Antioxidant and anti-inflammatory activity of *Polygonatum sibiricum* rhizome extracts

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1. Introduction

Many critical factors such as oxidative stress and inflammation are closely related to carcinogenesis and other degenerative disorders. The oxidative damage caused by reactive species to lipids, proteins and nucleic acids may trigger various diseases including coronary heart diseases, atherosclerosis, cancer and aging[1]. Antioxidants have the ability to inhibit or prevent the formation of reactive oxygen species (ROS) by donating hydrogen or electrons and play a crucial role in preventing oxidative cell damage[2].

Macrophages play very important roles in immune reactions, allergy, and inflammation[3]. During inflammatory diseases, different types of mediators such as nitric oxide (NO) and pro-inflammatory cytokines are produced by the macrophages[4,5]. The inducible nitric oxide synthase (iNOS) is the most important pro-inflammatory enzyme that catalyzes the production of NO from L-arginine[6].

**Objective:** To investigate the antioxidant and anti-inflammatory activities of distilled water extract of fresh *Polygonatum sibiricum* (*P. sibiricum*) rhizome.

**Methods:** The extracts were tested for antioxidant activity by using DPPH (1,1-diphenyl-2-picrylhydrazyl), and hydroxyl (OH•) radicals scavenging activity. The level of intracellular reactive oxygen species (ROS) was determined in H2O2 treated chang liver cells. Anti-inflammatory activity was evaluated by the inhibition of nitric oxide (NO), inducible nitric oxide synthase (iNOS), as well as tumor necrosis factor-alpha (TNF–α) protein expression in a lipopolysaccharide stimulated Raw 264.7 murine macrophages cell line.

**Results:** *P. sibiricum* water extracts scavenged DPPH, OH• radicals and decreased the level ROS. The extracts reduced NO reduction and inhibited the expression of iNOS and TNF–α proteins.

**Conclusions:** The findings indicate that water extracts of *P. sibiricum* could be considered as natural antioxidants and anti-inflammatory agents for food and drug industries.

**KEYWORDS**

Reactive oxygen species, Phenolic content, Lipopolysaccharide, iNOS
Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and has often been used in inflammatory response because it promotes the secretion of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) in many cell types, especially in macrophages[7]. The inhibitory activity of excess amounts of NO, iNOS and TNF-α produced by LPS-stimulated macrophages would therefore be a good indicator of anti-inflammatory activity of extracts.

*Polygonatum sibiricum* (P. sibiricum), also known as Huang Jing, is a perennial herbaceous plant belonging to the family Liliaceae. In Korea, these medicinal plants are widely consumed as tea. Different varieties of *Polygonatum* such as the methanol extracts of *Polygonatum odoratum* (Mill.) Druce, *Polygonatum falcatum* A. Gray, and *P. sibiricum* Redouté show hypoglycemic activity that has already been reported[8-10]. The plant is capable of reducing blood glucose and lipid levels, regulating and enhancing the immune system, and fighting aging[11]. However, there are no reports on the antioxidant and anti-inflammatory activity of the water extracts of fresh *P. sibiricum* rhizome. This study was therefore conducted to evaluate the antioxidant and anti-inflammatory activity of water extracts of *P. sibiricum*.

### 2. Materials and methods

#### 2.1. Materials and chemicals

DPPH, potassium persulfate, ascorbic acid (AA), gallic acid, Folín–Ciocalteu reagent (FC reagent), ethylenediaminetetraacetic acid (EDTA), and 5,5-dimethylpyrroline 1-oxide (DMPO) were purchased from Sigma Chemical Co., St. Louis, USA. Lipopolysaccharide (LPS) was purchased from Sigma, St. Louis, USA. Dulbecco’s modified Eagle medium (DMEM) and 3-(4,5 dimethythiazol2-yl)-2,5-diphenyltetrazoleum (MTT) were obtained from Wako Chemical Co., Tokyo, Japan. Fetal bovine serum (FBS) and antibiotics were purchased from Gibco-BRL, Gaithersburg, USA. All chemicals were used without any further purification.

#### 2.2. Plant material and preparation of extracts

*P. sibiricum* Redouté (Liliaceae) rhizomes were collected in Kyeonggi province of Korea in April, 2005. A voucher specimen (SNUPH-0328) is deposited in the Herbarium of the College of Pharmacy, Seoul National University.

Fresh *P. sibiricum* rhizome (300 g) was extracted with distilled water (DW) (100 g/700 mL) at 85 °C for 3 h[12]. The extract was removed from the marc by filtration and concentrated by vaporization under reduced pressure at 40 °C in a vacuum rotary evaporator (Hahn Shin Science Co., model HS2000N). The concentrated mass was dried and weighed to determine total extractable compounds. The percentage yield (w/w) was calculated as

\[
\text{Yield (\%)} = \frac{\text{Total extracted sample mass}}{\text{Total fresh sample mass}} \times 100
\]

The stock solution was prepared by dissolving the extracted mass into DW for different assays.

#### 2.3. Determination of total phenolic and total flavonoid contents

The phenolic content of the extract was determined by using FC (Folín–Ciocalteu) method[13]. Data were expressed as gallic acid equivalents (GAE) per 100 g dry mass. The total flavonoid contents were assessed based on the aluminum chloride colorimetric assay described by Marinova *et al*., with little modification[14]. Briefly, 10 mg/mL of sample stock solution and 0–25 µg/mL standard catechin were prepared. About 25 µL of each solution of sample/standard was mixed with 125 µL DW followed by 8 µL of 5% sodium nitrate and incubated for 5 min at room temperature. A total of 15 µL of 10% aluminum chloride solution was added to the mixture and absorbance measured at 513 nm. Data were expressed as the percentage of catechin equivalent (CE) per 100 g dry mass.

#### 2.4. DPPH radical scavenging activity

DPPH radical scavenging activity of the extract was carried out according to the method of Debnath *et al*[15]. Different concentrations (0.12 to 2.00 mg/mL) of sample and BHT (as positive control) were prepared. Then, 100 µL of each sample/standard solution was mixed with 100 µL of DPPH solution and the mixture was incubated at room temperature for 30 min. Absorbance of the solution was measured at 517 nm.

#### 2.5. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by using electron spin resonance (ESR) spectrometer (Jeol, Tokyo, Japan) according to the method of Debnath *et al*[15]. The extract or positive control (ascorbic acid) was mixed with 20 µL of DMPO (0.3 mol/L), 10 mmol/L H2O2 (in phosphate buffer solution, pH 7.4) and 10 mmol/L FeSO4 solution. Mixture was incubated for 2.5 min at room temperature and the hydroxyl radical scavenging activity was determined by using the following equation

\[
\text{Scavenging activity (\%)} = \left(1 - \frac{B-A}{B}\right) \times 100
\]

Where, A is the sample peak height and B is the peak height of control.

#### 2.6. Intracellular ROS detection

Intracellular ROS formation was detected by 2′,7′-dichlorofluorescin diacetate (DCF–DA, Sigma), which
is oxidized to fluorescent 2’,7’-dichlorofluorescin (DCF) by hydroperoxides. Treated chang liver cells (5×
10⁴) were washed three times with serum-free DMEM and incubated with 50 μmol/L DCF-DA (in serum-free DMEM) in the dark for 30 min at 37 °C. The cells were then washed with cold phosphate buffered saline (PBS) three times and resuspended in PBS. The intracellular levels of ROS were determined by measuring the mean fluorescence intensity by flow cytometry (BD FACSCaliburTM, USA). The excitation and emission were at 488 and 525 nm respectively.

2.7. Determination of cell viability

Raw 264.7 murine macrophages cells were plated (5×
10⁴/well) in 96 well plate containing 100 μL DMEM with 10% heat inactivated FBS and incubated overnight. Various concentrations of the extracts (0.25–2.00 mg/mL) were dissolved in PBS and applied to the cell cultures alone or with 10 μg/mL of LPS for a day. After incubation, the cells were washed once before adding 50 μL of FBS free medium containing 5 mg/mL MTT. After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue that formed in the cells was dissolved in 100 μL of dimethyl sulfoxide (DMSO). The optical density was measured at 540 nm.

2.8. Measurement of NO

NO was measured with cell supernatant by using the Greiss reagent. The Raw cells were cultured with DMEM and 10% FBS. A total of 5×10⁴ were put into a 96–well plate and incubated for a day. Various concentrations of the extracts (0.25–2.00 mg/mL) were dissolved in PBS and applied to the cell cultures alone or with 10 μg/mL of LPS for a day. After incubation, the cells were washed once before adding 50 μL of FBS free medium containing 5 mg/mL MTT. After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue that formed in the cells was dissolved in 100 μL of dimethyl sulfoxide (DMSO). The optical density was measured at 540 nm.

2.9. Western blot analysis

Total protein concentration was estimated by the Bio–Rad protein assay reagent according to the manufacturer’s instructions. A fixed amount (50 μg) of cellular protein from the treated and untreated cell extracts was separated using SDS–polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. The immunoblot was incubated overnight with a blocking solution, followed by incubation with dilution of polyclonal antibody against iNOS and TNF–α. The blots were washed twice with Tween 20/Tris–buffered saline (TTBS) and incubated for 1 h at room temperature with diluted solution of anti–rabbit secondary antibody (dilution ratio, 1:1,000) for both iNOS and TNF–α. The blots were washed three times with TTBS and then developed using enhanced chemiluminescence.

2.10. Statistical analysis

All experiments were performed in triplicate and all data were expressed as mean±standard deviation (SD). The statistical analyses were performed with GraphPad InStat. The observed differences were analyzed for statistical significance by one–way analysis of variance with Tukey’s multiple comparison as a post–hoc test. Differences of P<
0.05 were considered as significant.

3. Results

The yield was 30.53% (w/w) on dry mass basis. The total phenolic and flavonoid content were 48.62 mg and 13.56 mg expressed as a GAE and CE per 100 g dry mass, respectively (Table 1). The water extract of P. sibiricum showed strong DPPH radical scavenging activity in a concentration dependent manner in the range of 0.25–2.00 mg/mL and the highest scavenging activity was 82.05% at the concentration of 2 mg/mL (Table 1). The half maximal inhibitory concentration (IC₅₀) of the extract was 0.25 mg/mL. However, scavenging activity of the extract was significantly lower (P<0.05) than that of BHT (95.25%) at 2.00 mg/mL. The DPPH radical scavenging activity of the extract was highly correlated with its phenolic and flavonoid content (r²=0.9985, and 0.9860, respectively). Figure 1 shows the dose dependent hydroxyl radical scavenging activity of extracts. The highest activity was found 68.21% at the concentration of 2 mg/mL and IC₅₀ value was 0.31 mg/mL. However, AA was more effective (75% at 0.005 mg/mL) than the extracts.

Table 1
The total phenolic and flavonoid contents and DPPH radical scavenging with IC₅₀ values of water extracts of P. sibiricum.

| Sample     | Total phenolic(a) (mg GAE/100 g of dry mass) | Total flavonoid(b) (mg CE/100 g of dry mass) | IC₅₀ (mg/mL) | DPPH radical(c) Scavenging activity (%) at 2.0 mg/mL |
|------------|---------------------------------------------|---------------------------------------------|--------------|----------------------------------------------------|
| Water extract | 48.62±2.27                                   | 13.56±1.57                                  | 0.25±0.01   | 85.05±2.00                                        |
| BHT        | Np                                          | Np                                          | 0.10±0.02   | 95.25±1.40                                        |
| Ascorbic acid | Np                                         | Np                                          | Np          | Np                                                 |

(a)Each value is expressed as mean±SD (n=3). GAE: Gallic acid equivalent, CE: Catechin equivalent. (b)IC₅₀ (mg/mL): the concentration at which 50% is inhibited. Np: experiment not performed.
Figure 1. The dose dependent hydroxyl radical scavenging activity of extracts.
(a) Hydroxyl radical scavenging activity of *P. sibiricum* extracts at various concentrations by ESR. (b) ESR spectra of the extracts and control (without sample/standard). AA was used as a positive control. *P<0.05. Statistical comparisons were made with different concentrations of the water and ethanol extracts versus the AA (0.005 mg/mL). Each value is expressed as the mean±SD (*n*=3).  #: the peak of DMPO-OH. The conditions for ESR analysis as are follows: power: 1 mW; amplitude: 1×200; modulation width: 0.1 mT; sweep width: 10 mT; sweep time: 30 second.

Figure 2 shows the increasing mean fluorescence intensity in H$_2$O$_2$-treated cells as compared to the control. In contrast, pretreatment with the extract for 1 h significantly decreased the mean fluorescence intensity in a dose dependent manner.

Figure 2. Effect of *P. sibiricum* extracts on ROS production in H$_2$O$_2$-treated chang liver cells.

Figure 3 shows the inhibitory effect of *P. sibiricum* extracts on NO production from macrophages (RAW 264.7 cells) induced by LPS. The level of nitrite concentration in the culture supernatant was increased after LPS treatment, compared to control cells and *P. sibiricum* treatment inhibited the production of NO by dose depended manner. Only LPS treatment decreased cells viability compared to

Figure 3. The inhibitory effect of *P. sibiricum* extracts on NO production from macrophages (RAW 264.7 cells) induced by LPS.
(a) Nitrite scavenging activity of the *P. sibiricum* extracts on LPS treated RAW 264.7 cells. The optical density was measured at 540 nm. (b) Cell viability of Polygonatam sibiricum extracts on LPS stimulated RAW cells. Data were obtained from three independent experiments and expressed as mean±SD.

However, cells viability was increased by dose dependent manner after *P. sibiricum* treatment. *P. sibiricum* treatment significantly suppressed LPS–dependent production of iNOS and TNF–α respectively, in a dose response manner (Figure 4).

Figure 4. The effect of *P. sibiricum* water extracts on iNOS and TNF–α protein expression (mg/mL).
4. Discussion

Plants are an important source of active components that lead to the discovery and development of numerous agents that can be used as medicines[16]. Moreover, a diet rich in antioxidants and anti-inflammatory compounds derived from plant extract may lower the risk of developing neurodegenerative diseases, such as Alzheimer’s or Parkinson’s disease, associated with aging[17]. The present study therefore looked at the antioxidant and anti-inflammatory activities of P. sibiricum water extracts.

Phenolics are major components of plants and can scavenge free radicals. Scavenging activity on free radicals is one of principal functions of phenolic phytochemicals[15]. Flavonoids found in fruits, vegetables, nuts as well as in tea with various chemical structures and characteristics. They are also essential part of human diets. It is famous that flavonoids display numerous biological activities including anti-inflammatory, antiviral, antibacterial activities and also act as an antioxidant[15]. The water extract of P. sibiricum showed good phenolic and flavonoid contents. DPPH is synthetic nitrogen-centered free radical that is not biologically relevant, but it is famous to determine antioxidant activity. The free electron of the DPPH radical has a strong absorption maximum at 517 nm and is purple and/or deep violet in color. The reaction between DPPH free radical and antioxidant compound, the antioxidant compound gives an electron to DPPH and its color changes to light yellow. The extracts scavenged DPPH free radicals in a concentration–dependent manner. This activity was highly correlated with its phenolic and flavonoid contents. Hydroxyl is a very reactive radical among the ROS and therefore widely investigated. It is responsible for cell damage and various diseases such as arthritis, ischemia, AIDS, gastric illnesses, and cancer[18,19]. The evaluation of scavenging activity of the extract on these radicals was therefore very important. The extracts showed dose dependent activity on hydroxyl radical. H2O2 treated chang liver cell line is widely used as a model for detection of total ROS[20]. The extracts significantly decreased total ROS level in change liver cells. In addition, the rhizome extracts of Polygonatum odoratum and aerial parts of Polygonatum verticillatum also exhibited potent antioxidant activity, as reported by Wang et al. and Khan et al[21,22]. Our study is accordance to these analyses.

NO has an important role in the various types of inflammatory processes[23]. It can be produced in large amounts by iNOS from macrophages. As seen in many chronic inflammatory disorders, a variety of stimuli such as LPS produced massive amounts of NO by the activated macrophages, which can participate in more intense inflammatory responses[24,25]. P. sibiricum extracts reduced NO concentration by dose depended manner and had no effect on cytotoxicity of macrophages cells indicating that the inhibition of NO was not due to general cellular toxicity, at least up to the highest test concentration (2 mg/ml). Moreover, the P. sibiricum treated cell showed similar viability (P<0.05) compared with the untreated cells. There are three isoforms of NOS, including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS)–mainly catalyzed the production of NO. Various stimuli, like LPS, can promote the over-expression of iNOS, TNF-α is one of most potent pro-inflammatory cytokines usually released by macrophages and plays a crucial role in initiating and sustaining the inflammatory response. It was observed that the P. sibiricum extracts were able to suppress the iNOS and TNF-α protein expression in LPS–stimulated macrophage (Raw 264.7) cells line. In addition, aqueous extract of dried rhizomes of P. sibiricum showed potent anti-inflammatory activity on ear edema in mice by inhibiting the protein and mRNA levels of iNOS, cyclooxygenase–2 (COX–2), TNF-α, interleukin (IL)-1β, and IL-6[26]. Our results are well consistent with the previous report.

In conclusion, the P. sibiricum water extracts satisfactorily scavenged DPPH, hydroxyl radicals and intracellular ROS in chang liver cells. These data suggest that the P. sibiricum extract is a natural antioxidant. Interestingly, the extracts also inhibit the production of excessive amounts of NO that was supported by MTT assay. Based on the positive results from the effects on inhibition of iNOS and TNF–α expression, the extracts could potentially be used as food supplements with the purpose of providing anti-inflammatory effects. Nevertheless, further research is required on in vivo experiments to validate these results.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The sample extracts were tested for antioxidant activity by using DPPH and hydroxyl (OH•) radicals scavenging activity. The level of intracellular ROS was determined in H2O2 treated chang liver cells. Anti-inflammatory activity was evaluated by the inhibition of NO, iNOS, as well as TNF–α protein expression in a lipopolysaccharide stimulated Raw 264.7 murine macrophages cell line.

Research frontiers

P. sibiricum is a perennial herbaceous plant belonging to the family Liliaceae and is used in herbal infusions.

Related reports

Systematic studies on the antioxidative activity of P.
lipid levels, regulating and enhancing the immune system, antioxidant and anti-inflammatory activity. This study was conducted to evaluate the antioxidant and anti-inflammatory activity.

**Applications**

The study is a good addition to the basic data for application of an excellent natural source.

**Peer review**

This is a good and important study. Moreover, the manuscript is well written and the experiments were well designed.

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