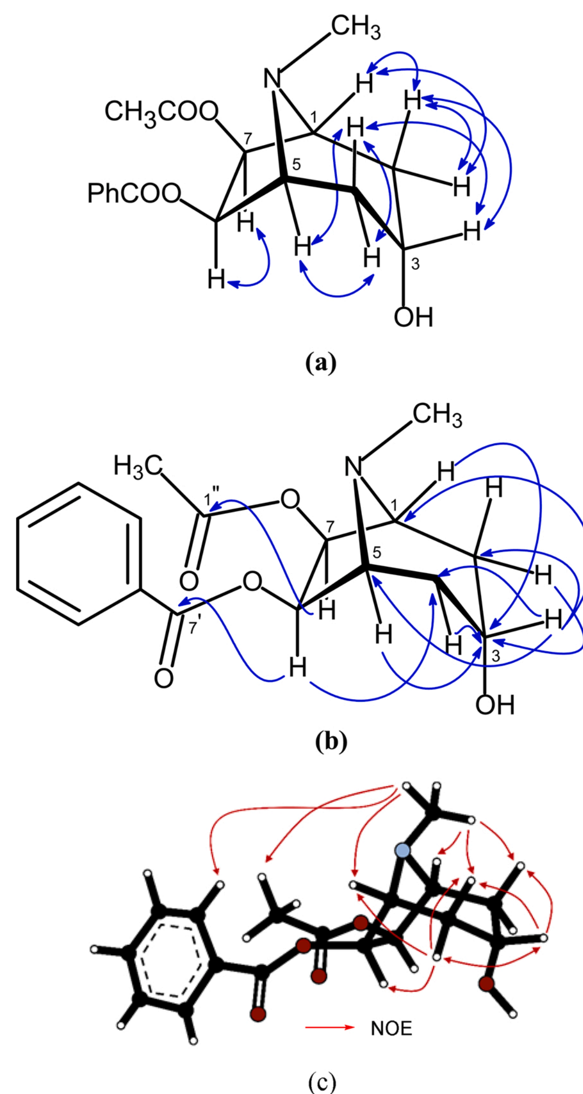

Fig. 2. Key correlations observed for compound **1** from: **(a)** ^{1}H-^{1}H COSY; **(b)** HMBC; and **(c)** NOEdiff spectra.

The findings permitted to confirm the identify of **1** as 7β-acetoxy-6β-benzoyloxy-3α-hydroxytropane, a novel tropane alkaloid.

The eight known constituents isolated included the tropane alkaloids 6β,7β-dibenzoyloxy-3α-hydroxytropane (**2**), 6β,7β-dihydroxy-3α-(phenylacetoxy)tropane (**3**) (Al-Said et al., 1986), 3α-benzoyloxy-6β,7β-dihydroxytropane (**4**) (Griffin, 1978), 6β-benzoyloxy-3α-(4-hydroxy-3,5-dimethoxybenzoyloxy)tropane (**5**) (Oliveira et al., 2011); the glycosylated flavonoid ombuin-3-rutinoside-5-glucoside (**6**) (González-Guevara et al., 2006); and the triterpenes lupeol (**7**), taraxerol (**8**) (Mahato and Kundu, 1994) and lupenone (**9**) (Nasser et al., 2013). These substances (**2-9**) were identified by comparing their ^{1}H and ^{13}C NMR data with those reported in the literature. In the case of compound **2,** X-ray crystallography was used to unequivocally prove its structure, previously determined by spectrometric methods (Cruz et al., 2016).

Compound **2** was isolated as a white crystalline solid (T_f = 170−171 °C). Analysis by gas chromatography coupled to mass spectrometry (GC-EIMS) revealed the molecular ion $[M]^{+\cdot}$ peak at m/z 381, compatible

with the molecular formula $C_{22}H_{23}NO_5$. The ^1H and ^{13}C NMR data of this substance (Table S2) were characteristic of 3,6,7-*O*-trisubstituted tropane alkaloid (Ribeiro et al., 2013), and closely related to the data reported for 6β,7β-dibenzoyloxy-3α-hydroxytropane hydrochloride, isolated from *E. subsessile* (Cruz et al., 2016). This is the second occurrence of 6β,7β-dibenzoyloxy-3α-hydroxytropane as a natural product so far, both in the genus *Erythroxylum*.

The ORTEP-3 view of **2** is presented in Fig. 3 and its crystal structure refinement data are shown in Table S3 (Supplementary data).

The X-rays diffraction study reveals that **2** forms a monohydrate crystal (Fig. 3a) when recrystallized in ethyl acetate. Although **2** has four stereocenters (at C1, C5, C6 and C7), it is considered a *meso* compound due to the internal mirror plane (through C9, N8, C3 and O3) that bisects the tropine ((3-*endo*)-8-methyl-8-azabicyclo[3.2.1]octan-3-ol) moiety. Therefore, the C1(*R*),C5(*S*),C6(*S*), C7(*R*) isomer, which was arbitrarily chosen as asymmetric unit (Fig. 3), is equivalent to the C1(*S*),C5(*R*),C6 (*R*),C7(*S*) one; and consequently **2** is optically inactive (Hoffmann, 2003). Moreover, since the space group adopted by compound **2** is centrosymmetric ($P2_1/c$), the crystal structure would be considered racemic even constituted by a true chiral molecule. Interestingly, the descriptors for the ring substituents (Luger and Bülow, 1983) have N8-C9 axial (angle with Cremer & Pople plane normal = 7.43(8)°) and equatorial (angle with Cremer & Pople plane normal = 88.36(8)°) for the 5- and 6-membered sub-rings, respectively. Intramolecular non-classical hydrogen bonds, having C9 atom as bifurcated donor and O1′ and O1″ as acceptors atoms, were observed (Fig. 3b).

2.3. Antiproliferative and antibacterial activities

Evaluation of the *in vitro* cytotoxic effect of compounds **1–9** was

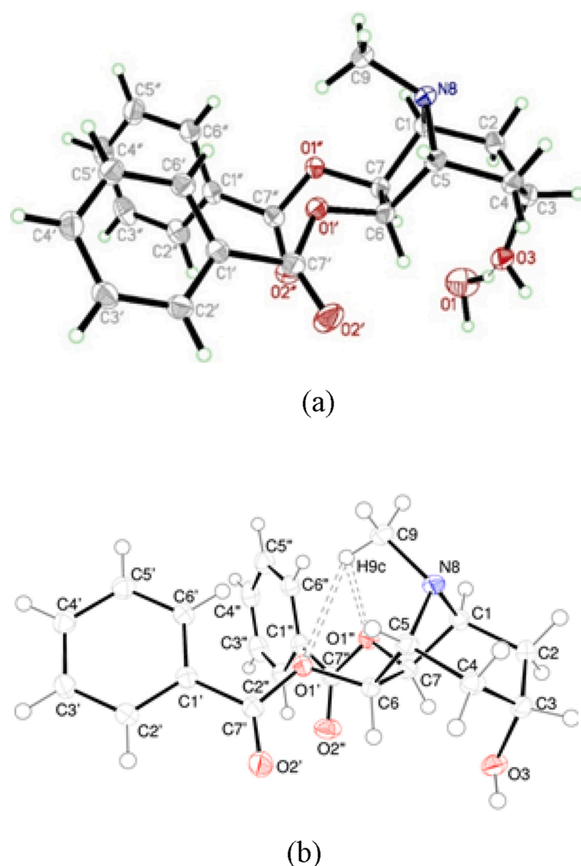

Fig. 3. ORTEP plot and atom numbering of **2**: (a) ellipsoids represent 50 %-probability level, showing water molecule of crystallization; (b) double-dotted lines represent intramolecular H-bond.

carried out with the following human tumor cell lines: hepatocellular carcinoma (HepG2), promyelocytic leukemia (HL-60), chronic myelogenous leukemia (K562), and non-malignant human lymphoblast (PBMC) and human lung fibroblast (MRC-5). All compounds were tested at a maximum concentration of 25 μg mL^{-1}.

Compounds **1**, **2**, **7**, **8** and **9** showed no activity against the cell lines tested (see Table S1).

Among the tropane alkaloids, **3** was the only one that have an effect on all three cancer cell lines, moderately decreasing their viability as observed by the IC_{50} of 13.72 μg mL^{-1} (49.83 μmol L^{-1}), 19.62 μg mL^{-1} (71.25 μmol L^{-1}) and 20.72 μg mL^{-1} (75.25 μmol L^{-1}), for HepG2, K562 and HL60, respectively. This compound is more toxic towards the non-malignant lymphoblast PBMC showing IC_{50} value of 4.22 μg mL^{-1} (15.33 μmol L^{-1}). From these data the selectivity index (SI) is < 0.3, indicating that **3** is not adequate to be further investigated as a potential anticancer drug. Substances with SI < 10 are not considered to possess therapeutic potential (Awouafack et al., 2013). This compound was previously isolated only from *Erythroxylum* species and had already been evaluated for its cytotoxicity against a panel of human cancer cell lines and were inactive ($ED_{50} \geq 20$ μg mL^{-1}) in all cases (Al-Said et al., 1986, 1989; Silva et al., 2001; Mi et al., 2002).

The other two tropane alkaloids **4** and **5** exhibited antiproliferative effect only against HepG2 cell line with IC_{50} values of 11.59 and 3.66 μg mL^{-1} (44.35 and 8.29 μmol L^{-1}) respectively. Conversely, these compounds had no cytotoxic effect on non-tumor cells (human lymphoblasts) at 25 μg mL^{-1}, and showed some selectivity (SI ≥ 2.2 for **4**; SI ≥ 6.8 for **5**). Although **4** and **5** have been previously isolated from other *Erythroxylum* species (Griffin, 1978; El-Imam et al., 1988; Khattak et al., 2003; Oliveira et al., 2011), to the best of our knowledge, there is no previous report on their cytotoxicity, therefore, further investigations are needed.

Finally, the glycoside **6** was toxic towards HepG2 cells (IC_{50} = 19.38 μg mL^{-1}) and had no effect on the human lymphoblast at 25 μg mL^{-1}. This compound has been previously isolated from three species of *Erythroxylum* (Iñigo and Pomilio, 1985; Iñigo et al., 1988; Gonzáles-Guevara et al., 2006; Albuquerque et al., 2014) and from *Porcelia macrocarpa* (Annonaceae) (Chaves et al., 2004), but no biological activity was reported.

The antibacterial activities of compounds **2–9** were investigated against two clinical relevant strains, *S. aureus* (ATCC 29,213) and *Pseudomonas aeruginosa* (ATCC 27,853). The antibacterial activity of a compound could be considered significant when MIC ≤ 10 μg mL^{-1} (Awouafack et al., 2013). However, none of the tested substances showed significant antibacterial activity (MIC = MBC > 100 μg mL^{-1}), when compared to tetracycline (MIC = MBC = 3.0 μg mL^{-1}) and ceftriaxone (MIC = 3.0 μg mL^{-1}; MBC = 10.0 μg mL^{-1}) that were used as standard drugs against *S. aureus* and *P. aeruginosa*, respectively. Though antibacterial activity has already been reported for extracts and fractions of *Erythroxylum* species (De Wet, 2011; Violante et al., 2012; Albuquerque et al., 2014), few studies have verified the activity of isolated compounds from these species. The synergistic effect between different molecules in the extracts and fractions and/or differences in ATCC strains might explain the lack of antibacterial activity of the isolated compounds herein reported. Since commercially available tropane alkaloids as scopolamine and atropine showed significant activity (MIC 2–16 μg mL^{-1}) against eight standard strains of Gram-positive and Gram-negative bacteria, including *S. aureus* (ATCC 25,923) and *P. aeruginosa* (ATCC 10,145) (Özçelik et al., 2011), we envisage that further investigation of *Erythroxylum* species could provide new bioactive compounds.

In conclusion, tropane alkaloids, one of the main taxonomic markers of the genus *Erythroxylum*, are the most abundant chemical constituents of the twigs and leaves of *E. macrocalyx*. The alkaloid 7β-acetoxy-6β-benzoyloxy-3α-hydroxytropane (**1**) is described for the first time in the literature. The leaves from *E. macrocalyx* proved to be a rich source (> 5%, w/w) of the alkaloid 6β,7β-dibenzoyloxy-3α-hydroxytropane (**2**),

which has been previously isolated of other species, its NMR and X-ray data are being reported for the first time. This compound, as well as other tropane alkaloids identified in this work, has only occurred in this genus so far, and most of them has received relatively little or no attention regarding their pharmacological properties. In this work, compound **5** [6β-benzoyloxy-3α-(4-hydroxy-3,5-dimethoxybenzoyloxy) tropane] showed the highest toxicity towards human liver cancer cell line with no toxicity on human normal cells. Therefore, the current study reinforces and expand the diversity of tropane alkaloids in the genus *Erythroxylum*, as well as, increases the possibility of using these substances as prototype in the synthesis of new antiproliferative agents.

3. Experimental

3.1. General experimental procedures

Column chromatography was performed on silica gel 60 (70–230 Mesh-ASTM, Merck). Thin layer chromatography was performed on silica gel F_{254} plates (Fluka) and visualized under UV light (λ = 254 and 365 nm), iodine vapor and/or Dragendorff's reagent. Uncorrected melting points were measured in an MQAPF-301 *Microquímica* apparatus. 1D and 2D NMR spectra were recorded on a Varian Mercury 300 (^{1}H NMR at 300 MHz and ^{13}C NMR at 75 MHz) and Bruker Avance III HD 600 (^{1}H NMR at 600 MHz and ^{13}C NMR at 150 MHz). The mass spectra (EI) were obtained at 70 eV, in a GC-LRMS system (Shimadzu GC17A/ QP2010 SE). High resolution mass spectrum was obtained in a Shimadzu HPLC system (pump LC-20 AD, CBM-20, DGU-20A-5 and "Photodiode Array" detector SPD-M20A) coupled with microOTOF Q II-ESI-TOF mass spectrometer (Brucker). IR spectra were recorded on Perkin Elmer infrared spectrometer, Spectrum Two ATR-FTIR model.

3.2. Plant material

Plant material of *Erythroxylum macrocalyx* Mart. (Erythroxylaceae) was collected from "Brejo Novo" farm (13°56′41.0″S 40°06′33.9″W), Jequié, Bahia - Brazil, by Guadalupe Edilma Licona de Macedo in 2003 (HUESB 1463), and identified by Maria Iracema Bezerra Loiola, from Federal University of Ceará (UFCE). For this research, Léia Alexandre Alves collected a sample of the same specimen, in April 2015, and the voucher specimen (HUESB 11,125) was deposited at the Herbarium of the Southwest Bahia State University (UESB). Authorization of access to Brazilian System for the Management of Genetic Heritage and Associated Traditional Knowledge – SISGEN (#A361C0C) has been granted.

3.3. Extraction and isolation

Dried twigs (347 g) and leaves (466 g) of *E. macrocalyx* were macerated with ethanol 95 %, at room temperature. The extracts from twigs and leaves were concentrated under reduced pressure to produce 22.6 g and 25.0 g of crude extracts, respectively. An aliquot of the twigs extract (21.0 g), adsorbed on silica gel 60, was fractionated on a filter column, eluting sequentially with hexane, ethyl acetate, ethanol and methanol, to yield the respective fractions: EMH (1.63 g), EMEA (1.70 g), EME (16.50 g) and EMM (0.96 g). After analysis of all fractions by thin layer chromatography (TLC), revealed by Dragendorff's reagent, the presence of alkaloids in the ethanol fraction (EME) was verified. This fraction was subjected to the methodology described by Quintans-Júnior et al. (2004), to afford the total alkaloid fraction from twigs (TTAF). TTAF (9.78 g) was fractioned by column chromatography (CC) on silica gel, using isocratically a mixture of $CHCl_3$/MeOH (1:1 v/v) as mobile phase, to afford five fractions (TTAF1- TTAF5). TTAF3 (5.23 g), rich in alkaloids, was subjected to silica gel CC, using $CHCl_3$/MeOH mixture with increasing polarity, to afford five fractions (TTAF3.1 to TTAF3.5). TTAF3.1 (445 mg), was subjected to silica gel CC, using $CHCl_3$/MeOH (9:1), to afford five other fractions (TTAF3.1.1 to TTAF3.1.5). Fractions TTAF3.1.4 and TTAF3.1.3 yielded, respectively, compounds **1** (2 mg) and **2** (133 mg). Fraction TTAF3.2 (1.94 g) yielded compound **3** (154 mg), after fractionation by silica gel CC, using $CHCl_3$/MeOH (7:3 v/v).

Methanol fraction (EMM, 0.96 mg) was partially soluble with a small volume of methanol. A light brown solid, poorly soluble in methanol, was separated from the soluble fraction by filtration and, after purification by successive washings with cold methanol, yielded compound **6** (60 mg). The soluble fraction (800 mg) was purified on silica gel column eluting with $CHCl_3$/MeOH (7:3 v/v) to yield compounds **4** (13 mg) and **5** (7 mg).

Hexane fraction (EMH, 1.63 g) was fractionated by silica gel CC, using hexane/EtOAc mixture with increasing polarity, to afford seven fractions (EMH1 to EMH7). EMH3 (300 mg) was subjected to silica gel CC, using CH_2Cl_2, to afford six fractions (EMH3.1 to EMH3.6). Fraction EMH3.4 (26 mg) was partially dissolved in a small amount of hexane, resulting in the separation of compound **7** (15 mg) soluble, and compound **8** (11 mg) insoluble in hexane. Fraction EMH1 (285 mg) was purified by silica gel column chromatography, eluting with hexane/ EtOAc (9.5:0.5) yielding compound **9** (5 mg).

The ethanol extract from the leaves showed a large amount of crystals, which were characterized as alkaloids by TLC analyses, revealed by Dragendorff's reagent. Leaves extract (1.00 g) was subjected to the procedure described by Quintans-Júnior et al. (2004), to afford the total alkaloid fraction from leaves (LTAF). LTAF (101 mg) was fractionated by silica gel CC, using EtOAc/MeOH (8:2 v/v) mixture, to afford six fractions. A pure sample of compound **2** (41 mg) was obtained from fraction 2 and a further amount (26 mg) slightly impure was isolated from fraction 3. Compound **1** (2 mg) was isolated from fraction 4, however, in the form of hydrochloride salt (**1a**), probably obtained from the addition of HCl in the procedure used to obtain the LTAF.

7β-acetoxy-6β-benzoyloxy-3α-hydroxytropane (**1**). Viscous pale yellow oil; HR-ESIMS (*m/z* 320.1494 $[M+H]^+$, calcd 320.1498 for $C_{17}H_{22}NO_5$); EI-MS *m/z* (rel. int.) 113 (100), 105 (58), 112 (54), 96 (39), 94 (25), 77 (24), 81 (17), 138 (15), 198 (12), 319 (7); For ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data see Table 1.

6β,7β-dibenzoyloxy-3α-hydroxytropane (**2**). White crystalline solid, m. p. 170−171 °C; EI-MS *m/z* (rel. int.) 105 (100), 77 (76), 113 (55), 94 (31), 96 (29), 112 (27), 42 (22), 51 (15), 381 (3); For ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) data see Table S2.

For compounds **1a**, **3**, **4**, **5** and **6** IR, MS, (1D and 2D) NMR spectra and spectroscopic data are presented in Table S5 and Figures S11, S12, S21, S22 and S23 (Supplementary information material).

3.4. X-ray diffraction studies

A suitable single crystal of **2** obtained by slow evaporation of ethyl acetate solution at room temperature after two days was selected for the X-ray experiment. The X-ray intensity data were collected at 105(2) K on a Bruker Venture diffractometer equipped with a Photon 100 CMOS Detector, a Helios MX optics, a Gallium Liquid Metal jet source (λ =1.34139 Å), and a Kappa goniometer. The programs APEX 2 (APEX 2, 2013) and SAINT (SAINT V8.34A, 2013) were used for data collection, cell refinement and data reduction. The structure was solved by direct methods and refined by full-matrix least-squares on F2 using SHELXL-2018/3 (Sheldrick, 2015). The hydrogen atoms of compound **2** were positioned stereochemically and were refined with fixed individual displacement parameters [Uiso (H) = 1.5 Ueq (C or O) for methyl and hydroxyl groups or 1.2 Ueq (C) for aromatic, methine and methylene groups] using a riding and rotating group model with fixed bond lengths (C–H or OH—) of 0.95, 0.98, 0.99, 1.00 and 0.84 Å for the aromatic, methyl, methylene, methine and hydroxyl groups, respectively. WINGX software was used to analyze and prepare the data for publication. Molecular graphics were prepared using ORTEP-3 for Windows (Farrugia, 2012). Crystallographic data for the structural analysis of the compound discussed here has been deposited at the Cambridge Crystallographic Data Centre (CCDC) as a supplementary publication under number CCDC 1994787. CCDC contains the supplementary

crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336, 033; e-mail: deposit@ccdc.cam.ac.uk).

3.5. Cells

HepG2 (human hepatocellular carcinoma), HL-60 (human promyelocytic leukemia), K562 (human chronic myelogenous leukemia), PBMC (human lymphoblast) and MRC-5 (human lung fibroblast) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in complete medium with appropriate supplements, as recommended by ATCC. All cell lines were tested for mycoplasma using a mycoplasma stain kit (Sigma-Aldrich Co., Saint Louis, MO, USA) to validate the use of cells free from contamination. Primary cell culture of peripheral blood mononuclear cells was obtained by standard Ficoll density protocol and, subsequently, incubated concanavalin A to obtain human lymphoblasts (Berthold, 1981; Brown and Lawce, 1997). The Research Ethics Committee of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) approved the experimental protocol (#031,019/2013). Cell viability was examined using Trypan blue exclusion assay for all experiments.

3.6. In vitro antiproliferative assay

Cell viability was quantified using Alamar Blue assay, as described previously by Ahmed et al. (1994) with minor modifications (Rodrigues et al., 2015). Briefly, non-tumor and tumor cells were placed in 96-well plates (7×10^4 cells mL^{-1} for adherent cells or 3×10^5 cells mL^{-1} for suspended cells in 100 μL of medium) and incubated with substances **2–9** at concentrations of 0−25 μg mL^{-1}, for 72 h at 37 °C, under a CO_2 (5%) atmosphere. Cell viability was quantified based on the ability of living cells to reduce Alamar Blue dye (52 μg mL^{-1}; Sigma Aldrich) to resorufin product and the absorbance was measured at 570 and 600 nm (DTX-880, Beckman Coulter) after 4 h at 37 °C, under CO_2 (5%) atmosphere. Doxorubicin (purity ≥ 95 %, doxorubicin hydrochloride, Laboratory IMA) was used as the positive control (0.08–5 μg mL^{-1}). Data were expressed as IC_{50} values with 95 % confidence intervals obtained by nonlinear regressions from three independent experiments using the GraphPad Prism (Intuitive Software for Science; San Diego, CA, USA).

3.7. In vitro susceptibility testing

All bacteria strains used for the biological evaluation of the compounds (**2–9**) isolated from *E. macrocalyx* were obtained from the ATCC (Manassas, VA). The strains were *S. aureus* (ATCC 29,213) and *P. aeruginosa* (ATCC 27,853). The susceptibility testing was implemented using a modified version of the CLSI (formerly NCCLS) methods as previously described (Santos et al., 2017; Mairink et al., 2019) by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2020.11.019.

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