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Antioxidant and anti-proliferative activity of *Rhizoma Smilacis Chinae* extracts and main constituents

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ABSTRACT

Rhizoma Smilacis Chinae (RSC) is a widely used herbal material in functional food and folk medicine. In this study, methanol extract (ME), water extract (WE), polysaccharide fraction and ethyl acetate fraction (EF) of RSC were prepared and the constituents were analysed by HPLC. Different antioxidant tests were employed to evaluate the antioxidant activities of RSC extracts and its main constituents, astilbin and chlorogenic acid. The results showed that RSC extracts possessed comparable antioxidant activity to butylated hydroxyanisole in a dose-dependent manner. The radical-scavenging capacity of ME and EF was even stronger than astilbin and chlorogenic acid. The EF and ME of RSC also showed stronger anti-proliferative activity on HepG2 cells than astilbin and chlorogenic acid, with IC_{50} values of 47 and 32 μg/mL for 24 h treatment, respectively. Flow cytometric analysis revealed that RSC extracts induced cell cycle arrest at G2/M phase and a late apoptosis of the cells.

1. Introduction

Plant-based foods contain significant amounts of phytochemicals, such as flavonoids, stilbenoids and phenolic acids. Epidemiological studies have demonstrated a positive correlation between the intake of edible plants (e.g., vegetables and fruit) and prevention of diseases like atherosclerosis, cancer and also ageing (Kaur & Kapoor, 2001). This association may be attributed to the antioxidant activity of natural antioxidants, which scavenge free radicals, thus saving the cell from different diseases. Hepatocellular carcinoma (HCC) is a global health problem today. The high occurrence and death rate of HCC require the continuous search of chemotherapeutic/chemopreventive agents from dietary sources. Human hepatoma HepG2 is a widely-used experimental model for chemopreventive agents screening *in vitro*. The cell line is easy to culture, is well-characterised, and closely resembles the human hepatocyte. Chu, Sun, Wu, and Liu (2002) investigated the anti-proliferative activity of common vegetables and fruits (Sun, Chu, Wu, & Liu, 2002) on HepG2. The cytotoxic and apoptotic effects of many herbal extracts on HepG2 cells have also been reported, such as *Smilax glabra* Roxb. (Sa, Gao, Fung, Zheng, Lee, & Wang, 2008), *Coix lacryma-jobi* (Lu, Zhang, Jia, Wu, & Lu, 2011), *Solanum nigrum* Linn. (Lin et al., 2007), etc.

Smilacis China L. is a climbing flowering plant that belongs to *Smilacaceae* family, *smilax* genera. Its tender stems are eaten as a vegetable in China, while in Korea its leaves are traditionally used to wrap rice cakes, to supply good flavour and antimicrobial activity (Jeon, Jin, Kim, & Park, 2006). *Rhizoma Smilacis Chinae* (RSC), the rhizome of *Smilacis China* L., also called Ba-Qia in Chinese, is a commonly used herbal material in traditional Chinese medicine for detoxification and relieving dampness (National Commission of Chinese Pharmacopoeia, 2010). It has been used to treat chronic pelvic inflammation, gout, rheumatism and skin problems. Many bioactive components, which may be responsible for these pharmacological effects, have been isolated from RSC. These included resveratrol, oxyresveratrol, astilbin, engeletin and steroidal saponins (Huang, Liu, & Shao, 2009; Shao et al., 2007a).

Pharmacological studies have revealed that the extracts of RSC have free-radical-scavenging and antioxidant-enzyme-fortifying activities on Chinese hamster lung fibroblast V79-4 cells (Lee, Ju, & Kim, 2001). The methanol extract of RSC could markedly inhibit neuronal cell damage in cultured rat cortical neurons (Ban, Cho, Koh, Song, Bae, & Seong, 2006). Further study showed that RSC has neuroprotective effect against focal cerebral ischaemic injury on rat, and the activity may be attributable to its components of oxyresveratrol and resveratrol (Ban et al., 2008). The aqueous extract of RSC had anti-nociceptive and anti-inflammatory effects on rat (Shu, Gao, & Yang, 2006). Steroidal saponins and sieboldogenin isolated from RSC were thought to be responsible for its anti-inflammatory activity (Shao, Guo, Cui, Ye, Han, & Guo, 2007b). Wu, Wang, Wang, Yang, Jia, and Ding (2010) isolated six polyphenols from RSC with cytotoxicity to breast tumour cell. Kaempferol 7-*O*-β-D-glucoside (KG), a flavonoid glycoside isolated from RSC could

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induce G2/M phase arrest and apoptosis on HeLa cells (Xu, Liu, Li, Wu, & Liu, 2008).

Lee et al. (2001) investigated the antioxidant activity of RSC extracts, using just the 1,1-diphenyl-2-picrylhydrazil (DPPH) radical-scavenging test. Furthermore, the correlation between the activity and its components was unclear. Although some phytochemicals with anti-tumour activity have been isolated from RSC, the studies are focused on the activity of individual components. There are still no studies on the anti-proliferative activity of different RSC extracts and their main constituents on HepG2 cell. Thus, in the present study, different extracts of RSC were prepared and the constituents were analysed by HPLC. Three antioxidant tests were employed to evaluate the antioxidant activities of RSC extracts and main constituents, astilbin and chlorogenic acid. Their antioxidant properties were compared with commonly-used synthetic antioxidant, butylated hydroxyanisole (BHA). Furthermore, the anti-proliferative properties of RSC extracts and main constituents on HepG2 cell were studied. The correlations between these activities of RSC and its components were discussed.

2. Materials and methods

2.1. Chemicals and materials

BHA, resveratrol(>99%), chlorogenic acid (>99%), DPPH, trichloroacetic acid, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), ammonium thiocyanate, potassium persulfate, ferrous chloride and ferric chloride were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC-grade acetonitrile was purchased from RCI Labscan Ltd. (Bangkok, Thailand). HPLC-grade acetic acid was purchased from International Laboratory (San Bruno, CA). Astilbin (>98%) and engeletin (>98%) were isolated from *Rhizoma Smilacis Glabrae* in our lab, and were characterised by UV, FTIR, MS and NMR. Throughout the study Milli-Q deionised water was used. It was prepared by a Milli-Q water system (Millipore, Billerica, MA,). All other chemicals used were analytical grade.

2.2. Plant materials and solvent extraction

The rhizome of *Smilacis Chinae L* (RSC) was collected from Fujian province, Ninghua county on Oct. 2009, and was authenticated by Dr. Zhi-Feng Zhang (College of Chemistry & Environment Protection Engineer, Southwest University for Nationalities, China). The rhizome was sliced into pieces and dried by sunlight. Dried RSC was finely homogenised and filtered through 40 mesh. Its HPLC chemical profile (qualitative analysis) was the same as a reference sample (lot No. 121466–200702) from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

To prepare water extract (WE), 5 g of sample mixed with 100 mL water was heated to boiling and refluxed for 1 h. Then, the mixture was centrifuged at 5000 rpm and 4 °C for 10 min. The supernatant was lyophilised and weighed after concentration by vacuum rotary evaporation at 60 °C.

To obtain the polysaccharide fraction, 5 g of sample mixed with 100 mL water was heated to boiling and refluxed for 1 h. Then, the mixture was centrifuged at 5000 rpm and 4 °C for 10 min. The supernatant was precipitated with four volumes of ethanol and left for 2 h. Then, the mixture was centrifuged at 5000 rpm and 4 °C for 10 min. The precipitate was lyophilised and weighed.

For methanol extract (ME), 5.0 g of sample mixed with 100 mL methanol was sonicated for 30 min. After filtration, methanol was removed by vacuum rotary evaporation at 50 °C. The residue in the flask was transferred using 10 mL boiling water and then lyophilised and weighed.

For ethyl acetate fraction (EF), 5.0 g of sample mixed with 100 ml methanol was sonicated for 30 min. After filtration, methanol was removed by vacuum rotary evaporation at 50 °C. The residue dissolved in 10 mL water was extracted three times with ethyl acetate (15, 15, 15 mL). The ethyl acetate was removed by vacuum rotary evaporation at 50 °C. The residue in the flask was transferred by 10 mL boiling water and then lyophilised and weighed. All extracts were stored at −20 °C until use.

2.3. HPLC analysis

HPLC analyses were performed on a Waters 600 HPLC system together with a photodiode array detector (DAD) (Waters Corporation, Milford, MA). An Agilent Zorbax SB C18 column (250 mm × 4.6 mm i.d., 5 μm; Agilent, Santa Clara, CA) was used. The mobile phase was acetonitrile (**A**) and 0.1% acetic acid aqueous solution (**B**) with a gradient program as follows: 0–15 min, 16–20% **A**; 15–40 min, 20–40% **A**. The flow-rate was 1 mL/min. Monitoring was performed at 290 nm, and injection volume was 10 μL.

2.4. Scavenging activity of DPPH radical

The DPPH radical-scavenging activity was assayed by the method described by Chu, Chang, and Hus (2000), with some minor modifications. Briefly, an aliquot of 200 μL of 0.12 mM DPPH radical solution dissolved in ethanol was mixed with 50 μL of extracts at various concentrations or water as negative control in a 96-well plate. The absorbance of the reaction mixture was measured at 517 nm 10 min later. The DPPH radical-scavenging activity (%) was calculated by the following equation:

$$\text{Scavenging activity } (\%) = (1 - A_{\text{sample}}/A_{\text{control}}) \times 100,$$

where A_{sample} is the absorbance in the presence of extracts and A_{control} is the absorbance of the control.

2.5. Antioxidant activity by the $ABTS^{\bullet+}$ assay

ABTS radical cation ($ABTS^{\bullet+}$) scavenging of the extracts was determined by the method of Siddhuraju and Becker (2007), with some minor modifications. Briefly, $ABTS^{\cdot+}$ was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate (final concentration). The mixture was left to stand in the dark at room temperature for 12–16 h before use. Prior to assay, the solution was 70 times diluted in water to give an absorbance of 0.75 at 734 nm for a volume of 200 μL in a 96-well plate. An aliquot of 200 μL of the diluted $ABTS^{\cdot+}$ solution was mixed with 25 μL of extracts at various concentrations or negative control (water). After 5 min at room temperature, the mixture was monitored at 734 nm. The radical-scavenging activity was calculated by the following formula:

$$\text{Scavenging activity } (\%) = (1 - A_{\text{sample}}/A_{\text{control}}) \times 100,$$

where A_{sample} is the absorbance in the presence of extracts and A_{control} is the absorbance of the control.

2.6. Reducing power

The reducing power of extracts was determined by the method of Gulcin, Oktay, Kirecci, and Kufrevioglu (2003). Briefly, an aliquot of 0.2 mL of extracts at various concentrations was mixed with phosphate buffer (0.5 mL, 0.2 M, pH 6.6) and potassium ferricya-