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Chemical profile and defensive function of the latex of *Euphorbia peplus*

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ABSTRACT

Plant latex is an endogenous fluid secreted from highly specialized laticifer cells and has been suggested to act as a plant defense system. The chemical profile of the latex of *Euphorbia peplus* was investigated. A total of 13 terpenoids including two previously unknown diterpenoids, (2*S**,3*S**,4*R**,5*R**,6*R**,8*R**,11*R**,13*S**,14*S**,15*R**, 16*R**)-5,8,15-triacetoxy-3-benzoyloxy-11,16-dihydroxy-9-oxopepluane and (2*R**,3*R**, 4*S**,5*R**,7*S**,8*S**,9*S**,13*S**,14*S**,15*R**)-2,5,8,9,14-pentaacetoxy-3-benzoyloxy-15-hydroxy-7-isobutyroyloxyjatropha-6(17),11*E*-diene), ten known diterpenoids, and a known acyclic triterpene alcohol peplusol, were identified, using HPLC and UPLC-MS/MS analyses and through comparison with the authentic compounds isolated from the whole plant. The diterpenoids exhibited significant antifeedant activity against a generalist plant-feeding insect, the cotton bollworm (*Helicoverpa armigera*), with EC_{50} values ranging from 0.36 to 4.60 μg/cm^2. In particular, (2*R**,3*R**,4*S**,5*R**,7*S**,8*S**,9*S**,13*S**,14*S**,15*R**)-2,5,9,14-tetraacetoxy-3-benzoyloxy-8,15-dihydroxy-7-isobutyroyloxyjatropha-6(17),11*E*-diene and (2*R**,3*R**, 4*S**,5*R**,7*S**,8*S**,9*S**,13*S**,14*S**,15*R**)-2,5,14-triacetoxy-3-benzoyloxy-8,15-dihydroxy-7-isobutyroyloxy-9-nicotinoyloxyjatropha-6(17),11*E*-diene had EC_{50} values of 0.36 and 0.43 μg/cm^2, respectively, which were approximately 7-fold more potent than commercial neem oil (EC_{50} = 2.62 μg/cm^2). In addition, the major peplusol showed obvious antifungal activity against three strains of agricultural phytopathogenic fungi, *Rhizoctonia solani*, *Colletotrichum litchi* and *Fusarium oxysporum* f. sp. *niveum*. The results indicated that terpenoids in the latex of *E. peplus* are rich and highly diversified, and might function as constitutive defense metabolites against insect herbivores and pathogens for the plant.

1. Introduction

Plant latex, a milk-like endogenous fluid, is stored in laticifer cells and secreted immediately after plant tissues being damaged (Agrawal and Konno, 2009; Konno, 2011). It has been found that over 20000 species from about 40 families of angiosperm plants could exude latex (Konno, 2011). The biological roles of latex were once thought as water reserve for plant, or the excretion of plant's waste metabolites (Pintus et al., 2010). Currently, increasing evidence indicated that latex provided defensive roles for plants against insect herbivores and pathogens (Agrawal and Konno, 2009; Farrell et al., 1991; Hunter, 1994; Konno, 2011). Plant latex was secreted from the damaged tissue rapidly, and can glue the mouthparts or mire the whole body of herbivorous due to the sticky trait of latex (Gu et al., 2014; Zalucki and Malcolm, 1999). Moreover, plant latex contains a great variety of proteins and specialized products including alkaloids, terpenoids, cardenolides, and many other components, most of which showed obvious toxicity against insects and pathogens (Agrawal and Konno, 2009; Hua et al., 2015;

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Huber et al., 2015; Konno, 2011; Konno et al., 2006). For example, sugar-mimic alkaloids in the latex of mulberry leaves were the potent inhibitors of sugar-metabolizing enzymes and toxic to caterpillars (Konno et al., 2006). Uscharin in the latex of *Calotropis procera* acted as defensive substance due to its molluscicidal activity against land snail *Thepa pisana* (Hussein et al., 1994). The major components in the latex of *Lactuca* species were guaianolide sesquiterpene lactones, among which lettucenin A served as a phytoalexin because of its potent antifungal activity against *Cladosporium herbarum* (Agrawal and Konno, 2009; Hussein et al., 1994; Sessa et al., 2000). Therefore, the lactic specialized metabolites in plants and their diversified defensive functions have long been intriguing research topics.

Euphorbia is the largest genus in the family Euphorbiaceae, comprising more than 2000 species (Shi et al., 2008). It is well known that most representatives contain toxic and highly skin-irritant latex (Falsone et al., 1993). *Euphorbia peplus* Linn. is a small annual weed with milky latex, which is originally native to Europe and North Africa and today occurs worldwide due to its rapid invasion (Jakupovic et al., 1998). Previous investigations have mostly focused on the specialized metabolites of the whole plant, and plenty of bioactive diterpenoids with diverse structures, including jatrophane, ingenane, and tetracyclic diterpenoids, have been isolated and identified (Hohmann et al., 1999a, 1999b; Jakupovic et al., 1998; Song et al., 2010). The latex of *E. peplus* has been reported to contain nonpolar triterpenoids, including lanosterol, cycloartenol, 24-methylenecycloartanol, and an acyclic triterpene alcohol (peplusol), together with a known diterpenoid 20-deoxyingenol 3-angelate (Giner et al., 2000). However, more detailed chemical profile of the latex of *E. peplus* has not been reported. In our preliminary experiment, we found that the latex of *E. peplus* displayed significant antifeedant effect against a generalist plant-feeding insect, the cotton bollworm (*Helicoverpa armigera*), but the responsible defensive compounds in the latex still remain unknown. Therefore, a detailed investigation on the chemical composition of the latex and the defense function of those metabolites were carried out. HPLC and UPLC-MS/MS analyses indicated 12 major specialized metabolites in the latex of *E. peplus*, which were carefully traced and isolated from the methanolic extract of the whole fresh plant, and were identified as twelve diterpenoids (**1**–**12**) including two previously undescribed compounds (**1** and **10**) through comprehensive spectroscopic analyses. Meanwhile, a major known acyclic triterpene alcohol (**13**) that was previously reported in the latex (Giner et al., 2000) was also isolated in this study. Verification of the existence of compounds **1**–**13** in the latex was achieved. The antifeedant activity against a generalist insect (*H. armigera*) and antifungal effect against phytopathogens of these latex metabolites were also reported in this paper.

2. Results and discussion

To determine the defensive function of the latex of *E. peplus*, the antifeedant activity of freshly collected latex against a generalist plant-feeding insect, the cotton bollworm (*H. armigera*), was tested. The larvae fed almost solely upon the control leaf discs, but never touched the latex treated leaf discs, which indicated that the latex was strongly antifeedant against the insect (Fig. 1).

To characterize the major active lactic metabolites, latex collected from the stem of *E. peplus* was suspended with methanol to remove the macromolecular substances such as proteins and polysaccharides. After centrifugation, the supernatant was directly analyzed by reversed-phase HPLC recorded at 238 nm. Twelve major peaks (1–12) were detected in the chromatogram (Fig. 2A). Because the amount of latex extract was insufficient for direct isolation and identification of these compounds, methanolic extract of the fresh whole plant of *E. peplus* was used to trace and isolate them. Consequently, thirteen target compounds were successfully isolated and identified.

Compound **1** was obtained as a white solid. Its molecular formula was determined to be $C_{33}H_{42}O_{11}$ on the basis of the molecular ion peak at *m/z* 614.2687 (M^+, $C_{33}H_{42}O_{11}$; calcd. 614.2727). Its IR absorption bands at 3441, 1739, 1638, 1604, and 1452 cm^{-1} indicated the presence of hydroxyl, carbonyl and benzoyl groups. The ^1H NMR spectrum of **1** (Table 1) displayed a typical benzoyl group [δ_H 8.13 (2H, d, J = 7.4 Hz), 7.62 (t, J = 7.4 Hz), and 7.49 (2H, t, J = 7.4 Hz)], three acetyl methyls [δ_H 2.13, 1.96, and 1.89, each 3H], three tertiary methyls [δ_H 1.33, 1.15, and 0.67, each 3H], and one secondary methyl [δ_H 1.06 (3H, d, J = 7.3 Hz)]. In addition, six proton resonances were also exhibited in the middle-field region between δ_H 6.03 and 3.67, corresponding to either oxy-methines or hydroxyl groups. The ^{13}C NMR and DEPT spectra (Table 1) exhibited 33 carbon resonances, including three acetoxy carbonyl groups (δ_C 171.5, 170.7, and 170.5) and one benzyloxy carbonyl group (δ_C 166.8). The 20 skeletal carbons included four methyls, four methylenes, six methines, and six quartenary carbons, which constituted a characteristic pepluane diterpenoid according to the reported diterpenoids from this plant (Jakupovic et al., 1998; Hohmann et al., 1999a). The ^1H and ^{13}C NMR spectra of **1** resembled those of (2*S**,3*S**,4*R**,5*R**,6*R**,8*R**,9*R**,l1*R**,13*S**,14*S**,15*R**,16*R**)-5,8,9,15-tetraacetoxy-3-benzoyloxy-11,16-dihydroxypepluane (**2**), a known pepluane diterpenoid also isolated in this study. The only difference between them was that an oxygenated methine (C-9) and an acetoxy group in **2** were replaced by a keto group (δ_C 206.7) in **1**. In the HMBC spectrum of **1** (Fig. 5), the simultaneous long-range ^1H-^{13}C correlations from H-13 (δ_H 4.93), H_2-10 (δ_H 2.75 and 2.31) and H-7β (δ_H 2.46) to the keto carbon at δ_C 206.7, indicated that the keto group was assignable to C-9. The ROESY spectrum of **1** (Supplementary data, Table S2) indicated that the relative configurations of the stereogenic centers in **1** were same as those in **2**. Consequently, compound **1** was determined as (2*S**,3*S**,4*R**,5*R**,6*R**,8*R**,l1*R**,13*S**,14*S**,15*R**, 16*R**)-5,8,15-triacetoxy-3-benzoyloxy-11,16-dihydroxy-9-oxopepluane (Fig. 4).

Compound **10** was isolated as a white solid and had a molecular formula of $C_{41}H_{54}O_{15}$, as determined from its ^{13}C NMR spectroscopic data and molecular ion at *m/z* 786.3467 (M^+, calcd. 786.3463). The ^1H and ^{13}C NMR spectra (Table 1) of **10** revealed the presence of seven ester residues attributable to a benzoate [δ_H 8.16 (2H, d, J = 7.4 Hz), 7.65 (t, J = 7.4 Hz), and 7.54 (2H, t, J = 7.4 Hz); δ_C 165.5, 134.0, 131.2, 130.5, and 129.4], an isobutyrate [δ_H 2.62 (m), 1.24 (d, J = 7.1 Hz), and 1.22 (d, J = 7.1 Hz); δ_C 175.6, 34.5, 19.6, and 18.8], and five acetates [δ_H 2.10, 2.09, 2.07, 2.03, and 1.94, each 3H of singlet; δ_C 170.9, 170.4, 170.2, 170.1, 169.6, 22.2, 21.1, 20.8, 20.7, and 20.5]. Furthermore, analysis of the ^1H NMR spectrum of **10** displayed four olefinic signals [δ_H 5.91 (d, J = 16.1 Hz), 5.72 (dd, J = 16.1 and 9.4 Hz), 4.92 (s), and 4.51 (s)], which were assigned to a *trans*-disubstituted double bond and a vicinal-disubstituted double bond, and were in agreement with the carbon resonances in the ^{13}C NMR spectrum [δ_C 146.2 (C-6), 134.7 (C-11), 132.2 (C-12), and 110.0 (C-17)]. A broad singlet at δ_H 3.66 with no HSQC correlation to any carbon should be from a hydroxyl group. In addition, one secondary and three tertiary methyls, one methylene, six acyloxymethines, two methines, two oxygenated quaternary carbons, and a quaternary carbon explained the remaining atoms. Taking into account previous observations, it can be deduced that this compound is a hydroxylated macrocylic jatrophane diterpenoid (Jakupovic et al., 1998). The NMR data of **10** closely resemble those of (2*R**,3*R**,4*S**,5*R**,7*S**,8*S**,9*S*,l3*S**,14*S**,15*R**)-2,5,9,14-tetraacetoxy-3-benzoyloxy-8,15-dihydroxy-7-isobutyroyloxyjatropha-6(17),11*E*-diene (**6**), a jatrophane diterpenoid also isolated in this study. The

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