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Purification of a polysaccharide from *Gynostemma pentaphyllum* Makino and its therapeutic advantages for psoriasis

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ABSTRACT

In current study, a water-soluble polysaccharide (GP-I), with a molecular mass of 33 kDa, was purified from *Gynostemma pentaphyllum*. Gas chromatography (GC) analysis suggested that it was composed of Glc, Gal, Man, Rha and Ara with a ratio of 5.3: 4.2: 3.0: 0.7: 0.8. The GP-I (25, 50, 100, 200 and 400 μg/ml) was found to have significant anti-proliferative effects on HaCat cells in a dose-dependent manner, as measured by MTT assay. On the contrary, Trypan blue exclusion experiment indicated that GP-I had no cytotoxicity to HaCat cells. Moreover, the decrease of mitochondrial membrane potential (MMP) in GP-I treated cells was also observed, indicating apoptosis in HaCat cells. Besides, tumor necrosis factor-α (TNF-α), a vital pro-inflammatory cytokine in psoriasis, in the supernatant of HaCat cells was dramatically reduced by GP-I. Collectively, these findings suggested that GP-I was a promising agent to be developed for psoriasis treatment in clinical therapy.

1. Introduction

Gynostemma pentaphyllum Makino (Cucurbitaceae) is a perennial liana and distributes widely in Southern China, Japan, India and Korea. It is a popular traditional Chinese medicine and is famous for their benefit to health and beauty, especially in Europe as an herb tea (Hu et al., 1996). Phytochemical studies on this plant have identified it is a saponin-rich plant and contains about 90 dammarane-type glycosides which are closely related to the component saponins in expensive ginseng, and hence, cheap *G. pentaphyllum* has attracted much interest as a substituted medicinal plant for ginseng and be regarded as "second ginseng" (Circosta et al., 2005). In recent years, *G. pentaphyllum* has attracted great attention owing to its wide bioactivities for the treatments of hepatitis, hypertension, chronic bronchitis, gastritis, cancer, and other diseases (Aktan et al., 2003; Attawish et al., 2004). For this reason, it is claimed that drinking herb tea of *G. pentaphyllum* could promote health and alleviate the severity of many disorders. In particular, the water-soluble polysaccharides have also been demonstrated to be partially responsible for some bioactivity of *G. pentaphyllum* herb. Recently, the polysaccharide components of *G. pentaphyllum* Makino also exhibit significant bioactivities, including anti-aging (Luo & Wang, 2005), anti-oxidant stress (Wang & Luo, 2007), improving immune competence (Qian et al., 1999) and anti-exercise fatigue (Fu, 2000).

Psoriasis is a chronic inflammatory skin disorder characterized by patches of thick, red skin covered with silvery scales, which affects approximately 2–3% of the population worldwide. Recently, conventional therapy in psoriasis are not satisfactory for most of the patients, largely due to the fact that many anti-psoriatic drugs have serious side effects and psoriasis is prone to developing drug resistance after long term exposure (Bos et al., 2005; Griffiths & Richards, 2001). Cytokine and anti-cytokine therapies seem to be a good approach to treatment (Numerof & Asadullah, 2006). However, biologicals have intrinsic challenges, such as limited administration route, side effects, quality control and production cost. Therefore, there is an ongoing research on new effective treatment options (Akama et al., 2009). Many studies reveal that Chinese herbal medicine has been extensively used to treat psoriasis and produce promising clinical results (Amenta et al., 2000; Feily & Namazi, 2009). However, its underlying mechanisms of action have not been systematically investigated. To the best of our knowledge, there was still no reported study on the therapy potential for psoriasis by polysaccharide from *G. pentaphyllum*. Therefore, the aim of this study was to isolate and identify the polysaccharide from *G. pentaphyllum* and to investigate its antipsoriatic activity and

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action mechanism in vitro using cultured HaCaT cells as a psoriasis-relevant experimental model.

2. Materials and methods

2.1. Materials

The whole *G. pentaphyllum* was purchased from Pingli Country Fiveleaf Gynostemma Institute, Shaanxi province, China. Sepharose CL-6B was purchased from Amersham (Sweden). T-series dextran, DEAE–cellulose, thiazolyl blue (MTT), trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO) and standard sugars were obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco Laboratories (Invitrogen Corporation, CA, USA). All other chemical reagents were analytical grade.

2.2. Extraction and purification of polysaccharide

The dried *G. pentaphyllum* was defatted with 95% alcohol and then decocted with distilled water at 90 °C for three times and 2 h for each time. The whole water extracts were collected and concentrated to 20% of the original volume under a reduced pressure and then centrifuged at 3000 rpm for 15 min. The supernatant was collected and 4 volume of 95% alcohol was added to precipitate the polysaccharides, and then kept at 4 °C overnight, and the polysaccharide pellets were obtained by centrifugation (3000 rpm for 10 min). The polysaccharide pellets were completely dissolved in appropriate volume of distilled water, deproteinated by freeze–thaw process for repeating seven times and centrifuged to remove insoluble material and then deproteinated by Sevag method (Staub, 1965). Finally the dialytic supernatant was lyophilized to give crude *G. pentaphyllum* polysaccharides (CGP).

The CGP was dissolved in distilled water, centrifuged, and then the supernatant was applied to a column of DEAE–cellulose anion-exchange chromatography column (3 × 30 cm), eluting at a flow rate of 1 ml/min successively with distilled water and a gradient of 0 → 1 mol/l NaCl. Fractions was collected and monitored with the phenol–sulfuric acid method at 490 nm absorbance. Three main fractions (CGP-A, CGP-B and CGP-C) were collected, dialyzed and lyophilized. CGP-A was further fractioned on a Sepharose CL-6B column (2.6 × 100 cm), eluted with 0.15 mol/l NaCl at a flow rate of 0.5 ml/min to yield one main peak (test tube nos. 36–40). Fractions containing a large amount of sugar were collected, dialyzed, and applied to a sephadex G-25 column to remove salts, and lyophilized to obtain purified polysaccharide, named as GP-I.

2.3. Molecular weight determination and chemical analysis

The homogeneity and the molecular weight distribution of polysaccharide were determined by gel permeation chromatography (), in combination with a high-performance liquid chromatography instrument (Angilent 1100, USA). The sample (2.0 mg) was dissolved in distilled water (2 ml) and passed through a 0.45 μm filter, applied to a gel-permeation chromatographic column of TSK-G3000 columns (7.8 mm ID × 30.0 cm), maintained at a temperature of 40 °C, eluted with 0.05 mol/L Na_2SO_4, at a flow rate of 0.5 ml/min and detected by a RID-10A detector. A 20 μl sample was injected in each run. The molecular weight was estimated by reference to the calibration curve made from a Dextran T-series standard of known molecular weight (T-2000, T-70, T-40, T-20, and T-10).

Total neutral sugar content was determined by the reaction with phenol in the presence of sulfuric acid at 486 nm (Dubois et al., 1956) using Glc as standard. Total uronic acid content was determined by photometry with m-hydroxybiphenyl at 523 nm (Blumenkrantz & Asboe-Hansen, 1973), using galacturonic acid (GalA) as the standard. The protein content of protein-bound polysaccharide was measured by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as the standard.

Polysaccharide was also analyzed for monosaccharide by gas chromatography. After hydrolysis with 2 M trifluoroacetic acid and conversion of hydrolysate into alditol-acetates as previously described method (Honda et al., 1981), the resulting alditol-acetates were analyzed by GC using a Vavian 3400 instrument (Hewlett-Packard, Component, USA), and detected with a flame ionization detector (260 °C), the column temperature was increased from 170 to 215 °C at a rate of 2 °C/min then hold on 5 min

2.4. Cell lines

HaCaT, which is a spontaneously transformed keratinocytes from histologically normal skin (Boukamp et al., 1988) and has been extensively used as an in vitro model for the studies of psoriasis (Farkas et al., 2003; Garach-Jehoshua et al., 1999; Thielitz et al., 2004), was provided by the Chinese Academy of Medical Sciences, Beijing, China. Hs-68, a human fibroblast cell line established from the foreskin of a normal Caucasian newborn male, was purchased from the American Type Culture Collection (USA). Both cell lines were cultured in DMEM medium supplemented with 10% FCS, 100 μg/ml of streptomycin and 100 U/ml of penicillin in a humidified atmosphere with 5% CO_2 at 37 °C. All cell culture experiments were carried out when the culture was approximately 60–90% confluent.

2.5. MTT assay

The inhibitory effects of GP-I on the HaCaT and Hs-68 cells were evaluated by the analysis in vitro using MTT assay (Yan et al., 2009). MTT is a tetrazolium salt that can be cleaved by active mitochondria of viable cells to form a dark blue formazan product which can be measured colorimetrically. Briefly, the cells were cultured on a 96-well cultivation plate at a concentration of 1×10^6 cells/ml. Each well was inoculated with 100 μl DMEM supplemented with 10% FCS solution containing the cells and 20 μl samples (at concentrations of 25, 50, 100, 200 and 400 μg/ml in PBS, respectively) under an atmosphere of 5% CO_2 at 37 °C for 48 h. After cultivation, the percentage of viable cells was determined by MTT assay, reading absorbance at 570 nm with a Benchmark microplate reader (Bio-Rad, California). PBS was used as negative control. The inhibitory rates of cells were calculated by the following formula: %Inhibitory rate = 1 − (mean absorbency in test wells)/(mean absorbency in control wells) × 100%.

2.6. Trypan blue exclusion assay

Cellular cytotoxicity induced by the GP-I treatment was measured using a trypan blue exclusion assay (Jang et al., 2005). Briefly, HaCaT cells were cultured in DMEM medium supplemented with 10% FCS under an atmosphere of 5% CO_2 at 37 °C for 48 h containing the polysaccharide fractions at concentrations of 25, 50, 100, 200 and 400 μg/ml in PBS, respectively. After incubation, the cells were stained with 0.4% trypan blue and approximately 100 cells were counted for each treatment. The survival rate of the cells was calculated as follows: cell survival rate (%) = viable cells/total cells × 100.

2.7. Assay for the changes of MMP

MMP were assessed by flow cytometry using the intramitochondrial dye JC-1 (Molecular Probes) using previously documented