In Vitro Anti-Cancer Alkaloid and Flavonoid Extracted from the *Erythrina variegata* (Leguminoseae) Plant

Tati Herlina¹*, Unang Supratman¹, Anas Subarnas², Supriyatna Sutardjo², Suseno Amien³, Hideo Hayashi⁴

¹Department of Chemistry, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Jatinangor, Indonesia
²Faculty of Pharmacy, Padjadjaran University, Jatinangor, Indonesia
³Faculty of Agriculture, Padjadjaran University, Jatinangor 45363, Serang, Indonesia
⁴Laboratory of Natural Products Chemistry, Division of Applied Biological Chemistry, Graduate School of Agriculture and Life Science, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan

Abstract

*Erythrina* plants, locally known as “dadap ayam”, are higher plant species and have been used as a folk medicine for treatment of cancer. To prove the effectiveness of the leaves and stem bark of *E. variegata* as an anti-cancer agent, the assay in this research was focused on in vitro test towards breast cancer cell T47D. In the course of our continuing search for novel anti-cancer agents from *Erythrina* plants, the methanol extract of the leaves and stem bark of *E. variegata* showed significant anti-cancer activity against breast cancer cell T47D in vitro using the Sulphorhoda mine B (SRB) assay. By using the anti-cancer activity to follow the separations, the methanol extract was separated by combination of column chromatography. The chemical structure of an anti-cancer compound was determined on the basis of spectroscopic evidence and comparison with the previously reported and identified as an erythrina alkaloid (1) and isoflavonoid (2). Compounds (1-2) showed anti-cancer activity against breast cancer cell T47D used with IC₅₀ of 1.0 and 3.3 µg/mL, respectively. This result strongly suggested that *E. variegata* is a promising source for anti-cancer agents.

Keywords: Anti-cancer, *Erythrina variegata*, Leguminoseae

INTRODUCTION

Breast cancer is the most commonly diagnosed invasive non-skin malignancy and second leading cause (after lung cancer) of cancer related deaths in the women. Both epidemiological and laboratory studies show that environmental and behavioral factors are more important than genetic factors in determining overall cancer frequency among populations. Currently, systemic cytotoxic chemotherapy approaches, controlling and treating breast cancer, are being used; which are not only less effective but are also non-selective and highly toxic to normal tissues (Tyagi *et al*., 2004). Cisplatin is an anticanine drug that has enjoyed remarkable success against testicular tumor, but dose limiting side-effects have limited its application against a broader range of cancer. An approach that is gaining attention in recent years is combination chemotherapy, where non-toxic or less toxic phytochemicals are being combined with chemotherapy agents to enhance the efficacy together with a reduced toxicity to normal tissues (Tyagi *et al*., 2002).

The search for anti-cancer medicinal plant depends on the accurate and specific ethno-botanical and ethno-pharmacological information obtained from the reference document. Recently, attention was focused on medicinal plants to provide new anti-cancer agents. *E. variegata* (Leguminoseae) is a famous medicinal plant widely distributed in tropical and subtropical region of the world. This plant is locally known as “dadap ayam” in Indonesia and the leaves of *E. variegata* are used as an anti-cancer agent (Heyne, 1987).

Previous studies have shown that the leaves of *E. variegata* contains alkaloids and flavonoids, which are unknown to display interesting biological activities (Chawla *et al*., 1987; Tanaka *et al*., 1988; 2000; 2002; 2003; Herlina *et al*., 2008).

*Corresponding author e-mail: tata_04her@yahoo.com*
Pharmacological report that steroid derivatives from the stem bark and the leaves of *E. variegata* showed an anti-cancer against on breast cancer cell T47D in vitro (Herlina, 2009). As part of our continuing search for novel anti-cancer compound from Indonesia *Erythrina* plants, we report here with the isolation, structure elucidation and anti-cancer activity.

**MATERIALS AND METHODS**

**General Experimental Procedure**

Melting points were uncorrected. The IR spectra were recorded with a Perkin-Elmer 1760 X FT-IR spectrophotometer, and the UV spectra were recorded with a Hitachi model U-3210. Mass spectra were recorded with JEOL JMS-DX300 instrument. The 1H- and 13C-NMR spectra were obtained with JEOL JNM GX 270 and JNM A-500 spectrometer. Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. Column chromatography was carried out using Merck Kieselgel 60 (70-200 mesh), and thin layer chromatography (TLC) analysis was performed on precoated Si Gel plates (Merck Kieselgel GF254, 0.25 mm 20 x 20 cm).

**Plant material**

Samples of the stem bark and the leaves of *E. variegata* was collected on June 2008, in Bandung District, West Java, Indonesia. The plant was identified by a staff at the Laboratory of Plant Taxonomy, Department of Biology, Bandung Institute of Technology, Bandung, Indonesia, and a voucher specimen has been deposited at the herbarium.

**Anti-cancer Assay**

National Cancer Institute developed an in *vitro* anticancer-drug method with the SRB (Sulforhodamine B) assay. This method measures the cellular protein content of adherent and suspension cultures in 96-well microtiter plates. Cultures fixed with trichloro acetic acid (TCA) are stained with 0.4% SRB dissolved in 1% acetic acid. Unbounded dye is removed by washing with 1% acetic acid, and protein-bound dye is extracted with 10 mM buffered tris base [tris (hydroxymethyl) amino methane] for the determination of optical density (515 nm) with a 96-well microtiter plate reader. Cisplatin functioned well as positive controls (Skehan *et al*., 1990).

**Extraction and isolation**

The dried leaves (2 kg) of *E. variegata* were soaked in MeOH. Evaporation of the MeOH gave an aqueous concentrate, which was extracted with CH3Cl2. The resulting CH3Cl2 extract was partitioned between n-hexane and MeOH containing 10% water, and then the lower layer was concentrated and extracted with EtOAc to afford residue (36.31 g). The methanol layer was partitioned between n-butanol-water (3:1). The n-butanol layer was subsequently dried over anhydrous sodium sulfate, filtered, evaporated to dryness, and assayed for anti-malarial activity. The n-butanol fraction (4 g) was chromatographed on Kieselgel 60 (70-230 mesh) by eluting with chloroform-ethyl acetate in an increasing ratio (1:1-1:5) to yield a 3 fractions (BA, BB, and BC). The BC fraction (1.5 g) was eluted with chloroform and 5% acetic acid were further flash-chromatographed on Kieselgel 60 to yield an isolate 1 (53 mg).

The dried stem bark (2.2 kg) of *E. variegata* was extracted by maceration technique three times with methanol. The crude methanolic extract was then assayed for antimalarial activity via the LDH method. Evaporation of the methanol extract gave concentrated aqueous extract, which was extracted with dichloromethane. The resulting dichloromethane extract was partitioned between n-hexane and methanol containing 10% water, and then lower layer was concentrated and extracted with ethyl acetate. The ethyl acetate layer was subsequently dried over anhydrous sodium sulfate, filtered, evaporated to dryness, and assayed for anti-malarial activity. The ethyl acetate fraction (15.8 g) was chromatographed on Kieselgel 60 (70-230 mesh) by eluting with n-hexane and an increasing ratio of ethyl acetate, and by ethyl acetate and an increasing ratio of methanol to afford the 20% methanol eluate (1.2 g). The fraction (683 mg) eluted with 10% and 20% methanol were further flash-chromatographed on Kieselgel 60 with 5% methanol in chloroform to yield a crude active compound (191 mg), which were crystallized from methanol to yield active compounds (21.3 mg) and 2 (25.5 mg).

**RESULTS AND DISCUSSION**

The methanolic extract of dried of the leaves and stem bark of *E. variegata* exhibited an anti-cancer activity against breast cancer T47D cell in *vitro* using the SRB method. The active methanol extract was partitioned between n-hexane, ethyl acetate, and n-butanol to afford an
active ethyl acetate and n-butanol. By using the SRB method to follow the separations, the ethyl acetate and n-butanol fraction were separated by combination of column chromatography on Kieselgel 60 to afford a two active isolates (1 and 2).

The isolate 1 was obtained as a yellow pale needleless crystal, m p 72-74 °C; UV (CHCl₃) λmax (ε) 288 (4500), 350 (50) nm; IR (KBr) νmax 3433, 2931, 1654, 1593, and 779 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 7.47 (1H, s, H-17), 7.26 (1H, s, H-14), 6.04 (1H, br d, J = 4.4, H-1), 4.40 (1H, t, J = 5.1, H-2), 4.32 (1H, m, H-8a), 3.98 (3H, s, OCH₃), 3.95 (3H, s, OCH₃), 3.42 (1H, m, H-9a), 3.35 (1H, td, J = 10.0, 6.3; H-8b), 3.16 (3H, s, OCH₃), 2.53 (2H, m, H-7), 2.15 (1H, dd, J = 12.1, 4.4eq), 2.08 (1H, dd, J = 11.7, 4.8; H-4ax), 2.08 (1H, dd, J = 11.7, 4.8; H-4ax); ¹³C NMR (CDCl₃, 100 MHz) δ 180.3 (C-11), 159.1 (C-10), 149.3 (C-16), 141.6 (C-6), 139.1 (C-13), 125.5 (C-1), 124.3 (C-12), 110.3 (C-17), 107.7 (C-14), 81.5 (C-3), 72.6 (C-2), 64.7 (C-5), 57.2 (OCH₃), 56.5 (OCH₃), 56.3 (OCH₃), 46.9 (C-4), 46.1 (C-8), 31.3 (C-7); EIMS m/z 359.

The isolate 2 was obtained as colourless needles crystal, mp 150-152°C; UV (CHCl₃) λmax (ε) 286 (16,000), 222 (80) nm; IR (KBr) νmax 3375, 2922, 1620, and 758 cm⁻¹; ¹H NMR (CDCl₃, 500 MHz) [δH 3.80 (3H, s), [δH 1.47 (6H, s), [δH 3.90 (1H, H-6eq)]; δH 4.20 (1H, H-6ax); δH 5.23 (1H, H-11a); δH 3.30 (2H, H-1’); δH 3.20 (2H, H-1’); [δH 5.31 (1H, H-2’); δH 5.27 (1H, H-2’)]. ¹³C NMR (CDCl₃, 125 MHz) δc 56.10, (δc 28), [δc 154 (C-4a) and 158.6 (C-1)]; δc 69.7, δc 69.7, δc 84.4, δc 29.4, δc 22.6, δc 121.9, δc 122.1. The HMBC and ¹H-¹H COSY spectra of isolate 2 are illustrated in Fig.1.

Anti-cancer activity of extract, fraction, and isolates

The percentage inhibition of methanol extract, ethyl acetate, n-butanol fraction and, isolates (1 and 2) against breast cancer cell T47D can be described to be in the following that isolate 1 (IC₅₀ 1.0 µg/mL) higher than isolate 2, n-butan fraction (IC₅₀ 10.5 µg/mL), ethyl acetate fraction (IC₅₀ 22.9 µg/mL), and methanol extract (IC₅₀ 43.7 µg/mL) (Table I).

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC₅₀ (µg/mL)</th>
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<tbody>
<tr>
<td>Methanol extract of the leaves</td>
<td>43.7</td>
</tr>
<tr>
<td>Methanol extract of the stem bark</td>
<td>40.5</td>
</tr>
<tr>
<td>Ethyl acetate fraction of the stem bark</td>
<td>22.9</td>
</tr>
<tr>
<td>n- butanol fraction of the leaves</td>
<td>10.5</td>
</tr>
<tr>
<td>erystagallin A (1)</td>
<td>3.3</td>
</tr>
<tr>
<td>10,11-dioxyoerthyratidine (2)</td>
<td>1.0</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Figure 1. The HMBC and ¹H-¹H COSY spectra of isolates (1 and 2)
The isolate 1 was obtained as a yellow pale needleless crystal, mp 72-74°C. The molecule formula was established to be C19H21NO5 by 1H- and 13C-NMR spectral data, thus requiring ten degrees of unsaturation. Its UV spectrum of isolate showed aryl and carbonyl absorption at 288 and 350 nm, respectively. The IR spectrum of compound isolate displayed some characteristic absorption for an aromatic ring, hydroxyl and carbonyl group. The 1H-NMR and 13C-NMR spectra of isolate showed signals assignable to a 1,2,4,5-tetrasubstituted benzene ring [δH 7.02 (1H, s) and 7.47 (1H, s)] and [δC 107.8; 110.4; 124.3; 139.2; 149.3; and 153.1] and two carbonyl groups [δC 160.0 and 180.4 ppm], indicating isolate to be a tetracyclic structure. Three methoxyls were also observed in the 1H-NMR and 13C-NMR spectra [δH 3.16 (3H, s); 3.95 (3H, s) and 3.98 (3H, s)] and [δC 57.2; 56.3 and 56.5]. To determine the connectivity of the partial structure, 1H-1H COSY, HMBC, and NOESY experiment for isolate was carried out, and the results are shown in Fig. 1. The NOESY spectra showed signals assignable to α-configurated equatorial of H-1, H-2, H-3, H-4, H-7, and H-8, while of 2-OH, 3-OCH3, H-4, H-7, H-8, H-14, 15-OCH3, 16-OCH3, and H-17 are β-configurated axial. The based on the spectral spectroscopic evidence, comparison with the previously reported and biogenetic point of view, the genus *Erythrina* seems to lack biogenetic ability to produce alkaloids (Chawla et al., 1987; Supratman et al., 2002) identified as 10,11-dioxyerythratidine.

The isolate 2, obtained as colourless needles crystal, mp 150-152°C, was shown to have a molecular formula of C26H39O5 based on 1H-and 13C-NMR (CDCl3, 500 and 125 MHz) spectral data with twelve double bond equivalents. The UV absorption maxima of 2 in MeOH at 286 nm (ε 16.000) and 222 nm (ε 80), suggested the presence of a flavonoid skeleton. IR absorption bands due to a hydroxyl and an aromatic ring were observed at 3375, 2922, 1620, and 758 cm⁻1, respectively. The 1H-NMR and 13C-NMR in combination with DEPT spectra of 2 showed signals assignable to a methoxyl group [δH 3.80 (3H, s); δC 56.1], a hydroxyl group [δH 1.47 (6H, s); δC 28], two oxy carbons [δC 154 (C-4a) and 158.6 (C-1)], three protons [δH 3.90 (1H, H-6eq)]; δC 69.7, δH 4.20 (1H, H-6ax); δC 69.7, and δH 5.23 (1H, H-11a); δC 84.4], and four protons [δH 3.30 (2H, H-1’); δC 29.4, δH 3.20 (2H, H-1’); δC 22.6, δH 5.31 (1H, H-2’); δC 121.9, δH 5.27 (1H, H-2’); δC 122.1], respectively, indicating that 2 to be pterocarpan derivative having two isoprenyl groups. The HMBC spectra of 2 showed correlations proton H-4 (δH 6.38) with C-2 (δC 121.6), between proton H-1’ (δH 29.4) with C-2 (δC 121.6) and C-3 (δC 155.7), between proton H-6ax (δH 3.90) with C-4a (δC 154.2) and C-11a (δC 158.6), and proton H-8 (δC 6.49) with C-6b (δC 120.6), respectively, indicating the position of hydroxyl groups at C-3 and C-6a. The correlation proton H-8 (δH 6.49) with C-9 (δC 159.9), indicating that methoxyl group at position C-9. Furthermore the correlation between proton H-1’ (δH 3.30) with C-1 (δC 132.2) and C-2 (δC 121.6), and H-1’ (δH 3.20) with C-10 (δC 113.7), indicating the position of isoprenyl groups at C-2 and C-10. The HMBC and 1H-1H COSY spectra of isolate 2 are illustrated in Fig.1. These observations together with a detailed comparison of there spectral data with those previously reported led us to identify the isolate 2 as erystagallin A (Tanaka et al., 1988).

The potency of methanol extract, ethyl acetate fraction, and 10,11-dioxyerythratidine against breast cancer T47D cell can be described to be in the following order; 10,11-dioxyerythratidine > erystagallin A > n-butanol fraction > ethyl acetate fraction > methanol extract, indicated that 10,11-dioxyerythratidine and erystagallin A to be potential as an anti-cancer agents.

**CONCLUSION**

The 10,11-dioxyerythratidine (1) and the crystagallin A (2) had been isolated from the leaves and stem bark of *E. variegata*. Our results strongly suggested that both compounds (1-2) are promising as anti-cancer agents.

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