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Immunomodulatory effects of crude phenylethanoid glycosides from *Ligustrum purpurascens*

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ABSTRACT

Ethnopharmacological relevance: *Ligustrum purpurascens*, named as "Ku ding cha", has been used as a kind of functional tea in southern China for about two thousand years, which has the effects on diuresis, anti-hypertension, weight-loss and anti-inflammation.
The aim of the study: This study was aimed to investigate the immune enhancement effects of the crude phenylethanoid glycosides (CPGs) from *Ligustrum. Purpurascens* on mice and analyze the chemical profiles of phenylethanoid glycosides in the CPGs.
Materials and methods: The immune functions enhancing potential of CPGs was determined using serum hemolysin antibody, phagocytosis, splenocyte antibody production, and NK cells activity assays. The contents of five major constituents in the crude glycosides of *Ligustrum purpurascens* were determined by using liquid chromatography, other five glycosides were deduced according to their UV and MS spectra compared with the literature as well.
Results: In the immunizing experiment, mice treated with different doses of CPGs showed an increase ($p < 0.01$) in the haemagglutination titre compared with the control group. The increases ($p < 0.05$) were found to be significant at doses of 440 mg/kg and 1.32 g/kg in the experiments of antibody production of spleen cells, MΦ phagocytosis of chicken RBCs and NK cell activity. Further chemical characterization yielded 10 constituents from CPGs, five glycosides were quantified by HPLC and the structures of other five compounds were speculated according to their UV and MS spectra.
Conclusion: The results suggested that phenylethanoid glycosides from *Ligustrum purpurascens* have immunomodulatory effects on mice.

1. Introduction

Ku Ding Cha is a folk health beverage in southern China for about two thousand years, which was first documented in the "Shen Nong's Herbal Classic". Its original materials contain 30 species in 12 families, and are mainly yielded by fermentation method (He et al., 2010a). Ku Ding Cha has been used for treating a variety of ailments including inflammation, hepatoprotective, hypertension, and also has the effects of antiobesity, diuresis and antioxidant (Huang et al., 2008; Wong et al., 2001; Lau et al., 2002; Chen et al., 2002; He et al., 2011, 2003). In addition, the research proved that Ku Ding Cha could also enhance physical fitness to resist the diseases (He et al., 2010b). *Ligustrum purpurascens* (Oleaceae) was often used as a kind of famous original plant of Ku Ding Cha. It was reported that *Ligustrum purpurascens* was rich in glycosides (He and Yang, 1989; He et al., 1992).

More and more attention was attracted to the research of immunomodulation to alleviate and cure diseases. Traditional Chinese Medicine (TCM) was reputed to promote physical and mental health, improve defense mechanism of the body and enhance longevity. These attributes were similar to the modern concept of adaptogenic agents, which were known to afford

Abbreviations: TCM, Traditional Chinese Medicine; CPGs, Glycosides from *Ligustrum purpurascens*; HA titre, haemagglutination antibody titre; SRBC, Sheep Red Blood Cell; LDH, lactate dehydrogenase; PI, phagocytic index; PC, conversion value of Percentage of phagocytosis; ESI, electrospray ionization; PFC, Plaque forming cells; MΦ, macrophage; RBC, red blood cell

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protection of the human physiological system against diverse stress (Bhattacharya et al., 2000). A number of medicinal plants have been claimed to possess immunomodulation activity. Some of them were known as immunomodulation agents in China, such as *Rehmannia glutinosa* and *Cistanche deserticola* (Chen et al., 1993; Zhang et al., 2011). In addition, the type of glycosides isolated from *Ligustrum purpurascens* was similar to that of *Rehmannia glutinosa* and *Cistanche deserticola* (Ma et al., 2008; Albach et al., 2007). So the glycosides from *Ligustrum purpurascens* might have the similar immune activity. However, there was paucity of data available on the immune effects of the extract of *Ligustrum purpurascens* in animals. Therefore, the present study was undertaken to evaluate the immunomodulatory effects of crude glycosides of *Ligustrum purpurascens*, and elucidation of its chemical constituents by UV and HPLC-MS^n.

2. Materials and methods

2.1. Medicinal plant and chemical reagents

Ligustrum purpurascens was purchased from Suijiang county, Yunnan province, China, and was authenticated by Peng Hua (The specimen voucher number: NPCR-Lipu-1, deposited with the herbarium of Kunming Institute of Botany, the Chinese Academy of Sciences). *Panax ginseng* was purchased from Eu Yan Sang in Hongkong, and was authenticated by Dong Tingxia (The specimen voucher number: NPCR-Lipu-2, deposited with School of Medicine, Shenzhen University). Methanol and acetonitrile (chromatographic grade) were purchased from Fisher Scientific (USA). Water was ultra pure grade, and all other chemicals used in the study were of analytical grade at least. RPMI-1640 medium was purchased from Hyclone (Hyclone, USA).

2.2. Preparation of reagents

The dried leaves (1.92 kg) of *Ligustrum purpurascens* were extracted with hot EtOH (20 l × 3 l), and the residue obtained by removal of solvent in vacuo was triturated with H_2O (3 l × 4 l). The insoluble and aqueous phase was parted, and the aqueous phase was directly subjected to chromatography column on the highly porous polymer Diaion (HP20, 1500 ml), eluting with H_2O, H_2O–EtOH (1:1) and EtOH. The eluates with 50% H_2O–EtOH (4 l) were concentrated in vacuo to give residues CPGs (230.4 g). CPGs were suspended in ultra pure grade water for administration to the experimental animals.

The root of *P. ginseng* (200.0 g), crushing, was extracted by circulation reflux twice with hot water (2 l × 3 l). Then the extracts were concentrated to specific gravity of 1.3 under reduced pressure concentration. 46.0 g yellow powder of H_2O extracts was acquired by spray drying. The powder was suspended in ultra pure grade water for the positive reagent.

2.3. Animals and cells

Female Kunming (KM) mice, weighing 18–22 g (8 weeks old), were purchased from the Animal Supply Center of Guangdong Academy of Medical Science. The animals were kept in an environmentally controlled breeding room (temperature: 25 ± 1 °C, humidity of 55 ± 5%, and a 12/12 h light/dark cycle) for at least one-week acclimatization before experiment. All mice were fed rodent laboratory chow with sterile water ad libitum. All the procedures were in strict accordance with the China legislation on the use and care of laboratory animals and with the guidelines established by the Institutional Animal Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals in China.

YAC-1 cells were purchased from Cell Bank of Chinese Academy of Science (Cell type: YAC-1 lymphoma cells from mice; Medium: RPMI-1640 with fetal bovine serum to a final concentration of 10%; Subculturing: Cultures can be maintained by addition or replacement of fresh medium. Start cultures at 3×10^5 cells/ml and maintain between 2×10^5 and 2×10^6 cells/ml; every 2 to 3 days to renew medium; 24 h before the experiment, renew the medium; stationary phase of cells used for the experiment; purchased from Shanghai, China).

2.4. The qualitative and quantitative analysis of CPGs

The qualitative and quantitative analysis of CPGs was performed using an Agilent Technology 6400 Series Triple Quadrupole LC/MS (1260 Infinity Binary LC, Agilent Technologies, USA).

1260 Infinity Binary LC was equipped with G1312B binary solvent-delivery system, G1379B. Thermostatted column compartment, G1316B vacuum degasser, G1367E autosampler, and G1315C diode array detector (DAD). Chromatographic separation was achieved on an Ultimate XB-C18 column (250 mm × 4.6 mm i.d., 5 μm; Welch, MD, USA) coupled with Agilent C18 pre-column (250 mm × 4.6 mm i.d., 5 μm; Palo Alto, CA, USA) at temperature of 25 °C. An initial eluting mixture of H_2O containing 0.1% formic acid and acetonitrile (82:18, v/v) was used at a flow rate of 1 ml/min. The initial mobile phase ratio was held for 20 min, and then the percentage of acetonitrile was increased to 20% from 20 min to 80 min. The sample injection volume was 10 μl. The detection wavelength was 227 nm.

A MS detector with an electrospray ionization (ESI) interface in positive ion mode (ESI^+) was used for qualitative analysis, with acquisition in Product Ion Scan mode. All the parent ions and product ions were recorded, respectively. The optimized electrospray conditions were: capillary voltage 4.00 kV; fragementor: 250 V; source temperature: 100 °C; desolvation temperature: 350 °C; collision energy 40 V for all peaks; gas flow: 13 l/min; nebulizer: 55 psi.

CPGs, acteoside, ligupurpuroside A, *cis*-ligupurpuroside B, *trans*-ligupurpuroside B, and osmanthuside B (CPGs, 20 mg/ml in methanol; all the pure compound, 1 mg/ml in methanol) were injected into the C-18 column, respectively for quantitative analysis. All chromatographic data were acquired and analyzed by using Agilent ChemStation software.

A typical HPLC chromatogram of CPGs and the spectra of UV and mass are shown in Fig. 1.

2.5. Effects of test extracts on haemagglutination antibody titre (HA titre)

The method described previously by Puri et al. (1994) was followed for this experiment. The mice were randomly separated into five groups ($n = 12$) as follows: Group I (control group) were daily gavaged with the vehicle for a period of 7 days; Group II (positive group) were daily gavaged with *P. ginseng* at a dose of 220 mg/kg body weight for 7 days; Groups III–V(treatment groups) were daily gavaged with CPGs at a dose of 220 mg/kg, 440 mg/kg and 1.32 g/kg body weight for 7 days, respectively. The mice were immunized by 200 μl of fresh sheep RBC suspension (2%) via abdominal injection on the 3rd day. Blood samples were taken from the eye socket vein of mice on day 7 and the serum was collected. Hemolysin (SRBC antibody) was determined by haemagglutination technique. Briefly, equal volumes of individual serum samples of each group were pooled. Two fold dilutions of pooled serum samples were made in 50 μl volumes of normal saline in microtitration plate, and added 50 μl of 0.5% suspension