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Nitric oxide inhibitory principles from *Derris trifoliata* stems

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Abstract

Nine rotenoids were isolated from the hexane and dichloromethane extracts of *Derris trifoliata* stems and were tested for nitric oxide (NO) inhibitory activity using RAW264.7 cells. The result indicated that 12a-hydroxyrotenone (**7**) possessed very potent NO inhibitory activity with an IC_{50} value of 0.002 μM, followed by **1** (deguelin, $IC_{50} = 0.008$ μM), **9** (12a-hydroxyelliptone, $IC_{50} = 0.010$ μM) and **2** (α-toxicarol, $IC_{50} = 0.013$ μM), respectively. In addition, the DPPH scavenging activity of rotenoids was also investigated. It was found that 6a,12a-dehydrodeguelin (**5**) possessed the highest activity against DPPH with an IC_{50} value of 7.4 μM, followed by deguelin (**1**, $IC_{50} = 27.4$ μM). All compounds did not show any cytotoxicity at their IC_{50} values for NO inhibitory activity.

Structure–activity relationships (SARs) of these rotenoids against NO release are as follows: (1) hydroxylation at C12a dramatically increased activity, (2) prenylation at furan ring increased activity markedly and (3) hydrogenation of a double bond at C6a–C12a conferred higher activity. For the DPPH radical scavenging effect, it was found that (1) introduction of a double bond at C6a–C12a increased activity and (2) hydroxylation of C11 at the D-ring decreased activity. As regards active compounds of *Derris trifoliata* stems, the isolated compounds are responsible for the NO inhibitory effect, especially **7**, **1**, **9** and **2**, whereas **5** and **1** are those for the DPPH scavenging activity.

Keywords: RAW264.7 cells; Nitric oxide; DPPH; *Derris trifoliata*; Rotenoids

Introduction

Derris plants are found throughout the tropical regions of Asia and East Africa and are widely used in cattle and sheep dips for the control of ticks and other ectoparasites. It is currently used in horticulture against aphids, caterpillars, sawflies, wasps, raspberry beetles and red spiders (Cremlyn 1978). *D. trifoliata* Lour. (syn: *D. uliginosa* Benth.) is one of the plants in the Leguminosae family, locally known in Thai as Tob-tab-nam. The stems of *D. trifoliata* have been used for laxative, carminative and expectorant (Bamroongrugsa 1999). The bark of this plant has been used to treat rheumatism and dysmenorrhea (Kirtikar and Basu 1987). The crude methanol extract of the seeds of this plant showed potent larvicidal activity against second-instar larvae of *Aedes aegypti* (Yenesew et al. 2006). Moreover, the ethanol extract of leaves given orally at doses 250 and 500 mg/kg produced a significant inhibition on acetic acid-induced writhing in mice (Ahmed et al. 2007).

Nitric oxide (NO) is one of the inflammatory mediators causing inflammation in many organs and it has potent antimicrobial activity (Goldsby et al. 2002). This inorganic free radical has been implicated in

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physiological and pathological processes, such as vasodilation, non-specific host defense and acute or chronic inflammation. NO acts as a host defense by damaging pathogenic DNA, and as a regulatory molecule with homeostatic activities (Kou and Schroder 1995). However, excessive production of this free radical is pathogenic to the host tissue itself, since NO can bind with other superoxide radicals and acts as a reactive radical which directly damages the function of normal cells (Moncada et al. 1991).

Since this plant has been used for the treatment of some inflammatory-related diseases such as rheumatism and dysmenorrhea and the crude extract of *D. trifoliata* also exhibited potent NO inhibitory activity with an IC_{50} value of 0.002 μg/mL in our preliminary experiment. The present study is therefore aimed to investigate the effect of compounds isolated from this plant against NO using RAW264.7 cells, as well as their radical scavenging activity on DPPH.

Materials and methods

Reagents

Lipopolysaccharide (LPS, from *Escherichia coli*), RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), indomethacin, L-nitroarginine (L-NA), caffeic acid phenethylester (CAPE) and phosphate-buffered saline (PBS) were purchased from Sigma. Fetal calf serum (FCS) was bought from Gibco. Penicillin–streptomycin was purchased from Invitrogen. Ninty-six-well microplates were obtained from Nunc. Other chemicals were from Sigma.

Plant materials

Derris trifoliata stems were collected in April 2002 at the Mangrove Research Station in Nakhon Si Thammarat province, Thailand. The voucher specimen is No. SC02. The plant material was identified by Prof. Puangpen Sirirugsa and the voucher specimen is kept at the Herbarium of the Department of Biology, Faculty of Science, Prince of Songkla University, Songkhla, Thailand.

Preparation of the plant extract and isolation

Ground-dried stems of *D. trifoliata* (16.2 kg) were immersed at room temperature in hexane, CH_2Cl_2 and MeOH (each 2×10 l, 5 days), successively. After evaporation, the viscous crude hexane (73.4 g), CH_2Cl_2 (118.1 g) and the MeOH (350.5 g) extracts were obtained. A portion of crude hexane extract (21.7 g) was subjected to QCC and eluted with gradient elution of hexane, EtOAc and MeOH which afforded ten fractions (A1–A10). Compounds **1** (12.7 mg), **2** (5.0 mg) and **6** (8.2 mg) were isolated from fraction A7 (0.94 g) by CC and prep. TLC with EtOAc-hexane (1:3, v/v). Fraction A8 (0.47 g) was subjected to CC (EtOAc-hexane, 1:3, v/v) and followed by reversed-phase prep. TLC (MeOH-H_2O, 1:3, v/v) to afford **3** (2.5 mg), **7** (9.7 mg) and **9** (16.7 mg) (Andrei et al. 1997; Carlson et al. 1973; Magalhães et al. 1996; Thasana et al. 2001).

The crude CH_2Cl_2 extract (118.08 g) was fractionated by QCC over silica gel using gradient elution of hexane-EtOAc and EtOAc-MeOH. The eluate was combined on the basis of TLC analysis to give five fractions (B1–B5). Fraction B2 (6.82 g) was separated by CC with EtOAc-hexane (1:4, v/v), followed by prep. TLC with EtOAc-hexane (1:4, v/v) to afford **4** (17.2 mg). Fraction B4 (31.35 g) was purified by QCC with hexane and increasing polarity with EtOAc and MeOH to give five subfractions (B4a–B4e). Subfraction B4c (4.96 g) was further separated by CC with CH_2Cl_2 followed by reversed-phase prep. TLC (MeOH-H_2O, 7:3, v/v) to yield **5** (2.2 mg) and **8** (101.1 mg) (Andrei et al. 1997; Carlson et al. 1973).

NO inhibitory activity assay

Inhibitory effects of compounds 1–9 (see Fig. 1) on the release of NO from RAW264.7 cells

Inhibitory effect on NO production by murine macrophage-like RAW264.7 cells were evaluated using a modified method from that previously reported (Banskota et al. 2003). Briefly, the RAW264.7 cell line [(purchased from Cell lines service (CLS)] was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/mL), streptomycin (100 μg/mL) and 10% FCS. The cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 200 μg/mL of LPS together with the test samples at various concentrations and was then incubated for 48 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric method. Briefly, after 48 h incubation with the test samples, MTT solution (10 μL, 5 mg/mL in PBS) was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the