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Major triterpenoids in Chinese hawthorn "*Crataegus pinnatifida*" and their effects on cell proliferation and apoptosis induction in MDA-MB-231 cancer cells

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ABSTRACT

The cytotoxicity and antiproliferative effect of phytochemicals presenting in the fruits of Chinese hawthorn (*Crataegus pinnatifida*) were evaluated. Shanlihong (*Crataegus pinnatifida* Bge. var. major N.E.Br.) variety possessed significant levels of flavonoids and triterpenoids, and showed potent antiproliferative effect against $HepG_2$, MCF-7 and MDA-MB- 231 human cancer cells lines. Triterpenoids-enriched fraction (S9) prepared by Semi-preparative HPLC, and its predominant ingredient ursolic acid (UA) demonstrated remarkably antiproliferative activities for all the tested cancer cell lines. DNA flow cytometric analysis showed that S9 fraction and UA significantly induced G1 arrest in MDA-MB-231 cells in a dose-dependent manner. Western blotting analysis revealed that S9 fraction and UA significantly induced PCNA, CDK4, and Cyclin D1 downregulation in MDA-MB-231 cells, followed by $p21^{Waf1/Cip1}$ up-regulation. Additionally, flow cytometer and DNA ladder assays indicated that S9 fraction and UA significantly induced MDA-MB-231 cells apoptosis. Mitochondrial death pathway was involved in this apoptosis as significantly induced caspase-9 and caspase-3 activation. These results suggested that triterpenoids-enriched fraction and UA exhibited antiproliferative activity through the cell cycle arrest and apoptosis induction, and was majorly responsible for the potent anticancer activity of Chinese hawthorn.

1. Introduction

It has been reported that cancer is the leading cause of death in the developed world and stands second most lethal disease in the developing countries (Jemal et al., 2011; Park et al., 2008). Moreover, drugs used in the treatment of cancer are highly expensive and also cause various side effects on the tissues of patients. Thus, the development of effective anticancer agents with least side effects on living system and strategies on the prevention of cancer incidence are required immediately (Tariq et al., 2015). More than 50% of all the drugs in modern therapeutics are based on natural products and their derivatives including various phytochemicals from herbal medicines (Pan et al., 2013; Schmidt et al., 2008). It is evident from various pharmacological studies that healthier dietary pattern (Jemal et al., 2011), such as regular consumption of fruits, vegetables, and whole grains had negative correlation with the risk of chronic diseases, including different types of cancer (Liu, 2013; Tariq et al., 2015). It has been recommended

Abbreviations: CC_{50}, half maximal cytotoxicity concentration; EC_{50}, half maximal effect concentration; UA, ursolic acid; CA, corosolic acid; OA, oleanolic acid; MA, maslinic acid; CDK4, cyclin-dependent kinase 4; PCNA, proliferating cell nuclear antigen.

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that consumers intake a variety of vegetables and fruits, especially whole fruits (USDA, 2015). This diet is a potential source of health beneficial nutrients and bioactive phytochemicals. Phytochemicals such as phenolics are an important class of compounds that act as effective antioxidants, which has been proven beneficial in cancer prevention (Chaudhary et al., 2015; Vanden Berghe, 2012). Triterpenoids, such as oleanolic acid, ursolic acid, 2α-hydroxyursolic acid, the main phytochemicals dominated in the fruits of *Rosaceae* family, play an important role in health benefits, including antioxidant and anticancer activities (He and Liu, 2007; Krukiewicz et al., 2015).

Chinese hawthorn (*Crataegus pinnatifida*) belongs to *Rosaceae* family, is a medicinal food product native to Northern China, and also widely distributes in Japan, South Korea, Europe and North America (Kwok et al., 2013). In the recent years, Chinese hawthorn has attracted more attention in the field of functional foods, nutritional supplements and medicines because of its various pharmacological effects on digestive, cardiovascular, and endocrine systems (Li et al., 2013b; Zhu et al., 2013). Fruits and leaves of hawthorn contain a variety of phytochemicals including phenolics compounds which appear to be primarily responsible for the wide health benefits (Wu et al., 2014), such as antioxidant (Cui et al., 2006b), neuroprotective (Chang et al., 2013), anticancer (Kao et al., 2007; Li et al., 2013a), antiviral, anti-inflammatory and antimicrobial activities (Tadic et al., 2008). Triterpenoids presenting in the fruits of hawthorn showed considerable antitumor activity, and may be responsible for the anticancer activity of hawthorn fruits (Qiao et al., 2015). However, previous study also showed that the main constituents, such as epicatechin, hyperoside, chlorogenic acid, and isoquercitrin, were most likely responsible for the active antiproliferative activity of hawthorn fruits extract (Li et al., 2013a).

Natural antioxidants have been proven to be beneficial in cancer prevention. In our previous study, Chinese hawthorn showed remarkable cellular antioxidant activity (Wen et al., 2015a), and was suggested as a promising source of healthy food for cancer prevention. However, the constituents possessing predominant antiproliferative activity of Chinese hawthorn fruits were remained unknown. Thereby, present study was aimed to elucidate the major phytochemicals presenting in different varieties of hawthorn, which have potent anticancer activities, and to analyze the possible mechanism of their antiproliferative effect.

2. Materials and methods

2.1. Chemicals

Gallic acid, Folin-Ciocalteu reagent, sodium borohydride ($NaBH_4$), aluminum chloride, chloranil, catechin hydrate, vanillin, procyanidine B_2, epicatechin, hyperoside, isoquercitrin, chlorogenic acid, ursolic acid (UA), corosolic acid (CA), oleanolic acid (OA), and maslinic acid (MA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). WME medium, DMEM medium, heparin, insulin, and other cell culture reagents were purchased from Gibco U.S. Biotechnology Co. (Grand Island, NY, USA). An annexin V-FITC apoptosis detection kit, RNase-A and propidium iodide were obtained from eBioscience, Inc. (San Diego, CA, USA). The pan-caspase inhibitor (Z-VAD-FMK) was purchased from Target Molecule Corp. (Boston, MA, USA). Methanol, ethyl alcohol (EtOH), acetone, acetic acid, tetrahydrofuran (THF), hydrochloric acid (HCl) were obtained from Guangzhou Reagent Co. (Guangzhou, China). All reagents used were of analytical grade. Additionally, HPLC-grade acetonitrile and methanol were obtained from CNW Technologies Gmbh (Dusseldorf, Germany).

The primary antibody against cyclin-dependent kinase 4 (CDK4), caspase-3, caspase-9, β-actin and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc., (Boston, MA, USA). The rabbit monoclonal anti-proliferating cell nuclear antigen (PCNA), *anti*-cyclin D1, and *anti*-$p21^{Waf1/Cip1}$ antibody were obtained from Abcam plc., (Cambs, UK). Kit for determination of bicinchoninic acid (BCA), Allergic ECL Chemiluminescence kit (BeyoECL Plus), and DNA marker used in DNA ladder analysis were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

2.2. Sample preparation and extractions

Fresh fruits of Chinese hawthorn varieties including Shanlihong (*Crataegus pinnatifida* Bge. var. major N.E.Br.), Shanzha (*Crataegus pinnatifida* Bge.), and Dajinxing (*Crataegus pinnatifida* Bge var. major N.E.Br.) were obtained from Shandong Institute of Pomology, Tai'an, Shandong, China. The fruits were cleaned with distilled water and the pulp were separated from the seeds using a knife to remove the seeds. Chemical extraction was carried out following the method of Wen et al. (2015a) with some modifications. Fresh seedless fruit pulp was cut into small pieces. Then 25 g of sample was blended with 150 mL of chilled (~4 °C) 80% acetone solution (1:6, w/v) using a Waring blender (DS-1, Shanghai, China) for 5 min, and then homogenized (12,000 rpm, 4 min) (T25, IKA Co., Staufen, Germany). After centrifugation (3500*g*, 10 min, 10 °C, Allegra X-15R, Beckman Coulter Co., Ltd., Le Brea, CA, USA), the supernatant was collected. Subsequently, the remaining residue was extracted twice by homogenizing, then centrifuged as above. The supernatants were combined and concentrated to dryness using a water generated vacuum rotary evaporator (Hei-VAP, Heidolph, Germany) at 45 °C. The extracts were reconstituted in 90% EtOH solution to a final volume of 25 mL and stored at −40 °C until use.

2.3. Determination of total phenolics and flavonoids contents

The total phenolics content was determined using colorimetric Folin-Ciocalteu method with some modifications (Wen et al., 2015a). Gallic acid was used as the standard and final concentration was expressed as milligram gallic acid equivalents per 100 g on dry weight basis (mg GAE/100 g fruit, DW). Additionally, the total flavonoids content was estimated as catechin equivalents through the sodium borohydride/chloranil protocol (Wen et al., 2015a). Measured values of total flavonoid contents were expressed as mg of catechin equivalents per 100 g on dry weight basis, (mg CE/100 g fruit, DW).

2.4. Identification and quantification of bioactive compounds

Major bioactive compounds were identified and quantified by reverse phase HPLC method. The analysis was carried out using a Sunfire C18 reversed phase column (4.6 × 250 mm, 5 μm of particle size, Waters Co., Milford, MA, USA) on Waters breeze (Waters, USA) HPLC system equipped with a binary HPLC pump (Waters 1525), an auto-sampler (Waters 2707), and a Photodiode Array detector (PAD) (Waters 2998). A gradient elution system, consisting of solvent A (0.1% trifluoroacetic acid, v/v) and solvent B (acetonitrile: methanol = 80:20, v/v), was employed, and the flow rate was 1.0 mL/min. The gradient elution program was as followed: 0–8 min with 10–18% solvent B, 8–12 min with 18–20% B, 12–36 min with 20–37% B, 36–40 min with 37–80% B, 40–45 min with 80–88% B, 45–56 min with 88–90% B, 56–60 min with 90–100% B, 3 min with 100% B, 63–67 min with 100–10% B, and 4 min with10% B. The sample injection volume was 10 μL at a column temperature of 35 °C. Phenolic compounds were identified from the comparison with the retention time of standard using PDA detection at 217, 280 and 340 nm, respectively (Cui et al., 2006a;