Antidermatophyte and antimelanogenesis compound from Eleutherine americana grown in Indonesia

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NOTE

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Abstract An active compound from the bulb of *Eleutherine americana* L. Merr. (Iridaceae) collected from East Kalimantan, Indonesia, was tested for its antidermatophyte and antimelanogenesis activity. Antifungal assay-directed fractionation of the *n*-hexane-soluble fraction of the methanolic extract of the bulb of *E. americana* led to the isolation of **1** as an active compound. The compound was identified as the naphthoquinone eleutherin by EI-MS and ^{1}H-, ^{13}C-, and two-dimensional NMR analyses. Antidermatophyte assay of **1** at concentrations of 10, 20, 40, 60, and 80 μg/disk and myconazole, a commercial antidermatophyte, at 10 μg/disk displayed 7, 8, 13, 16, 17, and 14 mm of inhibition zone against *Trichophyton mentagrophytes*, respectively. In a melanin formation inhibition assay, compound **1** displayed potent antimelanogenesis activity at 5 ppm with low toxicity compared with arbutin, a commercial skin-whitening agent. The results showed the high potential of **1**, an active compound from *E. americana*, to be applied as an antidermatophyte and antimelanogenesis agent.

Keywords Antidermatophyte · Antimelanogenesis · Bawang tiwai · *Eleutherine americana*

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Introduction

Bawang tiwai (*Eleutherine americana* L. Merr.) is a small plant that belongs to the Iridaceae, a botanical family that comprises 90 genera and about 1200 species [1]. The bulbs are used as diuretic, purgative, and antiemetic drugs [2]. Infusions of the bulbs are used as an astringent, to remedy intestinal infection, dysentery, liver and genital diseases, while the leaves are used as an antipyretic and antiemetic [3]. The bulb of this plant has long been used as a folk remedy for the treatment of cardiac diseases, especially coronary disorder [4]. Some quinones and carboxylic acid-type compounds with anticancer activity have been isolated from this plant [5, 6]. However, to the best of our knowledge, *E. americana* has never been thoroughly investigated in antidermatophyte assays. As a continuation of our research into antifungal active compounds, here we report the isolation and identification of a naphthoquinone compound from the bulbs of *E. americana*. The compound exhibits antifungal activity, especially against *Trichophyton mentagrophytes*. We also conducted an antimelanogenesis activity assay against B16 melanoma cells and the MTT assay to assess the potential of compound **1** as a skin treatment agent.

Materials and methods

General

The silica gel used for column chromatography was Wakogel C-200, 75–150 μm (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Thin-layer chromatography (TLC) aluminium sheets (silica gel 60 F_{254}, 20 × 20 cm) were obtained from Merck (Darmstadt, Germany). All other materials or solvents were of the highest purity or high-performance liquid chromatography (HPLC) grade. Melting point was measured on a Shimadzu DTA-60/DSC-60. The $[\alpha]_D$ value was measures on a JASCO DIP-370. Mass spectra were recorded on a Shimadzu GC–MS QP 5050A (Shimadzu Corp.) at an electron energy of 70 eV (direct inlet); ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a JEOL JNM-400 (JEOL Ltd., Tokyo, Japan).

Plant material

Bulbs of *E. americana* were collected in Samarinda (East Kalimantan, Indonesia, S0°30′–E117°09′) in May 2007. The plant was botanically identified Mr. Syafrizal (Department of Biology, Faculty of Mathematic and Natural Sciences, Mulawarman University, Indonesia). A voucher specimen is deposited in the Laboratory of Wood Chemistry, Faculty of Forestry, Mulawarman University, Indonesia, under the accession code KK-0705-BT103.

Extraction and isolation

E. americana dried powder bulbs (3 kg) from East Kalimantan, Indonesia, were extracted with MeOH (25 l) at room temperature for 6 days to give a methanolic extract (271 g). Part of the methanolic extract (133 g) was suspended in water and extracted successively with *n*-hexane, diethyl ether, and ethyl acetate to yield an *n*-hexane-soluble portion (33 g), diethyl ether-soluble portion (50 g), ethyl acetate-soluble portion (4 g), and a water-soluble portion (43 g). Each portion was subjected to agar diffusion antifungal assay against *Tricophyton mentagrophytes*. The *n*-hexane-soluble and diethyl ether-soluble portions displayed the strongest antidermatophyte activity in the bioassays, relative to control. Silica gel column chromatography of the *n*-hexane-soluble portion (D, 5 g, 15-mm inhibition zone) by using gradient elution of *n*-hexane–EtOAc (20:1–1:1) gave eight fractions (D1–D8). An active fraction D5 (450 mg, 13-mm inhibition zone) was subjected to column chromatography over silica gel by using gradient elution of C_6H_6–Me_2CO (50:1–1:1) to give more six fractions (D51–D56). Further silica gel column chromatography of an active fraction D53 (126 mg, 16-mm inhibition zone) by using gradient elution of C_6H_6–Me_2CO (50:1–1:1) guided by the antidermatophyte assay resulted in isolation of D535 (98 mg, 19-mm inhibition zone) as an active fraction. Purification of fraction D535 led to the isolation of compound **1** (60 mg, Fig. 1) upon recrystallization from ethanol.

Eleutherin (1)

Pale yellow needles, mp 175–177°C, $[\alpha]_D^{25}$ +348° ($CHCl_3$), positive in the color reaction with 2,4-dinitrophenylhydrazine, deep blue fluorescence under ultraviolet illumination at 365 nm. ^{1}H-NMR and ^{13}C-NMR (in DMSO-d_6) spectra were coincident with published data [7, 8]. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 1.22 (3H, d, J = 6.1 Hz), 1.38 (3H, d, J = 6.8 Hz), 2.10 (1H, dd, J = 9.9, 18.8 Hz), 2.41 (1H, dd, J = 3.4, 21.9 Hz), 3.89 (1H, s, OMe), 3.95 (1H, m), 4.81 (1H, q, J = 6.8 Hz), 7.53 (1H, d, J = 8.5 Hz), 7.60 (1H, d, J = 7.5 Hz), 7.78 (1H, t, J = 8.0 Hz). ^{13}C-NMR (400 MHz, DMSO-d_6) δ: 66.29 (C-1), 61.94 (C-3), 29.08 (C-4), 183.65 (C-5), 118.22 (C-6), 135.13 (C-7), 118.85 (C-8), 159.26 (C-9), 181.67 (C-10), 146.91 (C-11), 138.86 (C-12), 133.39 (C-13), 118.90 (C-14), 56.29 (OCH_3), 21.26 (CH_3), 19.38 (CH_3).

Fungal inoculum preparation

T. mentagrophytes, one of the most important dermatophytes in tropical climates, was used as a test fungus in agar diffusion antifungal assays. Stock inoculum suspension of the fungus was prepared from 7- to 15-day-old cultures grown on potato dextrose agar (PDA) at 28°C. Mature colonies were covered with approximately 10 ml of sterile saline (0.85%) by scraping the surface with the tip of a Pasteur pipette and then adjusted to 10^6 CFU/ml.

Agar diffusion assay

Ten-milliliter aliquots of sterile molten PDA were transferred to Petri dishes and allowed to solidify. The PDA plates were inoculated with 10 μl of fungal spore suspension spread uniformly on the surface of the plates. Sterile 7-mm-i.d. paper disks (Whatman no. 2, Japan) containing 100 μg/disk of methanolic extracts and the fractions or 10, 20, 40, 60, and 80 μg/disk of **1** or myconazole, a

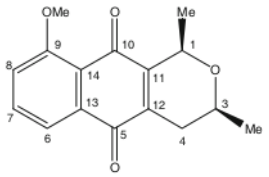

Fig. 1 Structure of compound **1** (eleutherin)

commercial antidermatophyte at 10 µg/disk, or 50 µl of extract were put on the surface of each inoculated plate. The plates were incubated in the dark at 25°C for 72 h. Zones of inhibition around the disks were measured in millimeters.

Cell culture

A mouse melanoma cell line, B16, was obtained from RIKEN Cell Bank. The cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.09 mg/ml theophylline. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Inhibition of melanin biosynthesis in cultured B16 melanoma cells

This assay was determined as described previously [9]. Briefly, confluent cultures of B16 melanoma cells were rinsed in phosphate-buffered saline (PBS) and removed from the plastic by using 0.25% trypsin/EDTA. The cells were placed in two plates of 24-well plastic culture plates (one plate for determining melanin and the other for cell viability) at a density of 1×10^5 cells/well and incubated for 24 h in media prior to being treated with the samples. After 24 h, the media were replaced with 998 µl of fresh media and 2 µl of DMSO was added with or without (control) the test sample at various concentrations ($n = 3$) and arbutin was used as a positive control. The cells were incubated for an additional 48 h, and then the medium was replaced with fresh medium containing each sample. After 24 h, the remaining adherent cells were assayed.

Determination of melanin content in B16 melanoma cells

The melanin content of the cells after the treatment was determined as follows. After removing the medium and washing the cells with PBS, the cell pellet was dissolved in 1.0 ml of 1 N NaOH. The crude cell extracts were assayed by using a microplate reader (Bio-Tek, USA) at 405 nm to determine the melanin content. The results from the cells treated with the test samples were analyzed as a percentage of the results from the control culture.

Cell viability

Cell viability was determined by using the microculture tetrazolium technique (MTT). The MTT assay provides a quantitative measure of the number of viable cells by determining the amount of formazan crystals produced by metabolic activity in treated versus control cells. Culture was initiated in 24-well plates at 1×10^5 cells/well. After incubation, 50 µl of MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide in PBS (5 mg/ml)] was added to each well. The plates were incubated in a humidified atmosphere of 5% of CO_2 at 37°C for 4 h. After the medium was removed, 1.0 ml isopropyl alcohol (containing 0.04 N HCl) was added into the plate, and the absorbance was measured at 570 nm relative to 630 nm.

Results and discussion

Dried bulb powder of *E. americana* was extracted with methanol and the extract obtained was subjected to solvent–solvent fractionation to give respective soluble fractions. An antidermatophyte assay against *T. mentagrophytes* confirmed that *n*-hexane- and diethyl ether-soluble portions exhibited the most activity, as evidenced by a 15- and 13-mm inhibition zone, respectively (Fig. 2). Bioassay-guided fractionation of the *n*-hexane-soluble portion of the methanolic extract of the bulb of *E. americana* resulted in isolation of compound **1** (Fig. 1) as an antidermatophyte compound. The compound was identified by spectroscopy and comparison with literature data. The MS, ^{1}H-NMR, and ^{13}C-NMR spectra of **1** were coincident data reported for eleutherin.

Antifungal activity of compound **1** against a dermatophyte, *T. mentagrophytes*, was evaluated in an agar diffusion assay. The results showed that compound **1** at the concentration of 10, 20, 40, 60, and 80 µg/disk and myconazole, a commercial antidermatophyte, at 10 µg/disk displayed 7, 8, 13, 16, 17 mm and 14 mm of inhibition zone against *T. mentagrophytes*, respectively (Fig. 3). The results also showed that at 10 µg/disk, compound **1** possessed lower antidermatophyte activity than miconazole; however, compound **1** has the advantage that it may cause less side effects. Based on the results, compound **1** seems to exert a concentration-dependent activity. Dermatophytes have the capacity to invade keratinized tissues (skin, hair, and nails), producing dermatophytosis [10]. Dermatophytoses rank among the

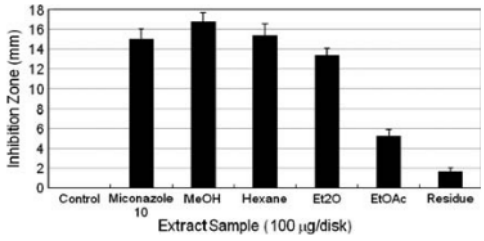

Fig. 2 Antidermatophyte activity of the methanolic extract and the soluble fractions from *E. americana* against *T. mentagrophytes* (all values are the mean of triplicates ± standard deviation (SD))

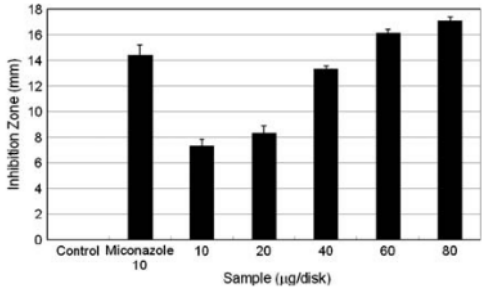

Fig. 3 Antidermatophyte activity of **1** against *T. mentagrophytes* (all values are the mean of triplicates ± SD)

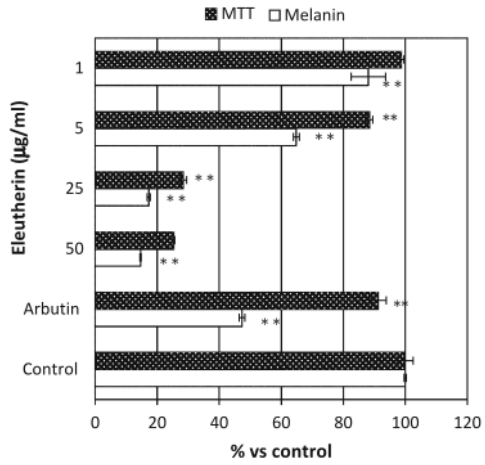

Fig. 4 Antimelanogenesis activity of **1** (arbutin concentration was 100 μg/ml). *Each column* represents mean ± SD of three independent test (Dunnett's test). **Significant difference from control group ($P < 0.01$)

most common and widespread infectious diseases worldwide, and *Trichophyton rubrum* and *Trichophyton mentagrophytes*, which cause infections of skin and nails, are two of the most frequently isolated dermatophytes [11]. Effective dermatophytosis treatment by application of topical antifungal preparations is a challenge. This has led to an intensive search for substances that are more effective, cheaper, have a wide-ranging antidermatophyte spectrum, are easier to use, and cause less side effects. Compound **1** (eleutherin) is a naphthopyran having a 1,4-naphthoquinone moiety, and several naphthopyran compounds were reported to occur as natural phytochemicals [12, 13]. Eleutherin also exhibits antitumor activity via a mechanism involving the inhibition of topoisomerase II [14].

In order to evaluate the potential of compound **1** to act as a skin treatment agent, an antimelanogenesis assay of the compound was conducted. Compound **1** concentration dependently inhibited melanin formation in B16 melanoma cells (Fig. 4); at 50 and 25 μg/ml, compound **1** inhibited 87% of melanin production with cytotoxicity. At 5 μg/ml, compound **1** inhibited 35% of melanin formation with less cytotoxicity. At 100 μg/ml, the positive control, arbutin, inhibited 53% of melanin formation with less cytotoxicity. At 1 μg/ml, compound **1** did not affect melanin inhibition. To the best of our knowledge, such antidermatophyte and antimelanogenesis activities of compound **1** have not been reported. In conclusion, our findings indicate that compound **1** might be a useful ingredient for skin treatment (both antifungal and as a skin-whitening agent) if its safety can be confirmed.

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