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Elaeodendron orientale as a source of cytotoxic cardenolides

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ABSTRACT

In the present study, we report six cardiac glycosides (**1**–**6**) along with four known ones (**7**–**10**) isolated from the leaves and fruits of *Elaeodendron orientale*. Their stereostructures were elucidated on the basis of spectroscopic analysis, including 1D and 2D NMR, and the absolute configuration of **1** was determined by X-ray diffraction analysis. The compounds were evaluated for growth inhibitory activity against a panel of human cancer cell lines, HeLa, A-549, MCF-7 and HL-60, and normal Vero cells. Four compounds from this series (**5** and **7**–**9**, IC_{50} values ranging from 0.01 to 0.07 μM) exhibited cytotoxicity against three of the cancer cell lines assayed that was similar to or higher than the well-known therapies digoxin and digitoxigenin. Taking into account the narrow safety range of cardiac glycosides used in clinic, this series shows a selectivity index higher than 3 for three of the cancer cell lines assayed, increasing their interest for further study.

1. Introduction

Cancer is still the second-leading cause of death worldwide, and as no curative therapy is available for many types of cancer, there is an ongoing need for novel leads with chemopreventive and chemotherapeutic properties (Gibbs, 2000). Currently, re-discovering the potential of decade-old drugs for novel anticancer therapeutics is providing a new route for drug development (Cragg et al., 2014).

Cardiac glycosides (CGs) are naturally occurring compounds, which have been used to treat cardiac failure for decades. However, within the past ten years, there has been a substantial increase in the number of studies that highlight the potential anticancer properties of these compounds (Mijatovic et al., 2007), including preclinical as well as phase I studies (Slingerland et al., 2013). It is now recognized that CGs are involved in complex cell signal transduction mechanisms (Mijatovic and Kiss, 2013), and that the use of selected CGs such as digitoxin (Lee et al., 2014), digoxin (Shraibom, 2012), reevesioside A (Leu et al., 2014), oleandrin (Yang et al., 2014) or ouabain (Jun et al., 2013) may represent a worthwhile approach towards the prevention and/or treatment of cancer, even despite their narrow therapeutic window. Additionally, further development of synthetic, semi-synthetic, or naturally occurring CGs might expand the possibilities of finding a CG with a wider therapeutic index.

Chemically, GCs are compounds with a steroidal framework nucleus, a lactone moiety at position 17 and a sugar moiety at position 3. The lactone at C-17 position defines two subgroups: cardenolides with an unsaturated butyrolactone ring, and bufanolides with an α-pyrone ring at such position. The steroid nucleus has a unique set of fused ring systems that makes the aglycone moiety structurally distinct from the other more common steroid ring systems. Generally, A/B and C/D rings are *cis* fused and B/C rings are *trans* fused; whereas in GCs produced by plants from the Asclepiadaceae, A/B rings are *trans* fused (Mijatovic et al., 2007). The steroidal skeleton can be substituted at position 3 by a sugar moiety (glycoside), leading to the classification of subfamilies such as glycosylated cardenolides or glycosylated bufadienolides, depending on the nature of the lactone moiety. GCs, currently used for the treatment of congestive heart failure and arrhythmia in humans, are isolated from plants, since their structural complexity makes synthesis difficult. Recently, insights into the biosynthesis of this type of metabolites have been reported (Munkert et al., 2014).

Celastraceae species are a rich source of cardenolides, mainly those belonging to *Elaeodendron* (Shimada et al., 1985; Tsujino et al., 1995; Cao et al., 2007; Hou et al., 2009; Butler et al., 2014) and *Euonymus* (Baek et al., 1994; Kitanaka et al., 1996) genera. Some cardenolides from this family are structurally remarkable, as they contain an unusual sugar linkage dioxane-type six-membered ring

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(Shimada et al., 1979; Shimada et al., 1981) and a methylendioxy moiety (Shimada et al., 1982; Shimada et al., 1990), such as elaeodendroside A isolated from the seeds of *Elaeodendron glaucum* (Kupchan et al., 1977), the first reported CG in Celastraceae species.

Elaeodendron orientale is an endemic shrub of Madagascar and the Mascareigne Islands, and is commonly known as the false olive (Islam et al., 2006). Traditionally, the bark is used for treating venereal diseases, skin infections, and scorpion fish poisoning. The leaves have astringent and emetic properties and are used to treat hypertension, and when mixed with those of *Kalanchoe pinnata* (Crassulaceae), which produce bufadienolides (Supratman et al., 2000), can be used against fish allergies (Gurib-Fakim et al., 1997). However, chemical investigation into *E. orientale* has not been previously reported.

As part of our search for bioactive metabolites from Celastraceae species, we report herein on the isolation, structure elucidation and antiproliferative activity of seven new (**1**–**6**) and four known (**7**–**10**) cardenolides from *E. orientale*. Their stereostructures were determined by application of 1D and 2D NMR techniques, including COSY, TOCSY, HSQC, HMBC, and ROESY experiments, and comparison with data reported in the literature. The absolute configurations of these compounds were established by biogenetic means, and the X-ray crystallographic analysis of compound **1**. Afterwards, all the compounds, except **3** due to the small quantity isolated, were evaluated against a representative panel of cancer cell lines: HeLa (carcinoma of the cervix), A-549 (lung carcinoma), MCF-7 (breast adenocarcinoma) and HL-60 (promyelocytic leukemia); together with Vero (African green monkey kidney) normal cells, searching for selectivity. Compounds **5**, **7**–**9** display higher growth inhibitory activities in various human cancer cell lines and also selectivity when compared to two cardenolides chosen as references, digitoxin and digitoxigenin. Compound **9** (IC_{50} 0.034 μM) showed a promising anticancer effect on MCF-7 cells as the effect was 4-fold and 29-fold higher than the reference drugs, respectively.

2. Results and discussion

2.1. Chemistry

The EtOH extract of leaves and fruits of *E. orientale* was partitioned into CH_2Cl_2–H_2O (1:1, v/v) solutions. The CH_2Cl_2 fractions, separately, were subjected to multiple chromatographic steps, involving Sephadex LH-20, vacuum-liquid chromatography (VLC), chromatography on chromatron centrifugal TLC, and preparative TLC on silica gel, to yield cardenolides **1–10** (Fig. 1). The structures of the new compounds (**1–6**) were deduced as described below.

Compound **1** was obtained as a colourless crystal (AcOEt, mp 298–300 °C), and its molecular formula was deduced as $C_{30}H_{38}O_{13}$ the HREIMS (*m*/*z* 606.2300). Its IR spectrum exhibited absorption bands for hydroxyl (3452 cm^{-1}) and carbonyl (1745 and 1705 cm^{-1}) groups, and the UV spectrum showing a maximum at 220 nm was consistent with the presence of an α,β-unsaturated carbonyl group. In the ^{13}C NMR spectrum the signals for 30 carbon atoms were observed, DEPT experiments revealed that these signals corresponded to three methyls, seven methylenes (one oxygenated, one dioxygenated carbon), eleven methines (one olefinic, one dioxygenated, six oxygenated carbons), and nine quaternary carbons (one lactone, one ketone, one olefinic, one dioxygenated, three oxygenated carbons). The multiplicities together with the twelve degrees of unsaturation suggested the presence of nine rings in a cardiac glycoside skeleton with one sugar moiety. The complete ^1H and ^{13}C NMR (Tables 1 and 2) signal assignments and connectivity were determined from a combination of COSY, TOCSY, HSQC, and HMBC data, and confirm the proposed structure. The ^1H NMR spectrum in combination with COSY and TOCSY experiments allowed the identification of spin systems in the steroidal skeleton and sugar moiety. Thus, COSY and TOCSY correlations established four spin systems in the aglycone: CH_2–CH–CH–CH for ring A (H_2-1β, H-2, H-3 H-4), CH_2–CH for ring B (H_2-7β, H-6), CH–CH-OH for ring C (H-9, H-11, OH-11), and CH–CH_2–CH_2 for ring D (H-17, H_2-16, H_2-15). A spin system was observed for the ring A′ in the sugar moiety, this formed by H_3-6′ (δ_H 1.29) through H-5′ (δ_H 3.91), H_2-4′ (δ_H 1.75, δ_H 2.07) to H-3′ (δ_H 3.86): CH_3-CH-CH_2-CH. Signal characteristics for a butyrolactone moiety [δ_H 4.80, 4.91 (*dd*, *J* = 18.2 Hz, H_2-21), δ_H 6.00 (*s*, H-22)], two methyl groups [δ_H 1.21 (H_3-18), δ_H 1.77 (H_3-19)], a methylendioxy group [δ_H 5.15, 5.23 (H_2-7′)] and an oxymethine [δ_H 4.75 (s, H-1′)] were observed in the ^1H NMR spectra. A ^1H NMR spectrum in D_2O confirmed the presence of four hydroxyl groups in the molecule [δ_H 2.68 (*br s*, OH-14), δ_H 3.67 (*d*, *J* = 2.54 Hz, OH-11), δ_H 3.70 (*d*, OH-4), δ_H 4.85 (*s*, OH-8)].

Connectivity of A, B, C, and D rings were assigned based on the interpretation of the HMBC spectrum. Long-range correlations from H_3-19 to C-1, C-5, C-9 and C-10 indicated the connectivity of A and B rings. The relationship between C and D rings was established by the observation of correlations from H_3-18 to C-17, C-12 and C-14 as well as those observed from H-17 to C-16, C-13 and C-20. Moreover, the α,β-unsaturated γ-lactone was deduced to be connected to C-17 by the observed correlation from H-17 to C-20, C-21 and C-22. The connectivity between C-1′ and C-3 through an oxygen bridge was confirmed by the observation of an HMBC correlation from H-1′ to C-3, indicating that the dioxane bridge consisted of [2-*O*-2′] and [3-*O*-1′]. These data indicated compound **1** is a glycosylated cardenolide linked to a 4′,6′-dideoxyhexosulose sugar moiety through a dioxane-type six-membered ring. The relative configuration of the aglycone was established on the basis of the coupling constants and confirmed by a ROESY experiment. The ROE correlations of H_3-18 to H-22, H_2-21 and H-11 indicated that the C-17 side chain and H-11 were all *β*-oriented, while the coupling constant of H-11 (*d*, *J* = 9.8 Hz) suggested that the adjacent H-9 occupied the α-oriented. The constant couplings $J_{3,4}$ (3.2 Hz) and $J_{6,7\alpha}$ (2.7 Hz) suggested *β*-oriented positions for the hydroxyl group at C-4 and the 5,6-epoxy group. NOE effect from H_3-19 to H-2 indicated a *cis*-fused A/B rings. The relative configuration of the sugar was determined by the observed ROE correlations of H-1′ with H-2, H-5′ and H-7′β, indicating a *β*-axial disposition of such protons and thus an α-equatorial orientation for H-3′ and H_3-6′, suggesting *cis*-fused 2′β,3′β-methylendioxy/A′ and A′/B′ rings. However, 2D NMR experiments could not provide sufficient information to determine the complete stereochemistry of compound **1**. Therefore, recrystallization of **1** from ethyl acetate afforded single crystals suitable for X-ray analysis (Fig. 2). X-ray diffraction led to an unambiguous determination of the absolute configuration of **1** as (*2R,3S,4S,5R,6R,8S,9R,10R,11S,13R,14R,17R,1′S,2′R,3′R,5′R*)-4,8,11,14-tetrahydroxy-5,6-epoxy-12-oxo-2-*O*,3-*O*-[(2′,3′-methylendioxy)-4′,6′-didesoxyhexosulose]-card-20(22)-enolide. This result has a special significance, as to date, there are only three reports of the absolute configuration by X-ray of cardenolide glycosides containing a single sugar in a dioxane attachment, those given for elaeodendroside A (Kupchan et al., 1977), affinoside B (Yamauchi et al., 1979), and humistratin (Nishio et al., 1982) isolated from *E. glaucum* (Celastraceae), *Anodendron affine* (Apocynaceae), and *Asclepias humistrata* (Asclepiadaceae), respectively, which were reported over three decades ago.

Compound **2** was obtained as a white amorphous solid, and its molecular formula was established as $C_{29}H_{36}O_{13}$ on the basis of its HRESIMS, corresponding to 15 units less than **1**. Comparison of their ^1H NMR and ^{13}C NMR spectra showed that compound **2** is closely related to **1**. The main differences in their RMN spectra were the absence of a methyl group (δ_H 1.29, *d*, *J* = 6.2 Hz; δ_C 20.8, q) and the replacement of a methine group (δ_H 3.91, *ddd*, *J* = 12.2,