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Cytotoxic and apoptosis-inducing activities against human lung cancer cell lines of cassaine diterpenoids from the bark of *Erythrophleum fordii*

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ABSTRACT

A phytochemical investigation into the bark of *Erythrophleum fordii* yielded four new compounds, two new cassaine diterpenoids (erythrofordin T and U, **1** and **2**) and two new cassaine diterpenoid amines (erythroformine A and B, **6** and **7**), as well as nine known compounds. We report for the first time the isolation of erythrofordin V (**3**) from a natural source and that of the remaining eight known diterpenoids (**4**–**5**, **8**–**13**) from *E. fordii*. All structures were elucidated using spectroscopic analysis. Cytotoxic activity of the isolated compounds (**1**–**13**) was examined *in vitro* against three non-small cell lung cancer cell lines (A549, NCI-H1975, and NCI-H1229) using the MTT assay. Cassaine diterpene amines (**6**–**10**, **12**, **13**) exhibited potent cytotoxic activity against all three cell lines with IC_{50} values between 0.4 μM and 5.9 μM. Erythroformine B (**7**) significantly induced apoptosis in all three cancer cells in a concentration-dependent manner.

Lung cancer, the leading cause of cancer-related deaths, is one of the most common malignant and damaging human tumors.[1] Although treatments have advanced over the past 20 years, the response rate and prognosis of lung cancer patients remain poor.[2] Lung cancer is classified into two major groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC is a very aggressive neuroendocrine lung carcinoma. NSCLC constitutes approximately 80–85% of lung malignancies, and the 5-year survival of this highly aggressive disease is only 15%. It consists of several subtypes, predominantly adenocarcinoma, squamous-cell carcinoma, and large-cell carcinoma, which are all treated in the same manner.[3,4] The current chemotherapy protocols for NSCLC are known to induce chemoresistance and result in a range of adverse effects.[5] Furthermore, only some tumor histotypes exhibit optimal results and many show a low or very low response to treatment. The search for new anticancer compounds that are more effective against unresponsive tumors, have fewer adverse effects, and act through new mechanisms for the treatment of NSCLC is therefore a clear necessity. Naturally-derived compounds, which are an abundant source of antitumor candidates, are especially relevant to this matter.[6,7] Apoptosis is a universal genetic program of cell death in higher eukaryotes, and the identification of compounds that activate and promote apoptosis is an attractive strategy for the discovery and development of potential anticancer agents.[8]

The genus *Erythrophleum* belongs to the tribe Dimorphandreae, subfamily Caesalpinioideae, and the family Leguminosae.[9] The genus consists of approximately 15 species, which are found in Africa, Asia, and Australia. Trees of this genus have long been known to contain alkaloids in their barks, which exhibit a digitalis-like action on the heart. The alkaloid content in the bark is generally 0.2–1%.[10–12] *Erythrophleum fordii* Oliver (Leguminosae) is a species with both medicinal and poisonous properties and is widely distributed throughout China, Vietnam, and Taiwan. It is used as a Chinese traditional medicine with predominant actions of "invigoration and promoting blood circulation".[13,14] Previous studies have shown that *E. fordii* contains alkaloids (cassaine

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diterpenoid amines and amides), triterpenoids, steroids, and miscellaneous structures, which exhibit various biological effects such as cytotoxic, antioxidant, anti-inflammatory, and anti-angiogenic activity.[11,15,16] A wide range of pharmacological functions has been attributed to cassaine diterpenes, including antitumor, anti-inflammatory, antiviral, antimicrobial, and antitrypanosomal activity, and they have been a popular topic of study in medicinal chemistry for many years owing to their excellent cardiotonic and antimalarial properties.[17] The main constituent of *E. fordii*, cassaine diterpenoid alkaloids, also exhibited potential cytotoxic activities against human cancer cell lines[11,18,19]; therefore, we continued to study the bark of *E. fordii* in anticipation of the discovery of new antitumor compounds. In this paper, we report the isolation and structure elucidation of compounds from this species, including four new cassaine diterpenoids and nine known compounds, and their cytotoxic and apoptosis-inducing effects on three NSCLC cells (A549, H1975, and H1229). To the best of our knowledge, this study is the first to report the isolation of compound **3** from a natural source and the remaining eight known diterpenoids (**4**–**5**, **8**–**13**) from *E. fordii*.

The methanolic extract of *E. fordii* bark was partitioned sequentially in *n*-hexane, CH_2Cl_2, and water. Repeated column chromatography (silica gel, RP-18, sephadex LH-20) of the *n*-hexane and CH_2Cl_2-soluble fractions resulted in the isolation of thirteen compounds (**1**–**13**) (Fig. 1).[20,21]

Erythrofordin T (**1**) was obtained as a colorless oil ($[\alpha]_D^{25}$ −18.7 (*c* 0.11, $CHCl_3$). The molecular formula of **1** was determined as $C_{27}H_{38}O_8$ from the molecular ion peak at *m*/*z* 490.2567 $[M]^+$ (calcd for $C_{27}H_{38}O_8$: 490.2566) in the HR-EI-MS spectra. The UV spectrum exhibited an absorption maximum at 238 nm. The IR spectrum of **1** showed absorption bands for hydroxyl (3412 cm^{-1}) and carbonyl (1712, 1647 cm^{-1}) functional groups.[21] The ^1H NMR spectrum of **1** revealed three methyl groups at δ 1.48 (3H, s, H-18), 1.09 (3H, d, *J* = 7.0 Hz, H-17), and 1.08 (3H, s, H-20), two methoxyl groups at δ 3.73 (3H, s, H-22) and 3.66 (3H, s, H-21), and one exocyclic vinyl proton at δ 5.70 (1H, d, *J* = 1.0 Hz, H-15). The ^{13}C NMR spectrum displayed resonances for three carbonyl signals at δ 209.5 (C-7), 174.3 (C-19), and 167.3 (C-16), and a pair of olefinic carbons at δ 164.5 (C-13) and 113.5 (C-15) (Table 1). All of the above NMR data were diagnostic of a cassaine diterpenoid skeleton with a β-carbomethoxy group at C-4, which was supported by analysis of the HMQC and HMBC spectroscopic data.[16,22] The HMBC correlations from H_3-22 (δ 3.73)/H-3 (δ 4.60)/H-5 (δ 1.49) to C-19 (δ 174.3) confirmed that the carbomethoxy group was attached at C-4.[16] The presence of a 6α-hydroxyl-7-keto group in **1** was substantiated by the correlation of the oxymethine proton signal at δ 4.37 (1H, dd, *J* = 12.5, 1.0 Hz, H-6) with the H-5 signal at δ 1.49 (1 H, d, *J* = 12.5 Hz) in the COSY spectrum, and the HMBC correlations between H-6 (δ 4.37) and C-5 (δ 58.0)/C-7 (δ 209.5)/C-10 (δ 37.6).[23] Furthermore, the proton signals at δ 6.82 (1H, dq, *J* = 7.0, 1.5 Hz, H-3′), 1.78 (3H, d, *J* = 7.0 Hz, H-4′), and 1.80 (3H, s, H-5′), together with the carbon signals at δ 167.7 (C-1′), 128.8 (C-2′), 137.9 (C-3′), 14.6 (C-4′), and 12.1 (C-5′) were characteristic of a tigloyloxy moiety ($C_5H_7O_2$).[24] The ^1H and ^{13}C NMR spectra of **1** (Table 1) closely resembled those of 6α-hydroxy-cassamic acid, methyl ester, except for the appearance of a tigloyloxy moiety.[23] The distinct HMBC cross-peak correlations from the remaining oxymethine proton at δ 4.60 (1H, dd, *J* = 12.5, 4.5 Hz, H-3) to C-19 (δ 174.3)/C-18 (δ 26.8)/C-1′ (δ 167.7) (Fig. 2) indicated that the tigloyloxy group was attached at C-3.

The α configuration of the C-6 hydroxyl group was further substantiated by NOE interaction between H-6β (δ 4.37) and H_3-20 (δ 1.08) (Fig. 2).[11] A large coupling constant (d, *J* = 12.5 Hz) indicated a diaxial configuration between H-5 and H-6; consequently, the proton H-5 was in the α-orientation. The α-orientation of H_3-18, H-9, and H-3 was confirmed by the NOE correlations between H-5 (δ 1.49) and H_3-18 (δ 1.48), H-5 (δ 1.49) and H-9 (δ 1.68), and H-3 (δ 4.60) and H-18 (δ 1.48) (Fig. 2). Additionally, NOE correlations were found between H_3-20 (δ 1.08) and H-8 (δ 2.36), and H-8 (δ 2.36) and H-14 (δ 2.98), which indicated that these groups were located on the β-face.[16] Finally, the olefinic proton at δ 5.70 (H-15) showed NOE cross-peaks to the methines resonating at δ 2.98 (H-14), which indicated the *E* geometry of the double bond at C-13/C-15. Based on the above analyses, the structure of **1** was elucidated as 3β-tigloyloxy-6α-hydroxy-cassamic acid, methyl ester, which was a new compound, and named as erythrofordin T.

Erythrofordin U (**2**) was obtained as a colorless oil. The HR-EI-MS of **2** exhibited molecular ion peaks at *m*/*z* 392.2199 $[M]^+$ (calcd

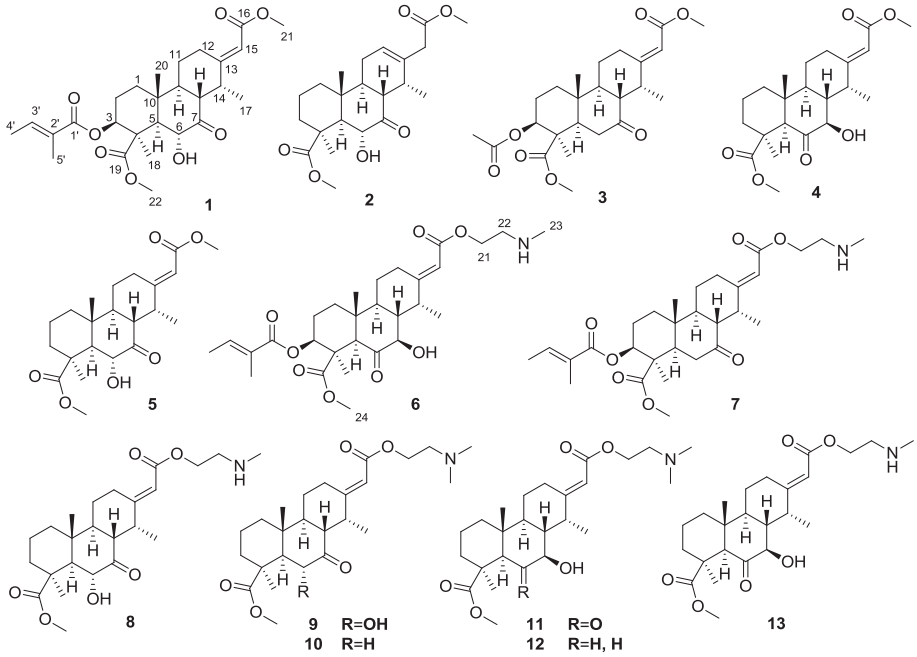

Fig. 1. Chemical structures of compounds of **1**–**13**.