Antioxidant Activity of Moutan Cortex Extracts by Multiple-Stage Extraction

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Abstract

Moutan cortex, a widely used traditional Chinese medicine for the treatment of various diseases, is the root bark of *Peaonia suffruticosa* Andrews (Paeoniaceae). In this study, the crude polysaccharides (CPS), paeonol and the crude total glycosides (CTG) of moutan cortex were extracted by the multiple-stage extraction. CPS was extracted by water, paeonol was distilled by steam distillation method, and CTG was extracted with ethanol after above two stages. The antioxidant activity of the extracts was evaluated with superoxide dismutase (SOD) and DPPH (1, 1-diphenyl-2-picryhydrazyl) radical scavenging activity assays. Among the three extracts, CTG exhibited the strongest SOD-like activity (510 U/mg) and DPPH radical-scavenging activity (EC50, 14.4 μg/ml), CPS had a weak SOD-like activity and DPPH radical-scavenging activity. Paeonol, however, was inactive. The different biological activities among the three extracts may be attributed to differences in their chemical composition, partially supported by polysaccharides, and polyphenolic contents.

Keywords: Moutan cortex, Multiple-stage extraction, Antioxidant activity, Polyphenolic content

1. Introduction

Moutan cortex, the root bark of *Paeonia suffruticosa* Andrews (Paeoniaceae), has been used extensively as a traditional Chinese medicine (TCM) for treating various diseases in eastern Asian countries. Most of the pharmacological investigations of moutan cortex have been addressed to its central nervous system activities,

anti-oxidative and sedative actions (Ma *et al.*, 1984; Li *et al.*, 1997; Rho *et al.*, 2005; Yoshikawa *et al.*, 1992). Paeonol (2-hydroxy-4-methoxyacetophenone), a major active component isolated from moutan cortex, possesses extensive pharmacological activities such as antioxidation, antiinflammation, and immunoregulation (Riley and Ren, 1989; Sun *et al.*, 2004). CPS is a heterosaecharide consisting of rhamnose，arabinose，xylose，mannose，glucose，galactose, etc, whose mean molecular weight was estimated to be 1.28×10^5. Studies indicated that CPS can reduce hyperglycemia caused by glucose and alloxan in mice and rats，and increase the SOD level in diabetes mellitus rats (Chen *et al.*, 2004; Liu *et al.*, 2002; Yang *et al.*, 2006; Hong *et al.*, 2003). CTG exhibits anti-inflammatory activity, immunomodulatory action and protective effect on liver injury (Tang *et al.*, 1999; Mei *et al.*, 1999a; Mei *et al.*, 1999b).

Reactive oxygen species (ROSs) exist in various forms, including free radicals such as superoxide ions, hydroxyl radicals, and peroxyl as well as non-free-radical species such as hydrogen peroxide (Squadriato and Pryor, 1998; Waris and Ahsan, 2006). These ROSs play important roles in degenerative or pathological processes in conditions such as aging, cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataract formation, and inflammation (Burns *et al.*, 2001; Ames, 1983; Gey, 1990; Diaz *et al.*, 1997; Aruoma, 1998). Oxidative stress occurs during an imbalance between ROSs and antioxidants. Excessive production of ROSs may lead to oxidative damage to DNA, proteins, and other macromolecules resulting in their accumulation with age (Fraga *et al.*, 1990; Harman, 1981).

Crude extracts of herbs rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Javanraedi *et al.*, 2003). Moutan cortex is in abundance in China, majority of which hasn't been made good use of except of export. On the other hand, after the extraction of paeonol, most of the material were disused. Along with the progressiveness of the study on moutan cortex, it has been essential to study the extraction of other active components in moutan cortex. In addition, no reports are available on the difference of the antioxidant activity of extracts from moutan cortex; therefore, in this experiment, the crude polysaccharides, paeonol and total glycosides of moutan cortex were extracted with the multiple-stage extraction, and evaluated in antioxidant activity through various in vitro assays.

2. Materials and methods

2.1 Materials

Moutan cortex was purchased from Luoyang Huayi Biotechnology Co. (Henan, China). Folin-Ciocalteu reagent and Gallic acid was purchased from Sigma Co. (St. Louis, MO, USA). SOD Assay Kit-WST was purchased from Dojindo Molecular Technologies, Inc. (Japan). DPPH (1,1-diphenyl-2-picryhydrazyl) and Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97%) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.2 Multiple-stage extraction

All dried materials of moutan cortex were ground in a mill and the powder was used in the following extraction preparation.

2.2.1 Preparation of crude soluble polysaccharide (CPS)

CPS was extracted according to the procedure reported by Zhang *et al.* (1995) with some modifications. The crushed powder of moutan cortex (50 g) was extracted with 500 ml of distilled water at room temperature (25°C) for 24 h. The extract was then centrifuged at 5000 rpm for 15 min and filtered through Whatman no.4 filter paper; the filtrate (400 ml) was then concentrated to 100 ml in an evaporator at 40°C, and mixed with 99% ethanol (1:4, v/v) overnight. The mixture was centrifuged at 5000 rpm for 15 min, then the precipitated fraction was dissolved with 50 ml of distilled water and filtered again, the filtrate (50 ml) was freeze-dried in a vacuum.

2.2.2 Preparation of paeonol

The residual fraction of above was dissolved in 400 ml of distilled water and the paeonol was extracted by steam distillation. The distillate was collected and placed in 4°C overnight, and the acicular crystal was dried in silica gel desiccator.

2.2.3 Preparation of crude total glycosides (CTG)

CTG was extracted according to the reported method with some modifications (Liao *et al.*, 2007). The residual fraction of extraction of paeonol was mixed with 400 ml of 99% ethanol (80%, final concentration), and extracted for 2 h by refluxing extraction. The extract was filtered through Whatman no.4 filter paper; the filtrate (400 ml) was then evaporated and lyophilized.

2.3 Determination of polysaccharide

The polysaccharide content was quantified with a modified phenol-sulfuric acid method according to Dubois *et al*. (1956). The extracted crude polysaccharide was dissolved in distilled water and used for polysaccharide analysis. The color reaction was initiated by mixing 1 ml of polysaccharide solution with 0.5 ml of 5% phenol solution and 2.5 ml of concentrated sulfuric acid, and the reaction mixture was kept in a 100°C water bath for 15 min. After cooling to room temperature, the optical density (OD) of the mixture was determined at 490 nm and the polysaccharide content was calculated with D-glucose as the standard. The results were expressed as μg of glucose equivalent per mg of extract.

2.4 Determination of SOD-like activity

The levels of SOD-like activity in the extracts were measured using the SOD Assay Kit-WST according to the technical manual provided by Dojindo Molecular Technologies, Inc. This assay relies on WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon reduction with O_2^-, a reaction inhibited by SOD. Briefly, in a 96-well microplate, 20 μl of sample solution (Sample well and Blank2 well) or double distilled water (Blank1 and Blank3) was mixed with 200 μl of WST working solution. For Blank2 and Blank3, 20 μl of dilution buffer was added. Then, 20 μl of enzyme working solution was added to each Sample well and Blank1 well. The plate was incubated at 37°C for 20 min and the OD was determined at 450 nm using a microplate reader (BIO-RAD Model 550, USA). SOD-like activity (inhibition rate, %) was calculated by the following equation:

SOD-like activity (inhibition rate, %)

$$= \{[(A_{Blank1}-A_{Blank3})-(A_{Sample}-A_{Blank2})]/(A_{Blank1}-A_{Blank3})\} \times 100$$

where A_{Blank1}, A_{Blank2}, A_{Blank3}, and A_{Sample} were the absorbances of Blank1, Blank2, Blank3 and Sample wells, respectively. One unit of SOD activity was defined as the amount of enzyme having a 50% inhibitory effect on WST-1.

2.5 Measurement of DPPH radical-scavenging activity

DPPH radical-scavenging activity was measured according to the procedure reported by Nakajima *et al*.(2007). The OD was measured at 570 nm with a microplate reader and DPPH radical-scavenging activity was assessed by the following equation:

DPPH-scavenging activity (%) = (1-A_{Sample}/$A_{Control}$) ×100

where A_{Sample} and $A_{Control}$ were the absorbances of the sample (tested extract) and control, respectively.

In this study, Trolox was used as the standard, and scavenging activity of the sample was expressed as a 50% effective concentration (EC50), which represented the sample concentration (μg/ml) inhibiting 50% of the DPPH radical activity.

2.6 Determination of total phenolic content

The total phenolic content of the extracts was estimated according to the Folin–Ciocalteu colorimetric method with some modifications (Singleton V. L. and Rossi J. A., 1965). A sample (0.5 ml) was mixed with 0.5 ml of the Folin–Ciocalteu reagent (Sigma, Saint Louis, MO, USA). Three minutes later, 0.5 ml Na_2CO_3 (20%) was added, and the mixture was made up to 5 ml with distilled water. After being kept in the dark for 90 min, the OD of the mixture was read at 725 nm. The quantification was determined based on a standard curve of gallic acid (25–250 μg/ml) (Sigma). Results were expressed as μg of gallic acid equivalent (GAE) per mg of extract.

2.7 Statistical analysis

All experiments were conducted in triplicate and results were expressed as mean ± SD. A two-tailed Student's t-test was used for the statistical analysis.

3. Results and discussion

3.1 Extraction yield

The average extraction yield of CPS, paeonol, and CTG was 1.42%, 1.26%, and 2.72% respectively (Fig.1) (n=3). Zhang *et al*. reported that the polysaccharide of moutan cortex extracted with hot water is inactive in lower blood sugar (Zhang *et al*., 1995), thus, we extracted CPS at room temperature. To extract paeonol from moutan cortex, the extraction method of steam distillation was considered to be optimum in laboratory (Li *et al*., 2007). CTG was extracted according to the reported method with some modifications (Liao *et al*., 2007). The results of CPS and paeonol were similar to the other reports (Li, 2005; Kang *et al*., 2006), however, the yield of

CTG was lower compared with that of Liu *et al*. (2006), it perhaps resulted from the loss of CTG during the process of extraction of CPS. The three main extracts of moutan cortex were obtained through the multiple-stage extraction, which has the advantages of simple and economic, saved time and greatly improved utilization of raw materials, also can be used in the large-scale preparation of extracts.

3.2 Polysaccharide content of CPS

In this study, the crude polysaccharide of moutan cortex was evaluated. The polysaccharide content of CPS was quantified with a modified phenol-sulfuric acid method (n=3). The glucose equivalent was 283.5 μg/mg of CPS. Zhang *et al*. (1995) reported that the polysaccharide of moutan cortex was homogeneous and a kind of acidic polysaccharide. Pharmacological test verified that it could significantly lower mice blood sugar (Zhang *et al*., 1995).

3.3 SOD-like activity

The levels of SOD-like activity in the extracts were measured using the SOD Assay Kit-WST (Fig.2) (n=3). One unit of SOD activity was defined as the amount of enzyme having a 50% inhibitory effect on WST-1. CTG exhibited the highest SOD-like activity among the three extracts (510 U/mg), and the SOD-like activity of CPS was 77 U/mg. Paeonol showed no activity in SOD assay.

3.4 DPPH radical-scavenging activity

Three extracts were determined in DPPH radical-scavenging activity (Fig.3) (n=3). The different extracts showed variable DPPH radical-scavenging activities. CPS and CTG exerted free radical scavenging effects in a dose-dependent manner. In particular, CTG exhibited a strong DPPH-scavenging activity (EC50, 14.4 μg/ml), and its effect approached that of Trolox used as a reference antioxidant. The EC50 of CPS was 100.6 μg/ml. Paeonol was inactive in DPPH radical-scavenging activity.

3.5 Total polyphenol content

The total polyphenol contents were estimated. As shown in Fig.4, among the three extracts, the highest total polyphenol contents was found in CTG (296.1 mg GAEs/g extract), and that of CPS and paeonol was less (52.9, 31.5 mg GAEs/g extract, respectively).

Superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes (Mates *et al*., 1999). DPPH is stable free radical used for measuring the electron-donating capacity (Jo *et al*., 2003).This study is the first compare to measure the DPPH radical-scavenging activity and SOD-like activity of three extracts from moutan cortex in vitro. As shown in above results, CTG exhibited the strongest SOD-like activity and DPPH free radical-scavenging activity among the three extracts from moutan cortex. It was revealed that moutan cortex can inhibit the production of reactive oxygen species (ROS) (Rho *et al*., 2005) and some paeonol glycosides exhibited radical scavenging effects (Matsuda *et al*., 2001). In the study of Matsuda *et al*. (2001), the isolation of five paeonol glycosides and their radical scavenging effects was reported. Paeonol is a main active compound isolated from moutan cortex, has also been suggested to have properties of scavenging free radicals and antioxidation (Zhang *et al*., 1999). Zhang *et al*.(1999) verified the effectiveness of structure-activity relationship (SAR) and theoretical calculation methods for antioxidants, among five phenolic antioxidants, such as ferulic acid, salvianic acid A, rutin, L-epigallocatechin gallate (main ingredient of green tea polyphenols), and paeonol, the activity of paeonol was the lowest. In this study, however, paeonol showed inactivity in SOD-like activity and DPPH radical-scavenging activity. It was possible that in the present *in vitro* experiment, paeonol appeared to have no ability in antioxidation by the two mechanisms above mentioned. It is needed to be clarified through different methods in the future study.

The polyphenol contents of herbs were suggested to be main antioxidants and have strong free radical scavenging activities (Hollman and Katan, 1999; Robert *et al*., 2001). When considering the correlation between chemical composition and antioxidant activity, the total polyphenol content in CTG and CPS correlate with the discrepancy in antioxidant activity. The abundant polyphenolic components of CTG significantly contributed to the strong antioxidant activity, CPS with small polyphenol contents were low active in the scavenging of DPPH radicals. Matsuda *et al*. (2001) examined the radical scavenging effects of seven paeonol glycosides and four monoterpene glucosides from the methanol-eluted fraction of Chinese moutan cortex, and suggested that the galloyl group was confirmed to be essential for the radical scavenging effect. The results of Liu *et al*. (1997) indicated that the free radical-scavenging activity of polysaccharides was dependant on the ratio of polysaccharide to protein. More specifically, the ratio of bound protein in the polysaccharide–protein complexes was considered essential to the scavenging activity. In their study, lentinan and schizophyllan, which contained

only a trace amount of protein in the polysaccharide samples, demonstrated almost no scavenging activities. In contrast, PSK (a protein-bound polysaccharide) and polysaccharide extracts form *Ganoderma* and *Grifola* which had lower polysaccharide/protein ratios showed the strongest scavenging activities (Liu *et al*., 1997). However, it was not clear whether the mechanism of superoxide and DPPH-radical scavenging by polysaccharide-protein complexes from moutan cortex were similar to that of plant phenolic compounds.

4. Conclusion

The multiple-stage extraction of CPS, paeonol, and CTG, saved time and greatly improved utilization of raw materials, also can become a new selection for the large-scale preparation of moutan cortex extracts. CTG of moutan cortex, the other main contents beside paeonol, include abundant polyphenol antioxidants, and maybe used in antioxidant additive.

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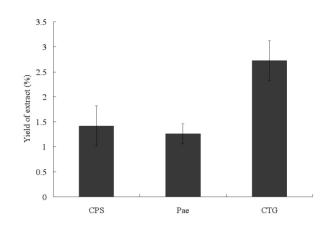
Figure 1. Yields of CPS, paeonol and CTG extracted from moutan cortex

Data are expressed as average percentages of dry weight of moutan cortex (n=3)
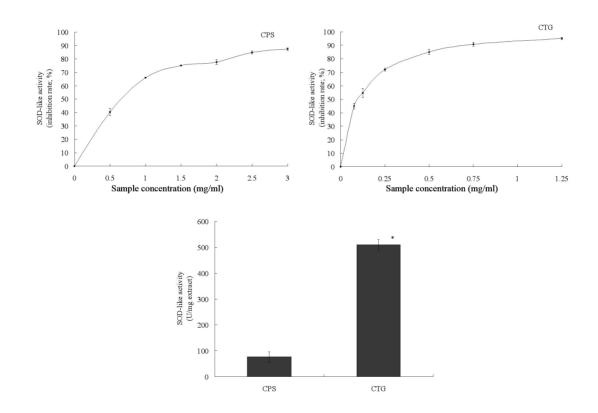

Figure 2. SOD-like activity of CPS and CTG extracted from Moutan Cortex

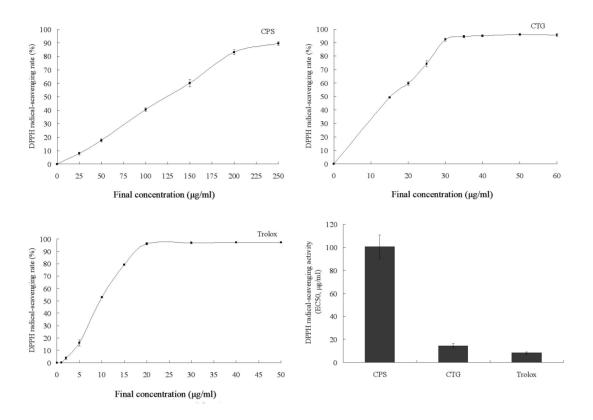Data are expressed as means±SD of three repeat experiments (n=3). (*p<0.01, vs. CPS)

Figure 3. DPPH radical-scavenging activity of the two extracts from Moutan Cortex

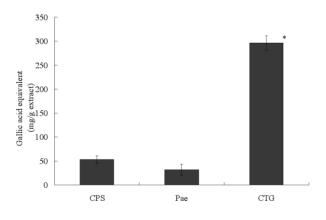Data are expressed as means±SD of three repeat experiments (n=3)

Figure 4. Total polyphenol contents of three extracts from Moutan Cortex

Data are expressed as means±SD of three repeat experiments (n=3). (*p<0.01, vs. CPS)