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Erythrofordins D and E, two new cassaine-type diterpenes from *Erythrophleum suaveolens*

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Abstract

Two new cassaine-type diterpenoids, namely erythrofordins D (**1**) and E (**2**), sourced from a Cameroon collection of *Erythrophleum suaveolens* were isolated and assessed for anti-tumor

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Supplementary Material

Supplementary material that *In-vitro* and *in-vivo* data for the crude extract and pure compounds, as well as NMR spectra for compounds **1** and **2** are available free of charge via the Internet

activity. In the NCI-60 cancer cell assay, erythrofordins D (**1**) and E (**2**) were found to be cytotoxic in the low micro molar ranges with a mean GI₅₀ value of 2.45 and 0.71 μ M, mean TGI value of 9.77 and 2.29 μ M, and a mean LC₅₀ of 26.92 and 11.48 μ M for **1** and **2** respectively. Using the COMPARE algorithm, the new compounds were found to have similar NCI-60 response profiles to the known cardiac glycosides hyrcanoside and strophanthin. In addition, in an assay examining the viability and contractile function in human cardiomyocytes derived from induced pluripotent stem-cells, erythrofordins showed cardiotoxicity effects at concentrations as low as 0.03 μ g/mL.

Graphical Abstract

Keywords

Erythrofordin; Natural product; Cardiotoxicity; COMPARE; NCI-60

The National Cancer Institute's (NCI) Natural Products Repository is one of the world's largest, most diverse publicly available collections of plant, marine invertebrate and microbial biota used for natural product-based research. As part of a screening campaign aimed at the identification of anti-cancer active natural product leads, this collection has been progressively screened in the NCI's 60 human tumor cell anticancer drug screen (NCI-60), initially as a one-dose prescreen at a nominal concentration of 100 μ g/mL, with select cytotoxic extracts and fractions undergoing further five-dose and, in some cases, in-vivo evaluations.¹ The NCI's Natural Product Repository plant collection has been a rich and prolific source of biologically active and chemically diverse small molecules, recently exemplified by the englerins, guaiane sesquiterpenes isolated from *Phyllanthus engleri* exhibiting potent selectivity against renal cancer cell lines;² and the schweinfurthins, prenyl stilbenes isolated from the plants of the genus *Macaranga* displaying selectivity for the central nervous system and leukemia cell lines.^{3,4} In continuation of work focused on the

identification of anti-cancer natural products from plants, the organic solvent extract of the plant *Erythrophleum suaveolens* (Fabaceae), displayed potent NCI-60 cell, hollow fiber and xenograft activity (see Supporting Information (SI), Figure S1–4 and Table S1) and was selected for scale-up bioassay-guided natural product isolation work. Herein, we outline the isolation, structural elucidation and biological activity of two new cassainetype diterpenoids, namely erythrofordins D (**1**) and E (**2**), sourced from a Cameroon collection of *E. suaveolens*. The genus is a well-known source of cassane- and cassaine-type diterpenes with reported anti-tumour, anti-microbial, anti-protozoal, anti-viral, and anti-inflammatory activities.⁵

A portion of the crude organic solvent extract was pre-fractionated on a normal-phase diol solid phase extraction column, with the NCI-60 cell activity concentrated in the most polar methanol fraction. The ¹H NMR fingerprint of the active fraction suggested the presence of tannin-like compounds and was subsequently further purified on a polyamide gel. The tannin-free, NCI-60 active fraction was then subjected to preparative reversed-phase C₈ HPLC to yield compounds **1** and **2**.⁶

Erythrofordin D (**1**)⁷ was isolated as an optically active oil ($[\alpha]_D = -88$, c 0.05, MeOH) with the molecular formula C₂₁H₃₂O₄ established from HRESIMS and ¹³C NMR spectra, representing six indices of hydrogen deficiency. The ¹H NMR spectrum in CD₃OD showed twenty one resonances, comprised of one sp² hybridized methine, five sp³ hybridized methines, five sp³ hybridized diastereotopic methylenes, and five sp³ hybridized methyls. The ¹³C NMR spectrum showed twenty one resonances: one ketone (δ_C 212.4), one other carbonyl (δ_C 168.6), an up-field shifted quaternary carbon (δ_C 167.5), an olefinic methine resonance (δ_C 113.6), two sp³ hybridized quaternary carbons (δ_C 40.0 and 37.2), an oxymethine (δ_C 79.2), four other sp³ hybridized methines (δ_C 54.8, 53.0, 49.0, and 40.9), five methylenes (δ_C 39.7, 38.1, 28.2, 28.0, and 25.1), a methoxy carbon (δ_C 51.4), and four other methylenes (δ_C 28.2, 15.6, 15.5, and 13.7). Interpretation of the 2D NMR data established the presence of a basic cassaine skeleton, characteristic of the natural products previously reported from the genus *Erythrophleum*.⁵ Crucial ¹H – ¹³C HMBC correlations (depicted in SI Figure S23) from the methoxy singlet to C-16 (δ_C 168.6), as well as the olefinic resonance at C-15 (δ_H 5.70, br d, J = 1.6 Hz; δ_C 133.6) to the C-16 carbonyl resonance, the C-12 (δ_C 25.1) methylene, the C-13 (δ_C 167.5) quaternary carbon, and the C-14 (δ_C 40.9) methine on the diterpene core established an *exo*- α,β -unsaturated methyl ester moiety connected to C-13. HMBC correlations from H-5 (δ_H 1.31, dd, J = 13.9, 3.9 Hz; δ_C 53.0), H-7 (δ_{Ha} 2.34, m, δ_{Hb} 2.37, m; δ_C 53.0) and H-8 (δ_H 2.27, dd, J = 12.6, 3.7 Hz; δ_C 54.8) to the carbonyl at δ_C 212.4 positioned the ketone group at C-6. Finally, HMBC correlations from the diastereotopic methylenes at C-1 (δ_{Ha} 1.24, m, δ_{Hb} 1.86, dt, J = 13.2,

3.5 Hz; δ_C 38.1) and C-2 (δ_{Ha} 1.17, br dd, J = 13.0, 3.9 Hz, δ_{Hb} 1.65, m; δ_C 28.0), the geminal dimethyl resonances at C-4 (δ_H 0.83, s, δ_C 15.6; δ_H 0.95, s, δ_C 28.2) and the methine resonance at C-5 to an oxymethine resonance (δ_H 3.22, m, δ_C 79.2), positioned the hydroxy group on C-3 to complete the planar structure of compound **1**. The geometry of the C-13, C-15 double bond was assigned to be *E*, based on a ROESY observation between the olefinic H-15 methine and the H-14 resonance. The relative configuration of **1** was established with a $^1H - ^1H$ ROESY experiment (τ_{mix} = 400 ms). The 1H NMR spectrum in CD_3OD was well resolved and ROESY correlations from H-8 to H-14 and H-20 established these resonances to be on the common β -face of the molecule, while ROESY correlations from H-3 to H-5 and H-9, as well as H-5 to H-9 positioned these resonances on the α -face. The placement of the C-3 hydroxy group on the β -face is consistent with other structurally-related analogues, exemplified by erythrofordins A-C⁸ and methyl and ethyl 3 β -hydroxyerythrosumate.⁹ Based on the structural similarity to the erythrofordins A-C⁸ and T-V¹⁰ reported from *Erythrophleum fordii* the new natural product **1** was given the trivial name erythrofordin D.

Erythrofordin E (**2**)¹¹ was isolated as an optically active oil ($[\alpha]_D = -84$, c 0.05, MeOH) with the molecular formula $C_{21}H_{30}O_4$ established from HRESIMS and ^{13}C NMR spectra, representing one additional index of hydrogen deficiency compared to that of **1**. The 1H and ^{13}C NMR spectra of **2** were similar to that observed for compound **1**, with the major differences centered on the C-3 resonance. The C-3 oxymethine in **1** was replaced by an up-field shifted carbonyl resonance (δ_C 214.7) in **2**. The placement of the ketone group at C-3 was confirmed via crucial HMBC correlations from H-1 (δ_{Ha} 1.57, ddd, J = 14.5, 13.5, 4.8 Hz, δ_{Hb} 2.16, ddd, J = 13.5, 9.0, 3.1 Hz; δ_C 38.2), H-2 (δ_{Ha} 2.31, m, δ_{Hb} 2.82, ddd, J = 15.3, 15.4, 6.1 Hz; δ_C 35.3), H-5 (δ_H 1.78, m; δ_C 53.7), H-18 (δ_H 1.04, s; δ_C 25.3), and H-19 (δ_H 1.09, s; δ_C 22.2) to the C-3 resonance. The relative configuration of **2** was established with a combination of $^1H - ^1H$ ROESY and selective 1D ROESY experiments (τ_{mix} = 400 ms). The geometry of the C-13, C-15 double bond was assigned to be *E*, based on a ROESY observation between the olefinic H-15 methine and H-14 resonance. 2D ROESY correlations from H-8 to H-14 and H-20 established these three well resolved resonances to be on the common β -face of the molecule, while the resonances for H-5 and H-9 were found to be within 0.05 ppm of each other in a number of different NMR solvents and required selective 1D ROE experiments in order to resolve the overlap associated with a $^1H - ^1H$ 2D experiment (SI Figure S15). Through specific irradiations at H-20, enhancements in the resonances associated with H-1a, H-2a, H-7, H-8, H-10a, H-11a, and H-18 resonances were observed, therefore assigning all of the resonances on the β -face of the molecule. Specific irradiation at H-1b, as the only well resolved resonance situated at the α -face of the molecule, showed an enhancement of the H-5 and H-9 resonances, therefore securing the relative configuration of compound **2** to be (5*R**, 8*S**, 9*S**, 10*R**, 14*R**)-**2**. The new natural product **2** was given the trivial name erythrofordin E.

In the NCI-60 cell panel, erythrofordins D (**1**) and E (**2**) were found to be cytotoxic in the low micro molar ranges with a mean GI₅₀ value of 2.45 and 0.71 μM , mean TGI value of 9.77 and 2.29 μM , and a mean LC₅₀ of 26.92 and 11.48 μM for **1** and **2** respectively (full NCI-60 data in SI Figures S5–10). The NCI-60 dose-response profile can be examined using

the COMPARE pattern recognition algorithm in order to establish similarities between NCI-60 response fingerprints.¹² The analysis is based on the mean graph concept, where an arithmetic mean of the logarithm of either GI₅₀, TGI or LC₅₀ of all cell line responses is calculated and then subtracted from the corresponding log transformed values of each cell line. The mean graph represents the visual fingerprint of the response to a compound which can then be analysed by the COMPARE algorithm. In our analysis, a strong correlation between compounds **1** and **2** with that of the source crude extract (GI₅₀ COMPARE of 0.79 and 0.63 for **1** and **2** respectively) (Table 1, Figure 1), led to the determination that the isolated erythrofordins were responsible for the NCI-60 activity of the crude *E. suaveolens* organic solvent extract. Furthermore, in order to account for the in-vivo activity observed by the crude extract, a hollow fiber experiment was done with an erythrofordin-enriched fraction and confirmed that the chemotype was responsible for the in-vivo activity of the extract (SI Table S2).

In order to gain insight into the possible mode of action of the erythrofordins D (**1**) and E (**2**), we conducted a standard agent COMPARE of the NCI-60 data. In this analysis, an NCI-60 profile can be matched against a set of prototypical seed compounds with distinct NCI-60 fingerprints and known mechanisms of action.¹³ Examples of COMPARE-guided identification of the mechanism of action of natural products include halichondrin B, an inhibitor of microtubule polymerization,¹⁴ and the spongistatins, tubulin-interactive antimitotics,¹⁵ both of which had their mechanism of action studies aided by the similarities of NCI-60 dose responses to that of known compounds with defined mechanisms. In a standard agent COMPARE analysis, erythrofordins D and E showed a strong correlation to the NCI-60 profile of known cardiac glycosides, namely the cardenolides hyrcanoside (NSC256926) and β -k-strophanthin (NSC4320) (Table 1). Cassaine and related compounds are known to be non-steroidal inhibitors of Na⁺/K⁺ ATPase with an effect similar to that of cardiac glycosides.⁵ The structural features proposed to be responsible for the pharmacological effect of cassaine-type diterpenes are a lipophilic perhydrophenanthrene core, an α,β -unsaturated ester group, and a basic amino residue.¹⁶ Notably, erythrofordins D (**1**) and E (**2**) lack the basic residue, yet still show a strong NCI-60 COMPARE correlation to two cardiac glycosides, suggesting that the amino sidechain may not be essential for the cardiac glycoside-like cytotoxic effect observed in the NCI-60 cell panel.

Cardiac glycosides are currently used in the treatment of congestive cardiac failure and atrial fibrillation, the primary mechanism of action being sodium pump regulation through reduction of Na⁺/K⁺ ATPase activity in the cardiac myocyte membrane, and a subsequent rise in the intracellular calcium.¹⁷ However, the therapeutic index for cardiac glycosides such as digoxin in humans is narrow (recommended dose 0.8 – 2 ng/mL),¹⁸ and it has been shown that mice can tolerate up to 100 fold higher serum digoxin concentration levels when compared to humans,¹⁹ suggesting mice-based xenografts are not a suitable animal model for the evaluation of the anti-tumor potential of cardiac glycosides. To further examine the potential cardiac glycoside-like cytotoxic effects of the erythrofordins, a series of experiments on human, induced pluripotent stem cell derived cardiac myocytes were conducted. Electrophysiology, contractile function, Ca²⁺ cycling and viability in human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) were measured. In the

assay, the impedance of the adherent cells, the extracellular field potential and cytosolic Ca^{2+} concentration were measured respectively with a real-time cellular impedance analyser (xCELLigence® RTCA Cardio), a multi-electrode array (MED-64 MEA) system and a Nikon Eclipse Ti fluorescence microscope on cells loaded with Cal520@dye, giving information on the spontaneous contraction of the cardiomyocytes, intracellular Ca^{2+} handling as well as integrated ion channel activity at the membrane.²⁰ Previously, the natural product jaspamide (NSC 613009) was shown to be cardiotoxic at a range of concentrations from 30 nM to 30 μM , inducing a decrease in cardiomyocyte viability as well as inhibition of activity of a number of cardiac ion channels.²¹ In this work, the effects of the crude *E. suaveolens* organic extract and the erythrofordinenriched fraction were compared against the two cardiac glycosides hyrcanoside and strophanthin that were identified to have strong COMPARE correlation coefficients to erythrofordins D and E. An additional cardiac glycoside, neriifolin (NSC 123976) was used as an experimental control. At concentrations between 0.3 $\mu\text{g}/\text{ml}$ and 30 $\mu\text{g}/\text{ml}$, both the crude extract and the erythrofordin-enriched fraction caused a time- and dose-dependent loss in cell viability (SI Figure S24), a loss of synchronised beating at 3 and 72 hours post treatment (Figure 2), and a reduction in the inter-spike interval and field potential duration. Before the loss of the synchronised beating the crude extract and the erythrofordin-enriched fraction induced a time- and dose-dependent increase in the basal level calcium as well as the peak amplitude of calcium transits (SI Figure S25). Notably, the concentrations at which the cardiac toxicity was observed for the crude *E. suaveolens* organic extract and the erythrofordinenriched fraction were tenfold higher than the IC_{50} doses detected in the NCI-60 assay (0.02 and 0.07 $\mu\text{g}/\text{mL}$ respectively). While the activity of the crude extract and the erythrofordin-enriched fraction was consistent with that of the cardiac glycosides, the detailed mechanisms underlying cardiac myocyte toxicity for the erythrofordins remains unknown.

At present, there is a clear challenge in identifying a suitable animal model that mimics human susceptibility to allow rapid screening and an early prediction of therapeutic index which complicates anti-cancer clinical development of this cardiotoxic group of natural products. Since the erythrofordins showed significant similarities in their NCI-60 cell assay response to the cardiac glycosides, as well as cardiotoxicity in the human cardiac myocyte assay, further in-vivo work on this class of compounds would need to focus on elucidation of what may be a tight safety margin for the anti-tumor effects and exposures that may elicit life threatening arrhythmias in vivo. Linking the in vitro safety margins to true positives in an in vivo physiological setting will likely require additional nonclinical assessments and confirmatory strategies beyond initial screening. If clinical development of these agents is in fact warranted, clinical concentrations that correlate with a change in cardiac contraction should be defined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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6. A portion of the organic extract (1.0 g) was pre-fractionated on a flash diol column (8g) eluting sequentially with hexane (1.0 mg), hexane/ethyl acetate (5:1), (5.8 mg), hexane/ethyl acetate (1:1) (15.0 mg), hexane/ethyl acetate (3:7) (9.2 mg), ethyl acetate (5.0 mg), ethyl acetate/methanol (7:3) (145.4 mg), and methanol (596.6 mg). A portion of the ethyl acetate/methanol (7:3) (126.4 mg) and methanol (556.8 mg) fractions were further purified on a polyamide column, eluting with methanol to give 17.0 mg and 151.0 mg respectively of tannin-free mid polarity pre-fractionated material. A portion of the combined polyamide gel fractions (144 mg) were then subjected to repeated C₈ preparative HPLC eluting with a steep gradient from methanol/water (6:4) to methanol, to yield erythrofordin D (1) 1.8 mg (0.24% crude organic extract weight) and erythrofordin E (2) 1.5 mg (0.19% crude organic extract weight).
7. Erythrofordin D (1) clear oil; [α]_D = –88 (c 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 225 (4.06) nm; IR (film) 2926, 1704, 1647, 1193, 1036 cm^{–1}; ¹H NMR (500 MHz, CD₃OD) data: δ_{H} 5.70 (1H, br d, J = 1.6 Hz, H-15), 3.74 (1H, dt, J = 14.0, 3.3 Hz, H12A), 3.64 (3H, s, H-21), 3.22 (1H, m, H-3), 2.96 (1H, m, H-14), 2.37 (2H, m, H-7), 2.27 (1H, dd, J = 12.6, 3.7, H-8), 2.04 (1H, ddd, J = 14.0, 5.0, 1.4, H-12B), 1.93 (1H, m, H-11A), 1.86 (1H, dt, J = 13.2, 3.5, H-1A), 1.69 (1H, m, H-11B), 1.67 (1H, m, H-9), 1.65 (1H, m, H-2A), 1.31 (1H, dd, J = 13.9, 3.9, H-5), 1.24 (1H, m, H1B), 1.17 (1H, br dd, J = 13.0, 3.9, H-2B), 1.04 (3H, d, J = 6.9, H17), 1.00 (3H, br s, H-21), 0.95 (3H, s, H-18), 0.83 (3H, s, H19); ¹³C NMR (125 MHz, CD₃OD) data: δ_{C} 212.4 (C-6), 168.8 (C-16), 167.5 (C-13), 113.6 (C-15), 79.2 (C-3), 54.8 (C-8), 53.0 (C-5), 51.4 (C-21), 49.0 (C-9), 40.9 (C-14), 40.0 (C-4), 39.7 (C-7), 38.1 (C-1), 37.2 (C-10), 28.2 (C-11, C-18), 28.0 (C-2), 25.1 (C-12), 15.6 (C-19), 15.5 (C-17), 13.7 (C-20); HRESIMS m/z [M+H]⁺ 349.23746 (calcd for C₂₁H₃₃O₄⁺, 349.23734).
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11. Erythrofordin E (2) clear oil; $[\alpha]_D = -84$ (c 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 222 (4.01) nm; IR (film) 1703, 1647, 1260, 1195 cm^{-1} ; ^1H NMR (500 MHz, CD_3OD) data: δ_{H} 5.72 (1H, br d, H-15), 3.77 (1H, br dt, $J = 14.4, 3.3$ Hz, H-12A), 3.65 (3H, s, H21), 2.99 (1H, m, H14), 2.82 (1H, ddd, $J = 15.2, 14.5, 6.1$ Hz, H2A), 2.56 (1H, dt, $J = 15.3, 0.7$ Hz, H-7A), 2.38 (1H, br dd, $J = 12.6, 3.5$ Hz, H-8), 2.31 (1H, m, H2B), 2.28 (1H, m, H-7B), 2.16 (1H, ddd, $J = 13.5, 9.0, 3.1$ Hz, H-1A), 2.06 (1H, ddd, $J = 14.4, 13.6, 5.3$ Hz, H-12B), 1.96 (1H, m, H-11A), 1.78 (1H, m, H-5), 1.81 (1H, m, H-9), 1.57 (1H, ddd, $J = 14.5, 13.5, 4.8$ Hz, H-1B), 1.26 (1H, m, H-11B), 1.23 (3H, s, H-20), 1.09 (3H, s, H-18), 1.05 (3H, d, $J = 6.9$ Hz, H-17), 1.04 (3H, s, H-19); ^{13}C NMR (125 MHz, CD_3OD) data: δ_{C} 214.7 (C-3), 211.2 (C-6), 168.8 (C-16), 167.2 (C-13), 133.8 (C-15), 54.7 (C-8), 53.7 (C-5), 51.4 (C-21), 48.5 (C-4), 48.0 (C-9), 40.9 (C-14), 40.2 (C-7), 38.2 (C-1), 37.3 (C-10), 35.3 (C-2), 28.5 (C-11), 25.3 (C-19), 25.1 (C-12), 22.2 (C18), 15.4 (C-17), 13.1 (C-20); HRESIMS m/z $[\text{M}+\text{H}]^+$ 347.22138 (calcd for $\text{C}_{21}\text{H}_{31}\text{O}_4^+$, 347.22169).
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Figure 1.

Mean bar graph of the NCI-60 cell GI_{50} data for erythrofordins D (**1**), E (**2**), and the *E. suaveolens* crude organic extract.

Figure 2. Beat rate and amplitude analysis for DMSO vehicle, β -kstrophanthin (NSC 4320), hyrcanoside (NSC 256926), neriifolin (NSC 123976), the crude *E. suaveolens* organic extract, and the erythrofordin-enriched fraction.

Table 1.

Pairwise COMPARE analysis¹² based on the GI₅₀ NCI-60 cell data for erythrofordins D (**1**) and E (**2**); the *E. suaveolens* crude organic extract; and the cardiac glycosides hyrcanoside and strophanthin. All samples show strong, positive correlations (significant at $p < 0.05$, $n = 47$).

	1	Crude extract	NSC256926	NSC4320
2	0.76	0.63	0.66	0.71
1		0.79	0.70	0.82
Crude extract			0.80	0.87