I.J. Engineering and Manufacturing **2011, 1, 7-12**
Published Online February 2011 in MECS (http://www.mecs-press.net)
DOI: 10.5815/ijem.2011.01.02

Available online at http://www.mecs-press.net/ijem

Characterization and Anti-proliferative Activity of Ethanol Extract of Tartary Buckwheat

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Abstract

Tartary buckwheat (Fagopyrum tataricum) has been consumed in China as health food and medicine. In this study, the ethanol extract (EE) of tartary buckwheat was prepared. The major flavonoid in EE was identified as rutin by HPLC-PDA-ESI/MS and the content of rutin in EE was determined as 27.4 % by HPLC. In the anticancer assay, it was found EE could inhibit the proliferation of the human hepatocellular liver carcinoma Hep G2 cell line in a dose-dependent manner. The increase of an early apoptotic population was observed by both annexin-FITC and PI staining, which suggested that EE could induce Hep G2 cells to enter into apoptosis.

Index Terms: Tartary buckwheat; extraction; antioxidant; apoptosis

1. Introduction

Common buckwheat (Fagopyrum esculentum) originates from Southwest China and has gradually been spread to all continents, while tartary buckwheat (Fagopyrum tataricum) is grown and used in the mountainous regions of Southwest China (Sichuan), in northern India, Bhutan, and Nepal [1]. Tartary buckwheat grain, as an important functional food material, contains proteins with high biological value and balanced amino acid composition, relatively high crude fiber and vitamins B1, B2, and B6 [2], and more flavonoids than common buckwheat [3]. Today, tartary buckwheat has been widely cultivated in Shanxi and Sichuan provinces, China. So, there is the possibility of large-scale application of tartary buckwheat as health food and medicine. In order to provide the corresponding development strategy, the ethanol extract (EE) of tartary buckwheat was prepared and its antioxidant and anti-proliferative activities was investigated.

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2. Materials And Methods

2.1. Materials and Chemicals

The tartary buckwheat (Fagopyrum tataricum cv. Jingqiao 2) for this study was purchased from Shanxi Academy of Agricultural Sciences (Taiyuan, China). Rutin obtained from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). A human hepatocellular liver carcinoma cell line (HepG2) was obtained from the Institute of Basic Medicine, Chinese Academy of Medical Sciences. Powdered Dulbecco modified eagle medium and trypsin solution were purchased from GIBCO (Grand Island, NY, USA). Foetal bovine serum (FBS) was purchased from Hyclone Laboratories, Inc. Propidium iodide (PI) and 3-[(4, 5)-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. An annexin V-FITC apoptosis detection kit was purchased from CLONTECH Company. Other chemicals were of analytical grade.

2.2. Preparation of the Ethanol Extract (EE)

Powdered tartary buckwheat (30 g) (moisture content 10.9%) was extracted with 450 mL of 70 % ethanol in a water bath at 70 ℃ for 3 h and then filtered. The filtrate was concentrated under vacuum at 45 ℃ and freeze-dried (Four-Ring Science Instrument Plant Beijing Co., Ltd, China), about 1.552 g of EE was obtained.

2.3. HPLC-PDA-ESI/MS

10 mg of the obtained above powder was dissolved and diluted to 100 mL with methanol. After filtered through 0.45 μm millipore filter, a 10 μL volume of the solution was injected for HPLC-PDA-ESI/MS analysis.

HPLC-PDA-ESI/MS analysis was performed using an LCQ Deca XP MAX system (Finnigan, USA). Separation was performed on an Eclipse×DB-C18 column (150 × 4.6 mm i.d.; 3.5 μm particle size). The mobile phase consisted of methanol and phosphate buffer of pH 4.5 (6:4). The flow rate was 0.45 mL/min. The first detection was by Photodiode Array Detector (PDA), and the second detection employed an Electrospray Ionization Mass Spectrometry (ESI-MS). Due to the acidic nature of flavonoids, the mass analyzer was set to record the negative spectra. And the scan range was set from 50 to 800 m/z.

2.4. Determination of the Content of Rutin in EE by HPLC

30 mg of EE was dissolved in methanol and diluted to 100 mL with methanol. After filtered through 0.45 μm millipore filter, a 10 μL volume of the solution was injected for HPLC analysis.

The sample was separated on a reversed phase column, SunFire^{TM} C18 column (4.6 × 250 mm; 5 μm particle size) made by Waters. The mobile phase consisted of water and methanol (8:2) with a flow rate of 1.0 mL/min. The column temperature was set at 30 ℃. The HPLC analysis was performed on a Waters Alliance HPLC system, which consisted of a Waters 2695 separations module and a Waters 2487 dual wavelength detector. The injection volume was 10 μL and the wavelengths for detection were set at 265 nm. The quantitative analysis of rutin in the sample was based on an external standard. The chromatographic data were recorded and processed by Empower 2 software.

2.5. Cell Culture and Drug Treatment

The human Hep G2 cells were cultured in DMEM medium with 10 % FBS, 100 UI/mL penicillin and 100 μg/mL streptomycin in humidified air at 37 °C with 5 % CO_2, respectively. The exponentially growing cells were collected and re-suspended in fresh medium for 4 h and then exposed to various concentrations of EE.

2.6. MTT Assay

Survival of cells was evaluated by using a system based on MTT, which was reduced by living cells to yield a soluble formazan product that could be detected colorimetrically. Cells were suspended in 96-well plates of 100 μL medium at a density of 2×10^4 cells/well and rutin at different concentrations. These were then incubated in humidified air at 37 ℃ with 5 % CO_2 for 48 h, exposed to 100 μL MTT (0.1 %, W/V) and incubated for another 4 h under the conditions mentioned above. The formazan precipitate was dissolved in 150 μL DMSO. IC_{50} values were tested through the MTT method [4]. The inhibition rate was calculated as follows:

Inhibition Rate % = (mean control absorbance-mean experimental absorbance)/mean control absorbance×100%.

2.7. Flow Cytometry Analysis

The flow cyctometry analysis was performed on an EPICS XL flow cytometer (Coulter, Australia). Cell pellets were fixed in 70 % ethanol at -20 ℃ for at least 12 h or overnight. After being washed twice with ice-cold PBS, they were incubated in RNase A/PBS (1 mg/mL) at 37 ℃ for 30 min, and stained with PI (0.5 mg/mL) at room temperature for 15 min. The intracellular DNA was then labeled with PI and the PI fluorescence of individual nuclei determined by the fluorescence-activated cell sorter at 488 nm excitation. Surface exposure of phosphatidylserine in apoptotic cells was measured by the annexin V-FITC apoptosis detection kit according to the manufacturer's instructions.

2.8. Statistical analysis

The data obtained in this study were expressed as the mean of three replicate determinations and standard deviation (SD). Statistical comparisons were carried out using student t test. P values of < 0.05 were considered to be significant.

3. Results And Discussion

3.1. Characterization of EE

By using HPLC-PDA-ESI/MS, the compound 1 eluted at 5.93 min (Figure 1) was identified as rutin. The UV and ESI-MS data (Figure 2) were following: UV, λ_{max}(nm) 255, 356; ESI-MS negative ion m/z: 609.08 ([M-H]-), 301.04 ([M-rutinose]$^-$), which coincided with our previous study [5]. And the content of rutin in EE was determined as 27.40±0.16 % by HPLC.

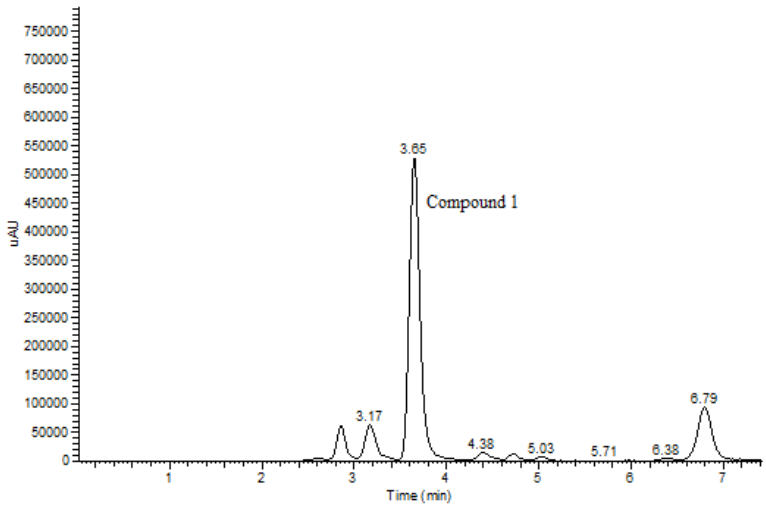

Fig. 1 HPLC-PDA profile of EE

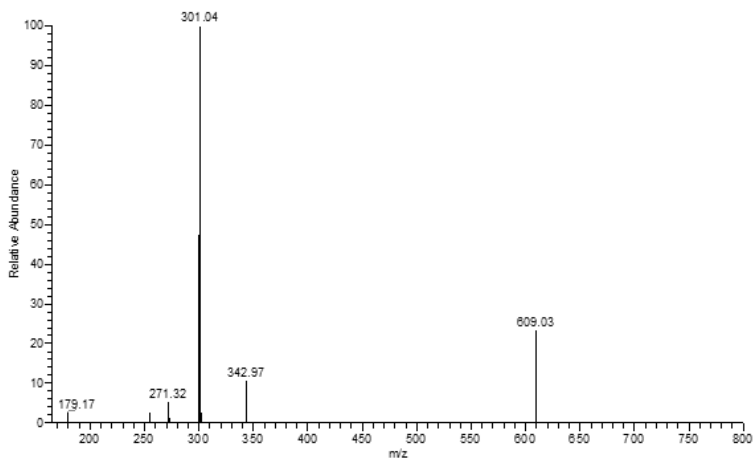

Fig. 2 ESI-MS spectrum of compound 1

3.2. Anti-proliferation of EE on Hep G2 cells

The inhibitory effects of EE on the proliferation of Hep G2 cells were tested at different concentration for 48 h and the IR % was determined (Figure 3). It induced a dose-dependent inhibitory effect. The inhibitory concentration 50% (IC_{50}) was about 27.1 μg/mL.

3.3. Flow cytometry analysis of cell cycle and apoptosis

Apoptosis is a highly regulated cell death process with characteristic biochemical features [6-7] and membrane-bond apoptotic bodies [8]. It occurs both during normal development and under certain pathological conditions in metazoans, and plays a crucial role in the maintenance of tissue homeostasis by the selective elimination of excessive cells [9]. The hallmark of early apoptotic cells is the transverse

redistribution of plasma membrane phosphatidylserine (PS) [10]; thus, the annexin V binding assay was performed to detect the surface exposure of PS. In this study, the Hep G2 cells were treated with EE (20 μg/mL) for 48 h. Figure 4 showed the FACS histogram with dual parameters including annexin V-FITC and PI. The dual parametric dot plots combining annexin V-FITC and PI fluorescence showed the viable cell population in the lower left quadrant (annexin V-negative/PI-negative), the early apoptotic cells in the lower right quadrant (annexin V-positive/PI-negative), and the late apoptotic cells in the upper right quadrant (annexin V-positive/ PI-positive).

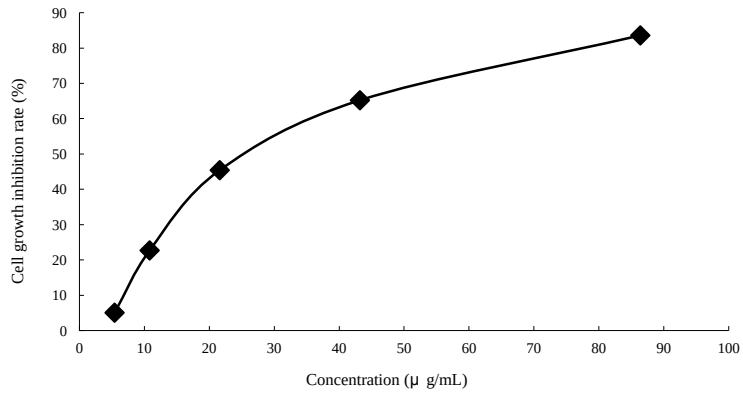

Fig. 3 Effect of EE on Hep G2 cell proliferation

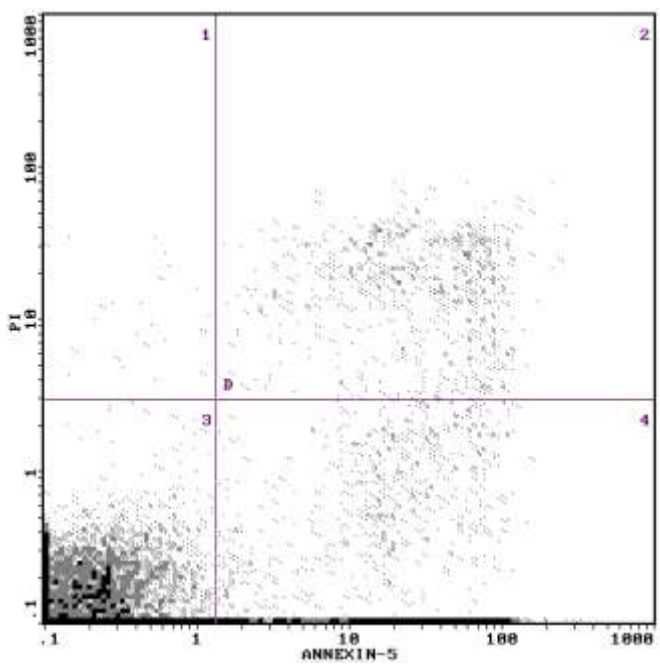

Fig. 4 Flow cytometric analysis of phosphatidylserine externalization (annexin V binding) and cell membrane integrity (PI staining) in the treated Hep G2 cells by EE

In untreated Hep G2 cells, 9.88 % of cells were the early apoptotic cells, 3.62 % of cells were the late apoptotic cells. The early and late apoptotic cells increased to 15.9 % and 6.96 %, respectively, after being treated with EE at a concentration of 20 μg/mL for 48 h. The result suggested that EE could induce cells into an apoptotic pathway resulting in the inhibition of proliferation of Hep G2 cells. Our efforts are underway to isolate and identify the main proliferative molecules in EE and their health promoting potential and mammalian safety.

4. Conclusion

In this study, it was found that the ethanol extract from tartary buckwheat could inhibit the proliferation of Hep G2 cells in a dose-dependent manner by inducing cells to enter into apoptosis. These results demonstrated the antioxidant and anti-proliferative potency of the ethanol extract which could be the basis for its alleged health promoting potential of tartary buckwheat. Tartary buckwheat could serve as a source of antioxidants or nutraceuticals with potential applications.

Acknowledgements

The financial support provided by Foundation of He'nan Educational Committee, No. 2009A550005 and the Science and Technology Plan of Henan institute of science technology (No.07040) was greatly appreciated.

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