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Anti-oxidant and inflammatory mediator's growth inhibitory effects of compounds isolated from *Phyllanthus urinaria*

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Abstract

Phyllanthus urinaria Linnea (Euphorbiaceae), is a traditional anti-hepatitis herb used in Taiwan. In continuation of our search for potent natural anti-inflammatory agents, from the ethanolic extract of this plant, nine compounds including phyllanthin (**1**), phyltetralin (**2**), trimethyl-3,4-dehydrochebulate (**3**), methylgallate (**4**), and rhamnocitrin (**5**), methyl brevifolincarboxylate (**6**), β-sitosterol-3-*O*-β-D-glucopyranoside (**7**), quercitrin (**8**), and rutin (**9**) were isolated. The structures of compounds **3** and **6** were established based on NMR and mass spectral studies. The isolates **1**–**9** were investigated for their antioxidant, and anti-inflammatory activities in vitro. In the antioxidant assay, the isolates **3**, **4** and **6** exhibited significant DPPH radical scavenging activity with an IC_{50} value of 9.4, 9.8 and 8.9 μM, respectively. On the other hand, in the inflammatory mediators growth inhibitory assay from LPS/interferon (IFN)-γ-activated peritoneal macrophages, all the isolates except **7**, significantly and dose-dependently inhibited the enhanced production of NO radicals, and such modulation was closely associated with the inhibition of tumor necrosis factor (TNF)-α and interleukin (IL)-6. In addition, 30 μM of isolates **3** and **6**, and 50 μM of **4**, significantly arrest the mitogen-stimulated spleen cells in G0/G1 stage. This is the first report on *Phyllanthus urinaria* isolates for their growth inhibitory activities against inflammatory mediators, in addition to spleen cell cycle arrest in G0/G1 stage. Therefore, these isolates from *Phyllanthus urinaria* may be useful for the treatment of cell-mediated immune diseases.
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Keywords: *Phyllanthus urinaria*; Isolated compounds; DPPH radical scavenging; Anti-inflammation; NO; IL-6; TNF-α; Cell cycle analysis

1. Introduction

Free radicals are implicated in the etiology of several diseases such as atherosclerosis, neurodegenerative disorders, some forms of cancer, and aging (Kupeli and Yesilada, 2007). Antioxidant bioactive compounds from plant sources and are commercially promoted as nutraceuticals, has been shown to reduce the incidence of these diseases (Hermans et al., 2007). Currently there is a great deal of interest in newer bioactive molecules from nature with health-promoting potential (Yesilada, 2005).

Macrophages play an important role in host defense against infection and cancer. Activation of macrophages by stimuli, such as lipopolysaccharide (LPS), a component of the Gram-negative bacteria cell wall, and interferon (IFN)-γ are well known as an effective stimulus in activation of macrophages to secrete pro-inflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, and secondary mediators, such as nitric oxide (NO) (Linton and Fazio, 2003). Under physiological conditions, these mediators are involved in anti-pathogenic processes and are well regulated. However, different pathological conditions, such as chronic inflammation, autoimmune diseases, and cancer, are closely associated with the production of excess amounts of NO and inappropriate expression of cytokines (Szekanecz and Koch, 2007). Hence, natural agents that regulate the production of various cytokines and suppress the overproduction of NO may have protective roles in inflammation-related diseases (Nam, 2006).

Phyllanthus urinaria Linnea, one of the herbal plants belonging to the genus *Phyllanthus* (Euphorbiaceae), is widely distributed in tropical and subtropical countries including Taiwan. The species of *Phyllanthus* have long been used in folk medicine to treat kidney and urinary bladder disturbances, intestinal infections, diabetes, and hepatitis B in several parts

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of the world (Calixto et al., 1998). Particularly, *Phyllanthus urinaria* is traditionally used in Taiwan to reduce heat, remove food stagnancy, improve eyesight, relieve inflammation, calm the liver, suppress *yang* hyperactivity of liver, detoxify poison from body and increase the flow of the urine (Chiu and Chang, 1998; Committee on Chinese Medicine and Pharmacy, 2003). Furthermore, the decoction from this species is commonly used as tea and juice in Taiwan for the treatment of inflammatory diseases (Kao, 1985). Previous phytochemical investigations on *Phyllanthus urinaria* have resulted in the isolation of lignans, flavonoids, tannins, and other benzenoid constituents (Yao and Zuo, 1993; Liu et al., 1999; Zhang et al., 2000a,b, 2004; Chang et al., 2003). Studies on the therapeutic effects of *Phyllanthus urinaria* in animal models have proven to be effective in protecting CCl_4-induced injuries of liver cells (Zhou et al., 1997), relaxing the histamine-induced contraction of trachea (Paulino et al., 1996a,b), producing pronounced systemic, spinal and supraspinal antinociception (Santos et al., 1995, 1999), inducing the contractile response in the urinary bladder (Dias et al., 1995) and decreasing the blood glucose level in streptozotocin-induced diabetic rats (Higashino et al., 1992). Further, inhibition of herpes simplex virus type 1 and type 2 infection (Yang et al., 2005, 2007), and anti-tumor effect on various tumor cells originated from different tissues (Giridharan et al., 2002; Huang et al., 2003, 2004a,b, 2006), and cardio protective (Chularojmontri et al., 2005) activities were also reported. However, only limited work has been carried out on the constituents and their anti-inflammatory activities of this plant. Due to the popularity of *Phyllanthus urinaria* among the Taiwanese, the objective of this study was to isolate the pure compounds from this species, and evaluate their radical scavenger activity in the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) assay, and on the production of various cytokines, including NO, TNF-α, and IL-6 in LPS/IFN-γ-activated murine peritoneal macrophage cells. In addition, to obtain some preliminary insights on the mechanism(s) of action, these isolates effect on the cell cycle progress of mice spleen cells was also examined. Results from this study may increase understanding of how compounds from *Phyllanthus urinaria* modulate immune function and how these compounds might be used in the treatment of inflammation-related diseases.

2. Materials and methods

2.1. General

Solvents for extraction and open column chromatography (CC) were reagent grade and used without further purification. ^{1}H NMR, ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer operating at 500.15 MHz, 125.20 MHz, respectively in DMSO-d_6 and $CDCl_3$. ESI-MS were recorded on Thermo-Finnigan LCQ Advantage system. CC separations were carried out by using silica gel 60 (0.063–0.200 mm) supplied by E. Merck. TLC was carried out in pre-coated silica gel 60 F_{254} plastic plates (Merck). Lipopolysaccharide (LPS, *Escherichia coli* 055: B5), bovine serum albumin (BSA), phosphate-buffered solution (PBS), concanavalin A (Con A), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Griess reagent were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Recombinant interferon-γ (IFN-γ) was purchased from PeproTech (Margravine, London, England). RPMI-1640 medium, Hank's balanced salt solution (HBSS), penicillin, streptomycin, L-glutamine and fetal calf serum were purchased from Gibco BRL (Grand Island, NY, U.S.A.).

2.2. Plant material

The whole plants of *Phyllanthus urinaria* Linnea (Euphorbiaceae) was collected in September 2006 from Wufeng, Taichung County in central Taiwan, and a botanically identified voucher specimen (YMT-06-03) was deposited in the Herbarium of the Institute of Biotechnology, Chaoyang University of Technology, Taiwan.

2.3. Extraction and isolation

The dried plant material (800 g) was pulverized using a milling machine and extracted with 80% aq. EtOH (4 1 × 5) under reflux. After exhaustive extraction, the combined extract was filtered, and the solvent was dried by rotary evaporation under reduced pressure at a temperature of maximally 35 °C to give dark brown syrup about 28 g (3.5% based on the dry weight), and then suspended in distilled H_2O. The water layer was portioned with *n*-hexane, chloroform and *n*-butanol, sequentially to yield the respective solvent extracts.

The chloroform extract (14 g, 1.75%, w/w) was further purified by silica gel chromatography (4 cm × 90 cm, 0.063–0.200 mesh) and eluted with a *n*-hexane/EtOAc gradient elution (the ratios of *n*-hexane/EtOAc were from 100:0 to 0:100). Twenty-five column fractions were collected and analyzed by TLC (*n*-hexane-EtOAc). Fractions with similar TLC patterns were combined, and rechromatographed on a silica gel column to yield, phyllanthin (**1**, 55 mg, 0.0069%), phyltetralin (**2**, 12 mg, 0.0015%), trimethyl-3,4-dehydrochebulate (**3**, 10 mg, 0.0008%), methylgallate (**4**, 75 mg, 0.0094%), and rhamnocitrin (**5**, 14 mg, 0.0018%). The concentrated *n*-butanol extract (11 g, 1.375%, w/w) was subjected to repeated column chromatography on a silica gel and eluted with CH_2Cl_2/MeOH with a gradually increased ratio of methanol. Methyl brevifolincarboxylate (**6**, 26 mg, 0.0033%) was obtained from the CH_2Cl_2/MeOH elution (20:1, v/v) as a yellow amorphous powder. β-sitosterol-3-*O*-β-D-glucopyranoside (**7**, 25 mg, 0.0031%) was obtained from CH_2Cl_2/MeOH (10:1) elution. Quercetin-3-α-L-rhamnopyranoside (quercitrin, **8**, 8 mg, 0.001%), and rutin (**9**, 32 mg, 0.004%) were obtained from CH_2Cl_2/MeOH, 8:2 and 7:3 elution, respectively (Fig. 1).

Phyllanthin (**1**): white needle-like crystals; ^{1}H NMR ($CDCl_3$) δ 2.02 (2H, m, H-8, 8′), 2.63 (4H, m, H-7, 7′), 3.27 (10H, m, H-9, 9′ and OCH_3-9, 9′), 3.79 (6H, s, OCH_3-3, 3′), 3.83 (6H, s, OCH_3-4, 4′), 6.59 (2H, d, J = 2.0, H-2, 2′), 6.62 (2H, dd, J = 8.0, 2.0, H-6, 6′), 6.73 (2H, dd, J = 8.0, 2.0, H-5, 5′); ^{13}C NMR ($CDCl_3$) δ

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