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Phytocompounds from *Vitis kelungensis* stem prevent carbon tetrachloride-induced acute liver injury in mice

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ABSTRACT

Vitis thunbergii, *Vitis flexuosa*, and *Vitis kelungensis* are three common wild grapes in Taiwan and have been used as a traditional medicine for inflammatory disorders. However, the potential bioactivities of these wild grapes have not been studied to date. In this study, anti-inflammatory activities and hepatoprotective properties of these three wild grapes were assessed by *in vitro* and *in vivo* assays, respectively. Results revealed that the methanolic extract of *V. kelungensis* stem (VKS) strongly suppressed NO production in lipopolysaccharide (LPS)-stimulated murine macrophages. And among all fractions derived from VKS, the EtOAc fraction exhibited the best inhibitory activity. In addition, VKS and its major resveratrol derivatives, (−)-ε-viniferin and 2-(4-hydroxyphenyl)-2,3-dihydrobenzo[*b*]furan-3,4,6-triol, can prevent CCl_4-induced liver injury and aminotransferase activities in mice, which were comparable to that of silymarin or resveratrol, the natural remedies for liver diseases.

1. Introduction

A number of researchers have pointed out that carbon tetrachloride (CCl_4) causes acute and chronic hepatic damage due to free radical generations (Hung, Fu, Shih, Lee, & Yen, 2006; Liao et al., 2007; Song & Yen, 2003; Tung et al., 2009). CCl_4 is metabolized by cytochrome P450 2E1 (CYP2E1) to become a trichloromethyl radical ($^{\cdot}CCl_3$) and a proxy trichloromethyl radical ($^{\cdot}OOCCl_3$), which are assumed to initiate free radical-mediated lipid peroxidation, leading to the accumulation of lipid-derived oxidation products that cause liver injury (Liao et al., 2007; Poli, Albano, & Dianzani, 1987; Recknagel, Glende, Dolak, & Waller, 1989; Shyur et al., 2008). A number of studies have shown that various herbals and natural remedies could protect liver against CCl_4-induced oxidative stress by altering the levels of increased lipid peroxidation and enhancing the decreased activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione-*S*-transferase (GST), as well as the decreased level of hepatic-reduced glutathione (GSH) (Hsiao et al., 2003; Hung et al., 2006; Lu & Liu, 1992; Song & Yen, 2003; Tung et al., 2009).

In Taiwan, *Vitis thunbergii*, *Vitis flexuosa*, and *Vitis kelungensis* are three common wild grapes, and they are traditionally used as medicinal plants. Aqueous extracts of wild grape stems are used in Taiwan for the treatment of diarrhoea, fracture, injury, jaundice, and hepatitis (Huang, Tsai, Shen, & Chen, 2005). It is well-known that plants in the genus *Vitis* commonly contain oligomers of resveratrol (Huang et al., 2005). Recently, many studies have shown that resveratrol possesses various pharmacological effects, such as cardiovascular protection (Berrougui, Grenier, Loued, Drouin, & Khalil, 2009) and chemoprevention of cancer (Jang et al., 1997). In addition, resveratrol has also been found to prevent or cure hepatotoxin (e.g., alcohol or CCl_4)-induced liver diseases, such as acute hepatitis and hepatic fibrosis (Ajmo, Liang, Rogers, Pennock, & You, 2008; Chávez et al., 2008; Rivera, Shibayama, Tsutsumi, Perez-Alvarez, & Muriel, 2008; Vitaglione et al., 2009). Thus, the objective of this study was to evaluate the potential protective effects of crude extract and its major resveratrol derivatives from the stem extract of *V. kelungensis* in CCl_4-induced acute liver injury in mice. Furthermore, the contents of major resveratrol derivatives from *V. thunbergii*, *V. flexuosa*, and *V. kelungensis* stems, in different seasons, were also addressed in this study.

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide (LPS), Griess reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), silymarin, and resveratrol were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased by Acros (Belgium). The other chemicals and solvents used in this experiment were of the highest quality available.

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2.2. Plant material

Fresh stems of *V. thunbergii, V. flexuosa*, and *V. kelungensis*, in different seasons, namely summer (June 2007), autumn (September 2007), winter (December 2007), and spring (March 2008), were sampled from the agriculture experimental station of National Chung Hsing University in Taichung County. The species were identified by Prof. Tzer-Kuan Hu (Department of Agronomy, National Chung Hsing University).

2.3. Extraction and isolation

The samples were cleaned with tap water and air-dried at ambient temperature (25 °C). They were then extracted with methanol by soaking each one for 1 week at ambient temperature twice. The extracts were decanted, filtered under vacuum, concentrated in a rotary evaporator, and then lyophilised. Of these, the resulting crude extract of *V. kelungensis* stem (72.0 g; 1.6%) was further fractionated, successively, with ethyl acetate (EtOAc), *n*-butanol (BuOH) and water, to yield soluble fractions of EtOAc (44.1 g; 61.3%), BuOH (10.0 g; 13.9%), and H_2O (16.8 g; 23.3%). The EtOAc fraction was separated by a silica gel 60 column (Merck, Darmstadt, Germany) eluted with a stepwise gradient of EtOAc/*n*-hexane 2/98 (v/v) to 100/0 (v/v), to give various subfractions (Fig. 1A). Ten major compounds were isolated and purified from the high yields of **A** (compounds **1**–**3**), **B** (compounds **4** and **5**), **C** (compounds **6** and **7**), **D** (compound **8**), **E** (compounds **8** and **9**), **F** (compound **9**), and **G** (compound **10**) subfractions by semipreparative HPLC, with a model PU-2080 pump (Jasco, Japan) equipped with a RI-2031 refractive index detector (Jasco, Japan) and a 250 × 10.0 mm i.d., 5 μm Luna silica column (Phenomenex, Torrance, CA). Separation was achieved using an isocratic system of EtOAc/*n*-hexane at a flow rate of 4 ml/min. NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer (Rheinstetten, Germany) in $CDCl_3$ (**1**–**7**), acetone-d_6 (**8** and **10**) or CD_3OD (**9**). Chemical shifts were determined in δ (ppm) relative to the solvent $CDCl_3$ (δ_H 7.26, δ_C 77.0), acetone-d_6 (δ_H 2.04, δ_C 29.8) or CD_3OD (δ_H 3.31, δ_C 49.1) as internal standard. ESI-MS spectra were measured on a Finnigan LCQ ion-trap mass spectrometer (San Jose, CA). The standard conditions employed included spray voltage 4.0 kV, capillary temperature 280 °C, and sheath gas flow rate 5 arbitrary units. The structures of compounds **1**–**8** and **10** (as shown in Fig. 1B) were identified by ESI-MS and NMR, and all spectral data were consistent with those reported in the literature (Chang, Chang, & Wu, 2000; Hisham, Kumar, Fujimoto, & Hara, 1996; Kuo & Lee, 1997; Kurihara, Kawabata, Ichikawa, Mishima, & Mizutani, 1991; Lin, Chen, & Kuo, 1991; Tanaka, Tabuse, & Matsunega, 1998; Tanaka et al., 2000). Compound **9** was, for the first time, isolated from nature and is reported here as a new natural product.

2-(4-Hydroxyphenyl)-2,3-dihydrobenzo[*b*]furan-3,4,6-triol (**9**): light-yellow amorphous solid; mp: 277–278 °C; $[\alpha]_D^{24}$ −0.456° (MeOH; *c* 1.00); UV (MeOH), λ_{max}, nm (log ε): 285 (3.77); IR (KBr), ν_{max}, cm^{-1}: 3321, 1604, 1512, 835; HREIMS *m*/*z*: 260.0674, $[M]^+$ calcd 260.0685 for $C_{14}H_{12}O_5$; ^1H NMR (500 MHz, CD_3OD): δ 6.93 (d, 2, *J* = 8.5 Hz, H-2, 6), 6.67 (d, 2, *J* = 8.5 Hz, H-3, 5), 6.53 (d, 1, *J* = 1.8 Hz, H-11), 6.11 (d, 1, *J* = 1.8 Hz, H-13), 4.47 (s, 1, H-7), 3.74 (s, 1, H-8); ^{13}C NMR (125 MHz, CD_3OD): δ 159.5 (C-12), 156.4 (C-4), 155.7 (C-14), 151.0 (C-10), 138.6 (C-1), 129.3 (C-2, 6), 124.0 (C-9), 116.1 (C-3, 5), 103.5 (C-11), 102.7 (C-13), 61.1 (C-8), 54.9 (C-7).

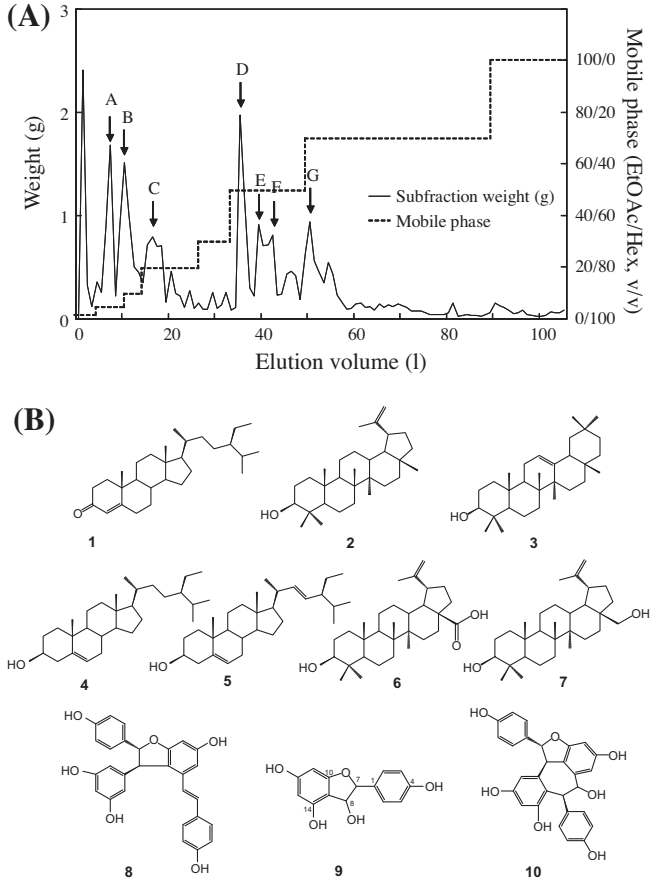

Fig. 1. (A) Subfraction weights of EtOAc fraction from *V. kelungensis* stem. (B) Phytocompounds isolated from EtOAc fraction of *V. kelungensis* stem. (**1**) β-Sitostenone, (**2**) lupeol, (**3**) β-amyrin, (**4**) β-sitosterol, (**5**) stigmasterol, (**6**) betulinic acid, (**7**) betulin, (**8**) (−)-ε-viniferin, (**9**) 2-(4-hydroxyphenyl)-2,3-dihydrobenzo[*b*]furan-3,4,6-triol, and (**10**) (+)-balanocarpol.

2.4. Cell cultures

The macrophage RAW 264.7 cell line, obtained from the ATCC (Manassas, VA), was grown in Dulbecco's modified Eagle's medium (DMEM) (Gibico/BRL, Grand Island, NY) supplemented with 10% heat-inactivated foetal bovine serum, 1 mM sodium pyruvate, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified 5% CO_2 incubator.

2.5. Measurement of NO production and cell viability

To investigate the anti-inflammatory activity of test samples, NO production in LPS-stimulated RAW 264.7 cells was examined. For NO determination, RAW 246.7 cells were seeded in 96-well plates at a density of 2×10^5 cells/well and grown for 4 h for adherence. The cells were treated with test samples for 1 h and then incubated for 24 h in fresh DMEM, with or without 1 μg/ml of LPS. The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction. Briefly, 100 μl of cell culture supernatant were reacted with 100 μl of Griess reagent (1:1 mixture of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in water and 1% sulfanilamide in 5% phosphoric acid) in a 96-well plate, and absorbance at 540 nm was recorded using an ELISA reader.

The cell viability assay was determined on the basis of MTT assay. After culturing, supernatants were collected for NO measurement, 100 μl of tetrazolium salt solution (1 ml MTT/10 ml DMEM) were added to each well, and then incubated for 1 h at 37 °C in a 5% CO_2 incubator. The medium was then aspirated and the insoluble formazan product was dissolved in 100 μl of DMSO. The extent of MTT reduction was quantified by measuring the absorbance at 570 nm.