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ORIGINAL ARTICLE

Isolation and characterization of bioactive compounds from *Lepisorus thunbergianus* (Kaulf.)

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Abstract *Lepisorus thunbergianus* (Kaulf.) (LET) is an evergreen fern found on rocks and tree trunks that is distributed in East and Southeast Asia. Our previous study showed that the methanol extract from LET had significant anti-oxidant activity, but the active components of LET are still unclear. In the present study, isovitexin, orientin, isoorientin and chlorogenic acid were isolated from LET under the guidance of antioxidant activity. In addition, the structure of isovitexin, orientin, isoorientin and chlorogenic acid was characterized using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) and nuclear magnetic resonance spectroscopy (NMR). Orientin and isoorientin presented similar activities toward the DPPH, with half maximal inhibitory concentrations (IC_{50}) of 15–17 μmol, and inhibition of reactive oxygen species (ROS) production by 50% at 100 μmol. Chlorogenic acid significantly inhibited intracellular ROS and nitric oxide (NO) production and had a strong effect toward DPPH. Furthermore, chlorogenic acid demonstrated decreased iNOS, COX-2, IFN-β and TNF-α gene expression. These findings demonstrate the potential anti-inflammatory effects of chlorogenic acid. Isoorientin, at a concentration of 100 μmol, showed 50% inhibition of human liver cancer cells (Huh7 and HepG2). These results suggest that compounds isolated from LET have potential to prevent liver cancer cell lines.

1. Introduction

Lepisorus thunbergianus (Kaulf.) (LET) is an evergreen fern found on rocks and tree trunks that is distributed in East and Southeast Asia. The plant has been reported to show topical anti-lipid peroxidative activity (Chung et al., 1993). Moreover, it was found that treatment of oral cavity cancer cells with LET extracts resulted in concentration-dependent growth inhibition (Jang, 2010).

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Table 1 DPPH free radical scavenging activity of compounds from *Lepisorus thunbergianus*.

Compounds	DPPH radical scavenging activity IC_{50} (μmol/ml)
Com. 1	126.38 ± 1.85 d
Com. 2	17.36 ± 1.07 c
Com. 3	15.22 ± 0.74 b
Com. 4	6.86 ± 0.56 a
Ascorbic acid	11.70 ± 0.82
α-Tocopherol	13.85 ± 0.46
BHA	81.73 ± 0.39
BHT	601.84 ± 0.29

[a]Mean values with the same letter are not significantly different at $p < 0.05$ by Duncan's multiple range test.

Free radicals and reactive oxygen species (ROS) have been proposed as causative factors in the toxicity of numerous chemicals and in the pathogenesis of many diseases, such as cardiovascular disease (Simonne et al., 1997), certain cancers (Ames et al., 1993), diabetes (Laaksonen and Sen, 2000), rheumatoid arthritis and cataracts (Taylor, 1992). Antioxidants are able to prevent the adverse effects of oxygen by helping to capture and neutralize free radicals, thereby eliminating free radical damage to the human body. Nitric oxide (NO) along with superoxide ($O2^{\cdot-}$) and the products of their interaction initiate a wide range of toxic oxidative reactions causing tissue injury (Hogg, 1998). Likewise, reactive oxygen intermediates are believed to be mediators of inflammation and responsible for the pathogenesis of tissue destruction in rheumatoid arthritis. Much attention has been directed toward the characterization of the antioxidant properties of plant extracts and their components in order to identify the constituents responsible for these activities. Previous studies have reported that among the many different groups of natural products, flavonoids are a group of chemical entities that are widely distributed in the plant kingdom. Flavonoids are a large group of polyphenolic compounds that are known to have antioxidative activity and to have scavenging effects on ROS (Nijveldtet al., 2001).

Preliminary assays carried out in our study show antioxidant activity of a methanol extract of LET. Therefore, in this study, the main antioxidants from LET were isolated, identified and quantified.

2. Materials and methods

2.1. Chemicals

All solvents used were of analytical grade. Methanol (MeOH), butanol (BuOH), *n*-hexane, ethyl acetate (EtOAc), chloroform ($CHCl_3$), benzene and acetone were obtained from Dae Jung chemicals & metals Co. Silica gel (70–30 mesh) and thin layer chromatography (TLC) plate (silica gel $60F_{254}$; 60RP-18 $F_{254}S$) was purchased from Merck Co. (Germany). Human liver cancer ($HepG_2$, Huh7) and RAW 264.7 macrophage cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea). 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, butylated hydroxyanisole (BHA), 2,6-di-tert-butyl-4-methylphenol (BHT), phosphate buffered saline (PBS) buffer and 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI medium 1640 and fetal bovine serum (FBS), and Penicillin were acquired from Thermo Scientific (USA).

2.2. Plant material and extraction

The entire LET plant was dried in the shade at room temperature and then powdered. 1.2 kg of sample powder was extracted with 100% methanol. The methanol extract was filtered and three sample replicates were extracted under the same conditions with new solvent. The methanol extracts were filtered and evaporated under reduced pressure using a vacuum rotary evaporator to produce a crude extract. The crude extract was suspended in deionized water and partitioned sequentially with *n*-hexane, ethyl-acetate (EtOAc) and butanol (water saturated BuOH) fractions were then evaporated using a vacuum rotary evaporator. The antioxidant profile of the extract and fractions were determined by means of the selected assays.

2.3. Purification

The BuOH fraction was the most active in preliminary assays and was therefore selected for the isolation and characterization of the main antioxidants from LET. Each fraction was assessed for the free radical scavenging effect using the DPPH decoloration test.

The BuOH fraction (19.1 g) was separated on silica gel (320 g, 0.263–0.2 mm) using stepwise gradient elution with benzene:acetone:methanol (9:1:0–0:0:100) to yield 11 fractions (Fr. 1–Fr. 11). Fraction Fr. 9 (7.5 g) was chromatographed on silica gel (120 g, 0.263–0.2 mm), and eluted with benzene:methanol:water (88:7:5) to yield four subfractions (Fr. 9-1–Fr. 9-4). Subfraction Fr. 9-2 was recrystallized from methanol to give compound 1 (27.6 mg). Subfraction Fr. 9-3 (2.0 g) was separated by column chromatography (CC) on an octadecylsilyl (ODS) gel (23 g, 12 nm S-75 μm) using stepwise gradient elution with methanol:water (0:100–100:0) to yield seven subfractions (Fr. 9-3-1–Fr. 9-3-7). Subfraction Fr. 9-3-5 was re-crystallized from methanol to give compound 2 (17.9 mg). Subfraction Fr. 9-3-3 was chromatographed on a silica gel column (12 g, 0.04–0.06 mm) by isocratic elution with benzene:methanol:water (100:50:3) to give compound 3 (10.1 mg). Subfraction Fr. 9-4 (2.5 g) was re-chromatographed on a Sephadex LH-20 gel (16 g, 40–120 μm) by gradient elution with methanol:water (0:100–20:80) and divided into six sub-fractions (Fr. 9-4-1–Fr. 9-4-6). Subfraction Fr. 9-4-3 (0.98 g) was purified by CC on an ODS gel (11 g) with 100% water to yield compound 4.

2.4. Structural identification of the compounds

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker spectrometer (Rheinstetten, Karlsruhe, Germany). Dimethyl sulfoxide (DMSO-*d*6) and methanol (CD_3OD) were used as solvents. MALDI-TOF MS data were recorded with a Maldi-top (Bio-Tek Instruments Inc, Winooski, USA).

2.5. DPPH radical scavenging activity

The DPPH radical scavenging activity of the extract and isolated compounds were determined by the method of