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

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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 agent 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 Sulphorhodamine 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 compounds were 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 IC_{50} of 1.0 and 3.3 µg/mL, respectively. This results strongly suggested that *E. variegata* is promising sources 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 anticancer 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* (Leguminosae) 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).

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Pharmacological report that steroid derivates 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 ^{1}H- and ^{13}C-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 GF_{254}, 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 CH_2Cl_2. The resulting CH_2Cl_2 extract was partitioned between *n*-hexane and MeOH containing 10% water, and then the lower layer was concentrated and extracted with EtOAc to efford 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 needless crystal, m p 72-74 ^{0}C; UV ($CHCl_3$) λ_{max} (ε) 288 (4,500), 350 (50) nm; IR (KBr) ν_{max} 3433, 2931, 1654, 1593, and 779 cm^{-1}; ^{1}H NMR ($CDCl_3$, 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, OCH_3-16), 3.95 (3H, s, OCH_3-15), 3.42 (1H, td, J = 10,0; 6,3; H-8b), 3.35 (1H, td, J = 12.1; 4,8; H-3), 3.16 (3H, s, OCH_3-3), 2.53 (2H, m, H-7), 2.15 (1H, dd, J = 12,1, H-4eq), 2.08 (1H, dd, J = 11,7; 4,8; H-4ax); ^{13}C NMR ($CDCl_3$, 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 (OCH_3-3), 56.5 (OCH_3-16), 56.3 (OCH_3-15), 46.9 (C-4), 46.1 (C-8), 31.3 (C-7); EIMS m/z 359.

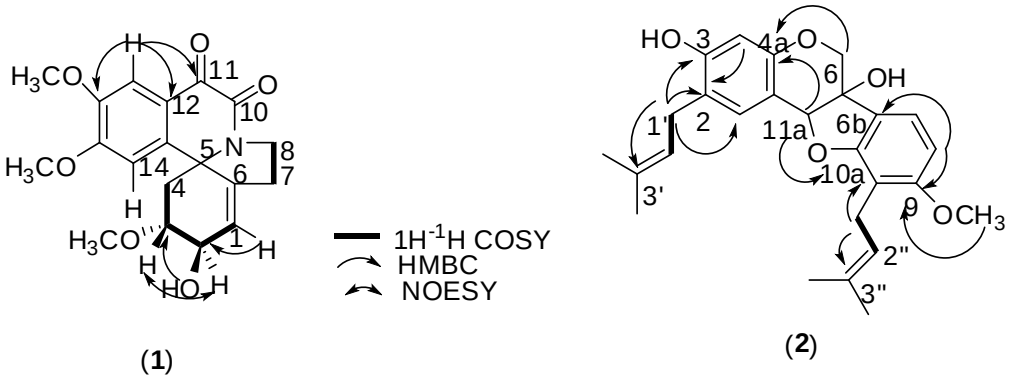
The isolate **2** was obtained as colourless needles crystal, mp 150-152^{o}C; UV ($CHCl_3$) λ_{max} (ε) 286 (16.000), 222 (80) nm; IR (KBr) ν_{max} 3375, 2922, 1620, and 758 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) [δ_H 3.80 (3H, s)]; [δ_H 1.47 (6H, s)], [δ_H 3.90 (1H, H-6eq)]; δ_H 4.20 (1H, H-6ax); δ_H 5,23 (1H, H-11a); [δ_H 3.30 (2H, H-1'); δ_H 3.20 (2H, H-1")]; [δ_H 5.31 (1H, H-2')]; [δ_H 5.27 (1H, H-2")]. ^{13}C NMR ($CDCl_3$, 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 ^{1}H-^{1}H 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** (IC_{50} 1.0 µg/mL) higher than isolate **2**, *n*- butanol fraction (IC_{50} 10.5 µg/mL), ethyl acetate fraction (IC_{50} 22.9 µg/mL), and methanol extract (IC_{50} 43.7µg/mL) (Table I).

Tabel I. IC_{50} values of methanol extract, ethyl acetate fraction, *n*-butanol fraction, and isolates obtained against breast cancer cell T47D

Sample	IC_{50} (µg/ml)
Methanol extract of the leaves	43.7
Methanol extract of the stem bark	40.5
Ethyl acetate fraction of the stem bark	22.9
n- butanol fraction of the leaves	10.5
erystagallin A (**1**)	3.3
10,11-dioxoerythratidine (**2**)	1.0
Cisplatin	3.2



(**1**) (**2**)

Figure 1. The HMBC and ^{1}H-^{1}H COSY spectra of isolates (1 and 2)

The isolate **1** was obtained as a yellow pale needless crystal, m p 72-74 ^{0}C. The molecule formula was established to be $C_{19}H_{21}NO_6$ by ^{1}H- and ^{13}C-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 ^{1}H-NMR and ^{13}C-NMR spectra of isolate showed signals assignable to a 1,2,4,5-tetrasubtituted benzene ring [δ_H 7.02 (1H,*s*) and 7.47 (1H,*s*)] and [δ_C 107.8; 110. 4; 124. 3; 139. 2; 149.3; and 153.1] and two carbonyl groups [δ_C 160.0 and 180.4] ppm, indicating isolate to be a tetracyclyc structure. Three methoxyls were also observed in the ^{1}H-NMR and ^{13}C-NMR spectra [δ_H 3.16 (3H, *s*); 3.95 (3H, *s*) and 3.98 (3H, *s*)] and [δ_C 57.2; 56.3 and 56.5]. To determine the connectivity of the partial structure, ^{1}H-^{1}H 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-OCH_3, H-4, H-7, H-8, H-14, 15- OCH_3, 16- OCH_3, 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-dioxoerythratidine.

The isolate **2,** obtained as colourless needles crystal, mp 150-152^{o}C, was shown to have a molecular formula of $C_{26}H_{30}O_5$ based on ^{1}H-and ^{13}C-NMR ($CDCl_3$, 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 ^{1}H-NMR and ^{13}C-NMR in combination with DEPT spectra of **2** showed signals assignable to a methoxyl group [δ_H 3.80 (3H, s); δc 56.1], a hydoxyl group [δ_H 1.47 (6H, s); δc 28], two oxycarbons [δc 154 (C-4a) and 158.6 (C-1)], three protons [δ_H 3.90 (1H, H-6eq)]; δc 69.7, δ_H 4.20 (1H, H-6ax); δc 69.7, and δ_H 5,23 (1H, H-11a); δc 84.4], and four protons [δ_H 3.30 (2H, H-1'); δc 29.4, δ_H 3.20 (2H, H-1"); δc 22.6, δ_H 5.31 (1H, H-2'); δc 121.9, δ_H 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 (δ_H 6.38) with C-2 (δ_C 121.6), between proton H-1' (δ_H 29.4) with C-2 (δ_C 121.6) and C-3 (δ_C 155.7), between proton H-6ax (δ_H 3.90) with C-4a (δ_C 154.2) and C-11a (δ_C 158.6), and proton H-8 (δ_H 6.49) with C-6b (δ_C 120.6), respectively, indicating the position of hydroxyl groups at C-3 and C-6a. The correlation proton H-8 (δ_H 6.49) with C-9 (δ_C 159.9), indicating that methoxyl group at position C-9. Furthermore the correlation between proton H-1' (δ_H 3.30) with C-1 (δ_C 132.2) and C-2 (δ_C 121.6), and H-1" (δ_H 3.20) with C-10 (δ_C 113.7), indicating the position of isoprenyl groups at C-2 and C-10. The HMBC and ^{1}H-^{1}H 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-dioxoerythratidine against breast cancer T47D cell can be described to be in the following order; 10,11-dioxoerythratidine > erystagallin A > *n*-butanol fraction > ethyl acetate fraction > methanol extract, indicated that 10,11-dioxoerythratidine and erystagallin A to be potential as an anti-cancer agents.

CONCLUSION

The 10,11-dioxoerythratidine (**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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REFERENCES

Chawla, A.S., Sood, A., Kumar, M. and Jackson, A.H., 1987, Alkaloidal constituents of *Erythrina variegata*, *Journal of Planta Medica,* **16**, 526-528.

Herlina, T., Nasrudin, Supratman, U., Subarnas, A., Sutardjo, S. and Hayashi, H., 2008, An isoflavonoid, warangalone from the stem bark of dadap ayam (*Erythrina variegata)*, *Journal of Basic Sciences,* **9**(1), 45-47.

Herlina, T., 2009, Anticancer compounds from Dadap Ayam (*Erythrina variegata*), *Indonesian Journal of Cancer,* **3**(4), 151-154.

Heyne, K., 1987, The Useful Indonesian Plants, Research and development agency, Ministry of forestry, Jakarta, Indonesia, pp. 1029-1031.

Skehan, P.R., Storeng, D., Scudiero, A., McMahon, D., Vistica, J.T., Warren, H., Boskesch, S., Kenney, and Boyd, M.R., 1990, *Journal Natural Product.,* **82**, 13.

Supratman, U., Fujita, T. and Hayashi. H., 2000, Paralytic alkaloid from *Erythrina subumbrans* (Leguminosae), *Applied Biological Science,* **6**, 7-16.

Tanaka, H., Toshihiro, T. and Etoh, H., 1998, Two Pterocarpans from *Erythrina orientalis*, *Phytochemistry*, **47**, 475-477.

Tanaka, H., Etoh, H., Shimizu, H., Makita, T. and Tateishi, Y., 2000, Two new isoflavonoids from *Erythrina variegate*, *Planta Medica.,* **66**, 578-579.

Tanaka, H., Hirata, M., Etoh, H., Watanabe, N., Shimizu, H., Ahmad, M., Terada, Y. and Fukai, T., 2002, Two diphenylpropan-1,2-diol syringates from the roots of *Erythrina variegate, J. Nat. Prod*., **65**, 1933-1935.

Tanaka, H., Hirata, M., Etoh, H., Shimizu, H., Sako, M., Murata, J., Murata, H., Darnaedi, D. and Fukai, T., 2003, Eryvarins F and G, two 3-phenoxychromones from the roots of *Erythrina variegate*, *Phytochemistry*, **62**, 1243-1246.

Tyagi, A.K., Singh, R.P., Agarwal, C., Chan, D.C.F. and Agarwal, K., 2002, Silibinin strongly synergizes human prostate carcinoma DU145 cells to Dox-induced growth inhibition, G2-M arrest and apoptosis, *Clinical of Cancer Research,* **8**, 3512-3519.

Tyagi, A.K., Agarwal, C., Chan, D.C.F. and Agarwal, K., 2004, Synergistic anti-cancer effect of silibinin with conventional cytotoxic agents doxorubicin, cisplatin and carboplatin against human breast carcinoma MCF-7 and MDA-MB468 cells, *Oncology Reports,* **11**, 493-499.