

Original Research Article

Quercetin - A Flavonoid Compound from *Sarcopyramis bodinieri* var *delicate* with Potential Apoptotic Activity in HepG2 Liver Cancer Cells

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Abstract

Purpose: To isolate and identify the cytotoxicity of the constituents of *Sarcopyramis bodinieri* var. *delicate*.

Methods: *S. bodinieri* var. *delicate* was extracted with hydrochloric acid-methanol and fractionated with ethyl acetate further. The chemical constituents of the ethyl acetate fraction were purified by a combination of D101 macroporous resin and Sephadex LH-20 column chromatography. The structure was characterized by ¹H-Nuclear Magnetic Resonance (NMR) and electrospray ionization tandem mass spectrometry (ESI-MS). Apoptosis was evaluated by fluorescence staining and Western blot analysis using 4,6-diamidino-2-phenylindole (DAPI) staining and poly (ADP-ribose) polymerase (PARP) SDS-PAGE tests in HepG2 liver cancer cells.

Results: One flavonoid with high purity was purified by the combination of D101 macroporous resin and Sephadex LH-20 column chromatography. The flavonoid compound was identified as quercetin by ¹H-NMR and ESI-MS analyses. DAPI staining and PARP SDS-PAGE tests showed 60 μM quercetin could induce potential apoptotic activity in HepG2 liver cancer cells.

Conclusion: Quercetin was the major cytotoxicity constituent in *S. bodinieri* var. *delicate*.

Keywords: Apoptotic activity, Quercetin, *Sarcopyramis bodinieri* var. *delicate*, HepG2 liver cancer cells, Electrospray ionization tandem

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INTRODUCTION

Sarcopyramis genus belongs to the family of Melastomataceae, containing of 4 species and 2 varieties in china [1]. Many of the species are medicinal plants used in clinics and/or as folk medicines to treat liver and other inflammatory diseases. *S. bodinieri* var. *delicate* is a traditional Chinese medicinal (TCM) plant, widely used as a hepatoprotective drug in China.

A previous study has showed that the water extract of this herb could reduce aminotrans-

ferase and cure choleplania and hepatoma *in vivo* [2]. However, the active constituents have not been isolated and identified. The ethyl acetate extract of of *S. bodinieri* var. *delicate* was reported to demonstrate promising antioxidant and free radical scavenging activities *in vitro* [3]. Our group is interested in isolating and identifying bioactive compounds from folk medicinal and edible plant species. As part of this research program, we recently carried out a completely investigation of the chemical constituents of *S. bodinieri* var. *delicate* [4-6]. Two new and many known flavonoid and

phenolic compounds were isolated, and their major structures identified as the glycosides and/or acylated glycosides of quercetin, isorhamnetin and kaempferol [4-6].

This paper herein describes a quick method to isolate quercetin, a major active flavonoid compound, from the methanol-HCl extract of *S. bodinieri* var. delicate and the evaluation of the apoptotic activity of quercetin *in vitro* using 4,6-diamidino-2-phenylindole (DAPI) staining and poly (ADP-ribose) polymerase (PARP) SDS-PAGE tests in HepG2 liver cancer cells.

EXPERIMENTAL

Plant material

The specimen of *S. bodinieri* var. delicate was collected from Fujian Province, P.R. China, in April 2007 and authenticated by Xiuhong Zhou (a senior gardener of Yongchun Forestry Bureau). A voucher specimen (RSC07) was deposited at the herbarium of the Medical College, Xiamen University. The plant material dried at 40 °C in an air oven for 48 h and finely powdered.

Equipment and chemicals

The ¹H-NMR spectra were recorded on a Bruker Avance-600 FT-NMR spectrometer, with tetramethylsilane (TMS) internal standard. ESI-MS spectra were recorded on 3200 Q-trap ESI-MS spectrometer (ABI, USA). Column chromatography was carried out with D101 macroporous resin (Cangzhou Bonchem Co., Ltd., China) and Sephadex LH-20 (Pharmacia Co., USA). HPLC-grade methanol was purchased from Merck Company (Darmstadt, Germany). All other organic solvents used were of analytical grade and purchased from Sinopharm Chemical Reagent Co, Ltd (Shanghai, China). A Shimadzu LC-20AT HPLC system was used to analyze and quantify the content of quercetin. 4,6-diamidino-2-phenylindole (DAPI), Anti-poly (ADP-ribose) polymerase (PARP) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Company. The cell culture medium (DMEM; high glucose, without sodium pyruvate) and fetus bovine serum (FBS) were purchased from Hyclone Company. Acrylamide/bis solution (30 %, 29:1) was bought from Bio-Rad laboratories, Inc. Protein content was tested using a BCA kit (Thermo Scientific).

Cell line and culture

The human hepatic cancer cells line, HepG2, was purchased from American Type Culture

Collection. HepG2 Cells were cultured in DMEM medium supplemented with 10 % heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified incubator with 5 % CO₂ (all reagents from Invitrogen, Carlsbad, CA). The cells were maintained in log phase by seeding twice a week at a density of 1 × 10⁵/ml, and the experiments were performed 1 day after trypsinization.

HPLC analysis

The dried plant material (1 g) was extracted with 20 ml 60 % methanol {with or without 10 % HCl (4:1, v/v)} in a conical flask and refluxed at 80 °C for 2 h. The acid extract was neutralized with Na₂CO₃. Both extracts were all conducted on HPLC analysis.

The HPLC system consisted of a Shimadzu LC-20A pump, a CTO-20A column oven and SPD-M20A dual absorbance detector (DAD). LC-solution software was used for controlling the analytical system and data processing. The analytical column is a shimpack VP-ODS column (150 mm × 4.6 mm, 5 µm) with a flow rate at 1.0 mL/min and injection volume of 20 µL. A gradient solvent system consisting of solvent A (0.1 % aqueous trifluoroacetic acid) and solvent B (MeOH) was used as follows: 0 - 15 min, from 10 to 30% B; 15-25 min, from 30 to 40 % B; 25 - 40 min, from 40 to 50 % B; 40 - 50 min, from 50 to 70 % B. The chromatograms for the *S. bodinieri* var. delicate extracts are shown in Figure 1A and B, respectively. The chromatograms were monitored at a broad spectrum window of 210 - 600 nm and are shown at 365 nm in Figure 1.

Extraction and isolation of quercetin

The dry plant powder (100 g) were extracted with 1000 ml a mixture of 60 % methanol and 10% HCl (4:1, v/v) in a conical flask and refluxed at 80 °C for 2 h. The extract was neutralized with Na₂CO₃ and then concentrated under vacuum to dryness. The concentrate was dissolved and suspended in 300 ml water and liquid-liquid extracted with ethyl acetate (300 ml, three times). The ethyl acetate extracts were purified by D101 macroporous resin column chromatography, eluted with a series of different concentration of ethanol (0%, 30% and 70%, respectively). Fraction 3 (70% ethanol eluted fraction) was subjected to a Sephadex LH-20 column (4.0 cm × 140 cm) eluted with methanol to yield compound **1** (41.3 mg).

Quantification of the total content of quercetin by HPLC

The determination of the total content of quercetin in *S. bodinieri* var. delicate was reported previously [7]. The mobile phase consists of methanol and water (0.1 % phosphoric acid buffer) (45:55), the flow rate was at 1.0 ml/min, separation temperature was at 40 °C and the detection wavelength was set at 365 nm. The injection volume was 20 µl.

Evaluation of cell apoptosis by DAPI staining

The nuclear morphological changes associated with apoptosis were analyzed using DAPI staining [8]. 10 ml HepG2 Cells suspension (1×10^5 /ml) were cultured in a 10 mm petri dish with 2-3 cover slips, allowed to attach overnight, then the cell exposed to vehicle (for control) or 60 µM quercetin (for experimental group) respectively. After 48 h, the cells were washed with PBS and fixed with 95% ethanol for 15 min at room temperature. The fixed cells were washed with PBS again and stained with 100 ng/ml DAPI solutions for 10 min at room temperature, then washed twice with PBS. Cells with condensed and fragmented DNA (apoptotic cells) were scored under a fluorescence microscope (Carl Zeiss, Germany) at $\times 40$ objective lens magnification.

Evaluation of cell apoptosis by Western Blotting

Cells treated and untreated (for control) with 60 µM quercetin were lysed by adding Lysis Buffer RIPA (150 mM NaCl; 50 mM Tris pH 8; 0.1 % SDS; 0.5 % sodium deoxicolate; 1 % Nonidet P40), centrifuged to separate non-soluble material. The protein content in the lysates was measured by BCA protein assay. Then 40 µg soluble proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The detection was achieved using the Immobilon Western Chemiluminescent HRP Substrate kit (Millipore). Primary antibodies are: PARP (Santa Cruz Biotechnology, Inc., sc-7150, 1:1000) and GAPDH (Sigma-Aldrich, G8795, 1:5000).

Statistical analysis

All experiments were performed in triplicate. Statistical analysis of the data was carried out by Microsoft Excel XP and differences were considered significant at $p < 0.05$.

RESULTS

Structure elucidation of compound 1

Compound 1 was obtained as yellow powder, UV-vis (MeOH) $\lambda_{\max} = 370, 256$ nm; ESI-MS m/z : 301 [M-H]⁻. ¹H-NMR (DMSO-*d*₆, 600 MHz) δ : 12.50 (1H, s, 5-OH), 7.68 (1H, d, $J = 2.2$ Hz, H-2'), 7.54 (1H, dd, $J = 2.2, 8.4$ Hz, H-6'), 6.88 (1H, d, $J = 8.4$ Hz, H-5'), 6.40 (1H, d, $J = 2.0$ Hz, H-8), 6.19 (1H, d, $J = 2.0$ Hz, H-6). ¹H-NMR spectrum showed two peaks at δ 6.19 (1H, d, $J = 2.0$ Hz) and 6.40 ppm (1H, d, $J = 2.0$ Hz) consistent with the meta protons H-6 and H-8 on A-ring and an ABX system at 7.68 (1H, d, $J = 2.2$ Hz, H-2'), 7.54 (1H, dd, $J = 2.2$ Hz, 8.4 Hz, H-6') and 6.88 (1H, d, $J = 8.4$ Hz, H-5') corresponding to the catechol protons on B-ring. ¹H-NMR data were consistent with those reported in the literature for the compound [4]. Compound 1 was identified as quercetin (1).

HPLC results for methanol extracts with or without HCl

As depicted in Figure 1, the HPLC chromatography of the methanol extract showed at least 6 peaks while only 3 dominant peaks were detected in the methanol-HCl extract. The peak 1 was identified as quercetin. The content of quercetin was higher in methanol-HCl extract than in methanol extract, which showed that many quercetin glycosides were presented in the plant.

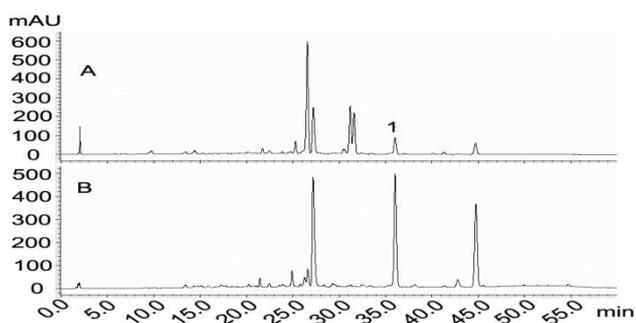


Figure 1: HPLC-DAD chromatogram of *S. bodinieri* var. delicate extracts. Peak 1 is quercetin; (A) Extracted with 60 % methanol; (B) Extracted with methanol-HCl (4:1, v/v).

Total content of quercetin

The standard curve of quercetin was $Y = 7.74 \times 10^7 X - 2.69 \times 10^5$ (X is the concentration of quercetin and Y is the peak area integral value of quercetin). Quercetin's linearity range was 0.025 - 0.4 mg/ml, with a mean correlation coefficient $r = 0.9998$. The content of quercetin in *S. bodinieri* var. delicate was 1.37 mg/g.

Quercetin induction of apoptosis of HpeG 2 cells

Quercetin is a promising cancer chemopreventive agent, we exploiting DAPI staining to observe nuclear morphological changes. As observed in Figure 2, we noticed that the untreated HepG2 cells displayed a normal, healthy nuclear shape, whereas the cells treated with 60 μM quercetin for 24 h demonstrated bright, condensed chromatin and a fragmented nucleolus, which are regarded as hallmarks of apoptosis.

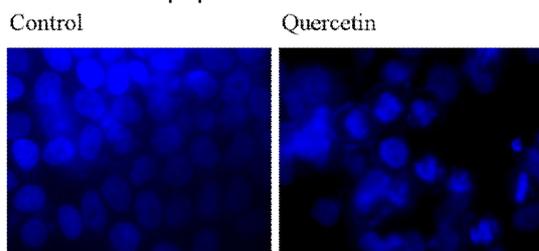


Figure 2: Apoptosis induced by quercetin in HepG2 cells. HepG2 cells treated with quercetin (60 μM /24 h) were immunostained with DAPI.

To further confirm the induction of apoptosis by quercetin, Western blot was conducted to detect the PARP cleavage. HepG2 cells treated with 60 μM quercetin at different time points and the cells collected for Western blot assay. Western blot result indicate that quercetin induced the cleavage of PARP in HepG2 cells after 24h (Figure 3). The results suggest that quercetin efficiently induced the apoptosis of HepG2 cells.

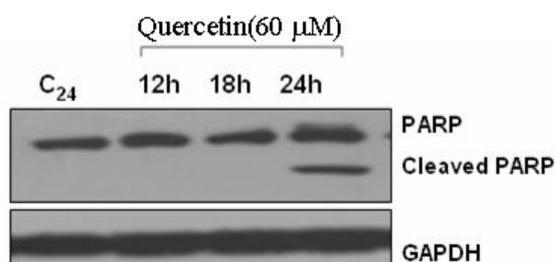


Figure 3: Apoptosis induced by quercetin in HepG2 cells. Western blot analysis of PARP in HepG2 cells treated with 60 μM for different periods of time (12, 18 and 24 h).

DISCUSSION

Flavonol glycosides were the predominant constituents existing in *S. bodinieri* var. *delicate*, especially the quercetin glycosides [4,5]. As a result, methanol-HCl is a powerful extracting solvent for quercetin from the medicinal plant. It is not surprising that the content of quercetin was higher in methanol-HCl extract than the methanol extract. Macroporous resin column is frequently

used to enrich the flavonoid compounds [9, 10]. Flavonol glycosides and other phenolic acids constituents were easily eluted by 30 % ethanol from the macroporous resin column, while elution of flavonol aglycones required 70 % or higher concentration ethanol. Hence, in the present study, we first used 30 % ethanol to elute the phenolic acids and the unhydrolyzed flavonol glycosides. The flavonol aglycones-enriched fraction was further isolated by the Sephadex LH-20 column eluted by methanol. High purity quercetin was obtained by combination of the macroporous resin and Sephadex LH-20 columns.

The apoptotic effect of quercetin was examined by DAPI fluorescence staining in HepG2 cells. Incubation of cells for 24 h with quercetin alone resulted in numerous cells that had smaller nuclei with chromatin condensation and perinuclear apoptotic bodies. PARP is a DNA repair enzyme activated by DNA damage and the cleavage of PARP has been widely used as a biochemical marker of apoptosis [11]. Thus, we further studied PARP levels in HepG2 cells after exposure to quercetin. Our results indicate that 60 μM quercetin can induce the PARP cleavage in a time-dependent manner. Quercetin showed potential apoptotic activity in the HepG2 cells.

CONCLUSION

A quick method to isolate high purity quercetin from *S. bodinieri* var. *delicate* has been developed for the first time. Successful evaluation of the isolated compound's anti-proliferative potential and induction apoptosis activities in HepG2 cancer cells has been achieved.

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