Carbohydrate Polymers 89 (2012) 1232–1237

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

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

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ARTICLE INFO

Article history:
Received 3 March 2012
Received in revised form 26 March 2012
Accepted 2 April 2012
Available online 7 April 2012

Keywords:
Gynostemma pentaphyllum
Polysaccharide
Purification
Psoriasis

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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http://dx.doi.org/10.1016/j.carbpol.2012.04.001

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

methodology (Bortner & Cidlowski, 1999). If MMP depolarizes, JC-1 becomes a monomer and emits green fluorescence; when MMP polarizes, JC-1 becomes a polymer and emits red fluorescence. A decrease in the red to green fluorescence intensity ratio indicates mitochondrial depolarization of the cells, thus the occurrence of apoptosis. Briefly, 1×10^6 HaCaT cells/well were seeded in 6-well plates and incubated with GP-I at 25, 50, 100, 200 and 400 μg/ml for 48 h. After incubation, both floating and adherent cells were harvested, pooled together and washed with PBS. Then 10 mM JC-1/ml of culture was added 30 min prior to flow cytometric analyses. Each sample was examined using 530 nm (FL-1) versus 590 nm (FL-2) fluorescence. A decrease in the fluorescence intensity ratio of FL-2 to FL-1 indicates the occurrence of apoptosis. Results were analyzed in terms of FL-2 to FL-1 ratio and compared with the ratio of the PBS control.

2.8. Cytokine assays

HaCaT cells were plated in 96-well flatbottomed tissue culture plates (2×10^5 cells/well) in culture medium with TNF-α (5 ng/ml), testing polysaccharides (25, 50, 100, 200 and 400 μg/ml) or PBS. Cells were cultured at 37 °C in 5% CO_2 humidified incubator for 24 h and supernatants were harvested and the concentration of TNF-α was determined using an ELISA kit (R&D Systems), according to the manufacturer's instructions.

2.9. Statistical analyses

Data are expressed as the mean ± S.D. Significance between experimental groups was tested by Student's *t*-test, and $p < 0.05$ was considered to be statistically significant.

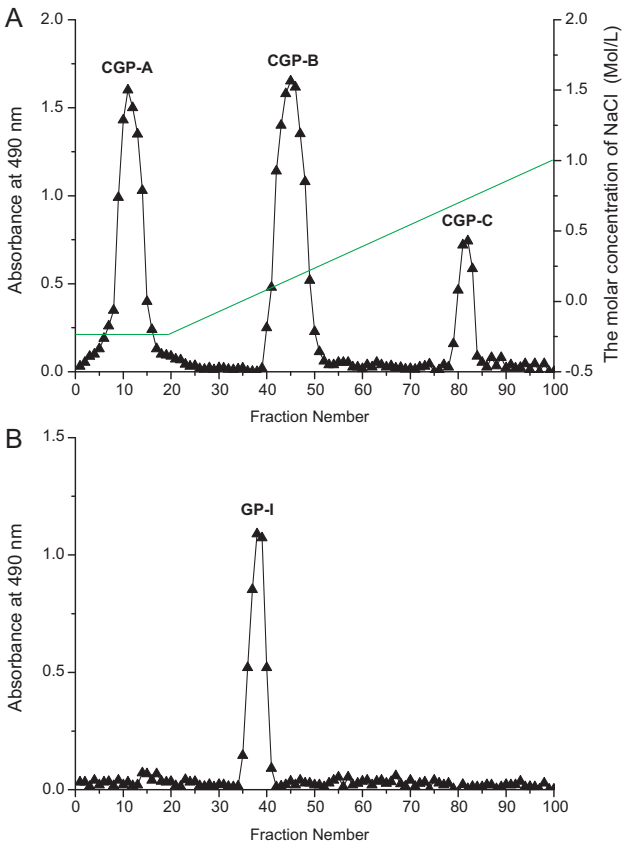

Fig. 1. Elution profiles of *G. pentaphyllum* polysaccharides on DEAE-cellulose ion exchange chromatography (A) and Sepharose CL-6B gel filtration chromatography (B).

3. Results and discussion

3.1. Preparation of GP-I and its chemical properties

After the dried *G. pentaphyllum* was defatted with 95% EtOH for three times, the residue was dried in air followed by extraction with boiling water. The concentrated supernatant was precipitated with 4 volumes of 95% EtOH to achieve crude protein-polysaccharide complex. Then the combined protein and free protein in complex was removed by freeze thawing and Sevag method, yielding crude polysaccharide CGP. As seen in Fig. 1, CGP was subjected to a DEAE–cellulose chromatography and eluted stepwise with 1 column volume (CV) of distilled water and 3 CV of 0 → 1 mol/l NaCl, respectively. Three main fractions (CGP-A, CGP-B, and CGP-C) were separated according to the ion charge difference. The fraction CGP-A, eluted with water, was lyophilized and purified successively on a Sepharose CL-6B column with 0.15 M NaCl at a flow rate of 0.5 ml/min, giving GP-I. On high-performance gel-permeation chromatography (HPGPC), GP-I showed a symmetrical peak (Fig. 2), indicating its homogeneity. According to the calibration curve with standard dextrans and glucose by HPGPC, the average molecular weight of GP-I was determined as 33 kDa. It had a negative response to Bradford's method. The fact that no absorption was detected by the UV spectrum at either 280 or 260 nm indicated the absence of protein and nucleic acid. The total carbohydrate content, uronic acid and protein of GP-I were 94.3%, 2.5%, and 0.6%, determined by phenol–sulfuric acid method, m-hydroxydiphenyl colorimetric method and Bradford method, repectively. GC analysis indentified that GP-I was composed of Glc, Gal, Man, Rha and Ara with a ratio of 5.3:4.2:3.0:0.7:0.8 (Table 1).

3.2. Inhibitory effects of GP-I on HaCaT cells and its cytotoxicity to Hs-68 cells

To evaluate the effect of GP-I on cell growth, MTT assays were performed on HaCat and Hs-68 cells, using increasing drug concentrations (25–400 μg/ml). Fig. 3 showed that GP-I has differential antiproliferative property on the HaCat and Hs-68 cell lines tested. For these assays, cultured cells were exposed to GP-I or PBS for 48 h. GP-I significantly reduced proliferation of HaCat cells, in a dose dependent manner, starting from 25 to 400 μg/ml, with inhibitory rate of 34–75%. In order to determine whether GP-I have unspecific cytotoxicity on any cell type, we tested them on the growth of Hs-68, a line of normal human fibroblast cells. It was evident that GP-I did not exert significant inhibitory effect on the proliferation of Hs-68 cell line, and these results were in stark contrast to the significant cytotoxic effects exerted by GP-I on HaCaT cells.

To the best of our knowledge, MTT assay only proved that GP-I treatment possessed a good anti-proliferation effect on HaCat cells. But we do not know whether this effect is cytotoxic or cytostatic. In the light of this reason, Trypan blue exclusion experiment was introduced to determine how GP-I exerted its inhibitory effect on the growth of HaCaT cells. As seen from Fig. 4, HaCaT cells exhibited the highest degree of cell survival ranging from 84.5 to 93.0% when exposure to GP-I at all concentrations, which demonstrated GP-I had a negligible cytotoxic effect on HaCaT cell line. Given that the significant inhibitory effect of GP-I on the proliferation of HaCat cells determined by MTT assay, we can draw a conclusion that this anti-proliferation effect probably arose from the apoptosis induced by GP-I.

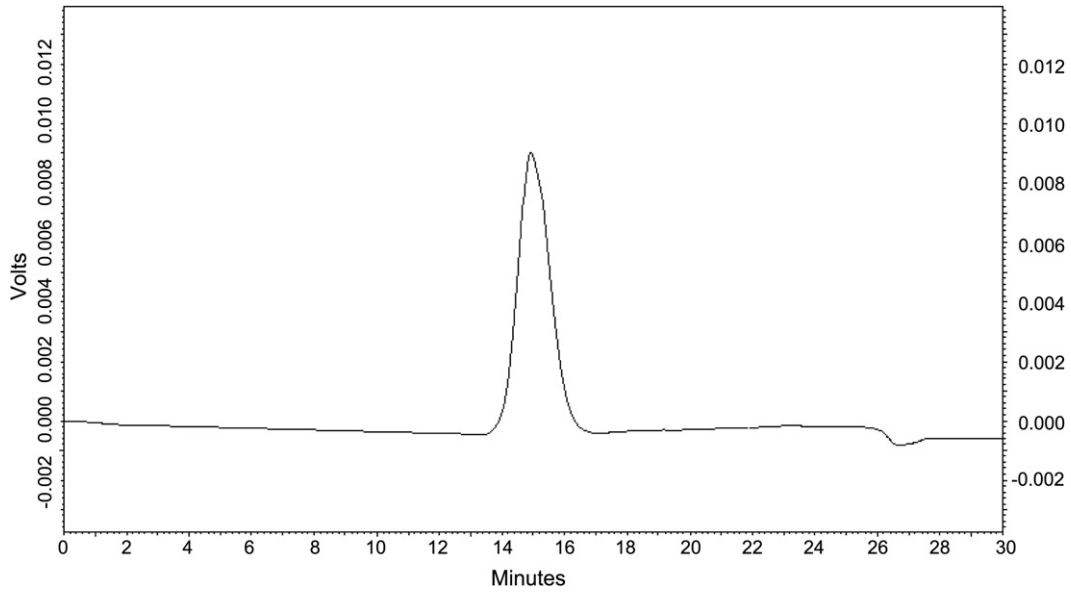

Fig. 2. Profile of GP-I in HPGPC.

Table 1
Physicochemical properties of GP-I.

Sample	Molecular weights (KDa)	Carbohydrate content (w/w%)	Uronic acid (w/w%)	Protein (w/w%)	Contents of the sugar residues (mol.%)[a]				
					Glc	Gal	Man	Rra	Ara
GP-I	33	94.3	2.5	0.6	5.3	4.2	3.0	0.7	0.8

Glc: glucose; Gal: galactose; Man: mannose; Rha: rhamnose; Ara: arabinose.
[a] (mol.%): Quantities of the carboxy-methylated uronic acid are given in mol.%.

3.3. Effects of GP-I on the MMP changes

Mitochondrial homeostasis is critical in regulating apoptosis (Green & Reed, 1998; Kroemer et al., 1998). In particular, it has been documented that some or all of the following mitochondrial changes are associated with apoptosis: mitochondrial membrane hyperpolarization and depolarization, matrix swelling, and permeability of the outer membrane, resulting in the release of proapoptotic proteins such as cytochrome *c*, apoptosis-inducing factor, and potentially other proteins. In many instances, such mitochondrial events are a prerequisite for the activation of a family of aspartic acid-specific, cysteine-containing proteases (caspases) that are known to be important mediators of apoptosis (Rathmell & Thompson, 1999; Thornberry & Lazebnik, 1998). Mitochondrial dysfunction has been shown to participate in the induction of apoptosis and has even been suggested to be central to the apoptotic pathway (Ly et al., 2003). With respect to that of control, the MMP in GP-I-treated HaCaT cells decreased dramatically from 96.5% to 28.5% at 48 h at the concentrations of GP-I ranged from 25 to 400 μg/ml in a dose-dependent manner (Fig. 5). Treatment with 50 μg/ml Gp-I significantly reduced MMP in HaCaT cells for 48 h, which proved that such low dose could enough produce a significant effect. These results indicated that attenuation of MMP by GP-I fraction in cultured HaCaT cells was well in agreement with the Trypan blue exclusion assay. These

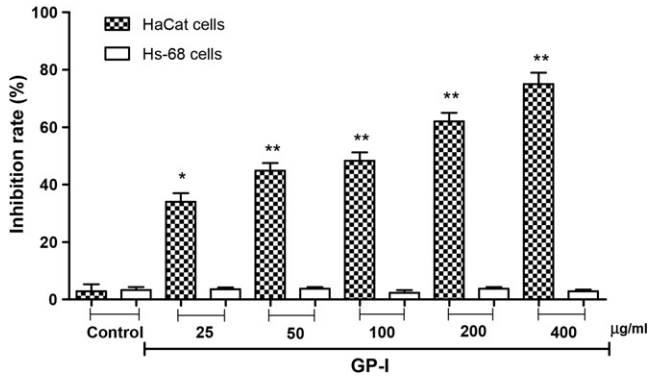

Fig. 3. Effect of Gp-I on the proliferation of HaCat and Hs-68 cells by MTT assay. The results are presented as the mean ± S.D. of at least three independent experiments. The asterisk (* or **) indicates significant difference compared to the control group ($p < 0.05$ or $p < 0.01$).

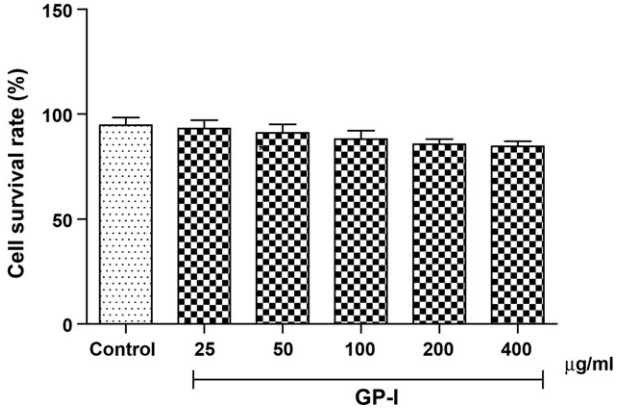

Fig. 4. The cytotoxicity of GP-I on HaCaT cells by Trypan blue dye exclusion assay. The results are presented as the mean ± S.D. of at least three independent experiments.

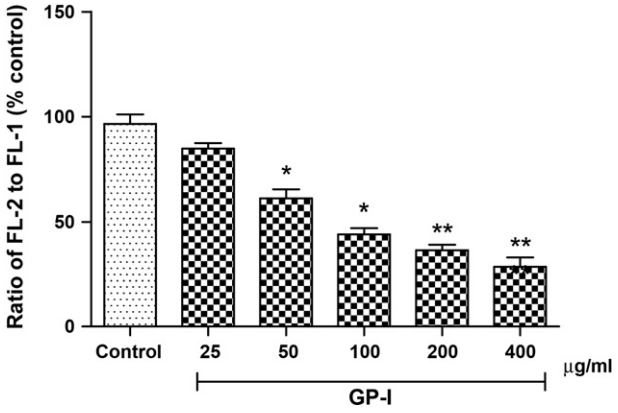

Fig. 5. Effect of GP-I on the change of MMP in HaCaT cells. The results are presented as the mean ± S.D. of at least three independent experiments. The asterisk (* or **) indicates significant difference compared to the control group ($p < 0.05$ or $p < 0.01$).

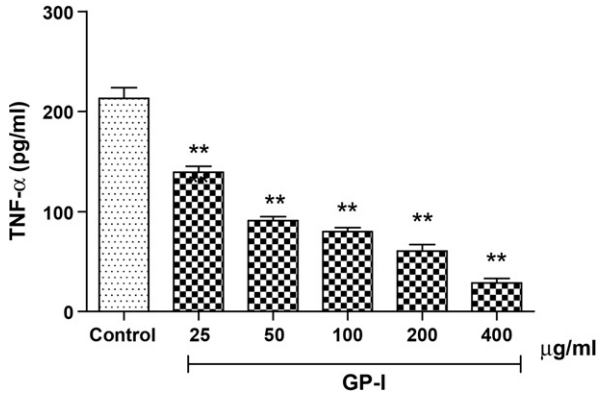

Fig. 6. Effect of GP-I on the production of TNF-α in the culture of HaCaT cells. The results are presented as the mean ± S.D. of at least three independent experiments. The asterisk (**) indicates significant difference compared to the control group ($p < 0.01$).

findings provided experimental evidence that GP-I fraction was capable of inducing cellular apoptosis in HaCaT cells.

3.4. Effects of GP-I on the TNF-α secretion in culture of HaCaT cells

TNF-α is a key proinflammatory cytokine in various skin diseases, especially in psoriasis (Gaspari, 2006). To study the effect of GP-I treatment on the TNF-α secretion in the culture, HaCaT cells were exposed to GP-I at doses ranging from 25 to 400 μg/ml, and then cells were harvested 24 h after treatment for ELISA analysis. As shown in Fig. 6, GP-I suppressed the production of TNF-α, exhibiting a dose-dependent manner. The amounts of TNF-α produced by GP-I in all test doses were lower than that in the negative control ($p < 0.05$).

4. Conclusion

In this study, a water-soluble polysaccharide GP-I was successfully purified from the whole plant of *G. pentaphyllum* by DEAE-cellulose ion exchange chromatography and Sepharose CL-6B gel filtration chromatography, with an average molecular weight of 33 kDa. Its content of carbohydrate, uronic acid and protein were 94.3%, 2.5%, and 0.6%, respectively. GC analysis indentified that GP-I was a heteropolysaccharide, composed of Glc, Gal, Man, Rha and Ara with a ratio of 5.3:4.2:3.0:0.7:0.8. MTT assay preliminarily identified that GP-I had an effective anti-proliferation activity against an in vitro psoriasis-relevant HaCaT cell line. On the contrary, GP-I did not exert a cytotoxic effect on human fibroblast Hs-68 cell line.

The fact that there was no difference in the survival of HaCat cells between the GP-I-treated and control group in Trypan blue exclusion experiment indicated the apoptosis may contribute to its anti-proliferation effect. Moreover, the MMP potential decreased dramatically along with the increase of GP-I concentration, which supported the fact that GP-I mainly induced cellular apoptosis to inhibit the proliferation of HaCaT cells. In addition, TNF-α produced in the culture of HaCaT cells was markedly attenuated by addition of GP-I, suggesting its usefulness to combat inflammatory dermatoses, including psoriasis and atopic dermatitis. This study also provided evidences to support the therapeutic effects of GP-I for treatment of psoriasis. At present, detailed work on the mechanism is in progress.

Acknowledgements

The study was supported by the Natural Science Foundation of China (No. 30901297), the Doctoral Fund of Youth Scholars of Ministry of Education of China (20090201120074), the Fundamental Research Funds for the Central Universities (2009, 2012).

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