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The anti-inflammatory effects of *Pyrolae herba* extract through the inhibition of the expression of inducible nitric oxide synthase (iNOS) and NO production

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Abstract

The extract of *Pyrolae herba* (PH), which has been used as an anti-inflammatory folk remedy in Korea and China, was investigated for its anti-inflammatory action using arachidonic acid, 12-*O*-tetradecanoylphorbol 13-acetate or carrageenan-induced edema assays. The anti-nociceptive activity of PH was also tested in mice using the acetic acid-induced writhing model. PH showed dose-dependent and significant ($P < 0.05$ at 100–400 mg/kg) anti-inflammatory and anti-nociceptive activities in the animal assays. The mechanism of the activities of PH was examined by testing the extract to determine if it inhibits the expression of inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO) from the murine macrophages, RAW 264.7 cells. Similar to the *in vivo* activities, both the iNOS expression and NO production were significantly suppressed by PH in a dose-dependent manner. PH also inhibited the activating phosphorylation of p38 MAP kinase and NF-κB in these cells. These results provide a scientific basis to explain the effects of PH as an anti-inflammatory folk remedy in Asian countries.

Keywords: Anti-inflammatory; Inducible nitric oxide synthase; Macrophages; Nitric oxide; *Pyrolae herba*

1. Introduction

Pyrolae herba, Nok-Je-Cho in Korea and Luxiancao in China, is the whole dried plant of *Pyrola japonica* Klenze (Pyrolaceae). The *Pyrolae herba* extract (PH) contains chimaphilin, acetovanillon, and toluhydroquinone (Kagawa et al., 1992), and has been used traditionally as a folk remedy in Korea and China to treat cancer, excessive menstrual flow, chronic phthisical cough, and various inflammatory diseases including rheumatoid arthritis and arthralgia (Bae, 2000). Although some constituents of PH have been identified, there have been a few studies on the pharmacological effects of a PH extract for proving its pharmacological activities (Kagawa et al., 1992).

It is widely known that nitric oxide (NO), synthesized by nitric oxide synthase (NOS), is involved in diverse physiological processes (Radomski et al., 1987; Moncada et al., 1991; Snyder and Bredt, 1992; Yap and Sher, 1999). An excess in NO production is largely thought of as causing a variety of inflammatory diseases, such as sepsis, psoriasis, arthritis, multiple sclerosis, and systemic lupus erythromatosus (Clancy et al., 1998; Kröncke et al., 1998). Three isoforms of NOS – types I, II, and III – have been identified in mammalian cells, according to the physical and biochemical characteristics of the purified enzymes. Type I (neuronal NOS, nNOS) and type III (endothelial NOS, eNOS), referred to as the constitutive NOS (cNOS), are permanent fixtures in cells. Type II, on the other hand, an inducible-type NOS (iNOS), comes about only

Abbreviations: PH, *Pyrolae herba*; iNOS, inducible nitric oxide synthase; NO, nitric oxide; MAP kinase, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; SDS-PAGE, sodium dodesyl sulfate-polyacrylamide gel electrophoresis; COX-2, cyclooxygenase-2

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after one has been exposed to specific stimulants in some cells, such as cytokines, bacterial lipopolysaccharides, and calcium ionophores to induce inflammatory reaction (Vodovotz et al., 1993; Chesrown et al., 1994; Weisz et al., 1996; Denlinger et al., 1996). Thus, the expression and activity of iNOS is the major therapeutic target to treat various inflammatory diseases.

This study was undertaken to validate the use of PH as an anti-inflammatory medicine. The *in vivo* anti-inflammatory activity was investigated using arachidonic acid, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), or carrageenan-induced edema animal models. The anti-nociceptive activity using the acetic acid-induced writhing model was tested in mice. The effects of PH on the expression of iNOS and NO production were also investigated using LPS-stimulated RAW 264.7 macrophages. As a result, PH was identified as a potential medicinal herb for curing inflammatory diseases.

2. Materials and methods

2.1. Reagents

The arachidonic acid, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), carrageenan, and acetic acid were purchased from Sigma–Aldrich (St. Louis, MO). The cell culture reagents were obtained from GIBCO/Invitrogen (Carlsbad, CA). The tris-glycine polyacrylamide gels were purchased from Novex (San Diego, CA). The antibodies against iNOS, phospho-p38, NF-κB, and actin were obtained from Santa Cruz (Santa Cruz, CA). The anti-phospho-NF-κB p65 (Ser 536) antibody was supplied by Cell Signaling (Danvers, MA). Lipopolysaccharide (LPS) and Griess reagents were acquired from Sigma–Aldrich (St. Louis, MO). As the positive control, Joins, a combination herbal drug containing the extracts of *Trichosanthes Kirilowii*, *Prunella vulgaris*, and *Clematis mansshurica*, commonly used for the treatment of rheumatoid arthritis in Korea, was obtained from Sun Kyung Pharmaceutical Co. Ltd. (Seoul, Korea).

2.2. Preparation of the ethanol extract of Pyrolae herba

The *Pyrolae herba* extract (PH) was purchased from Hunan Guohua Pharmaceutical Co., Ltd. in China. The whole plant of *Pyrola japonica* was collected from Changsha area in Hunan of China and the plant was authenticated by Dr. Yaojinpeng in Hunan Guohua Pharmaceutical Co., Ltd. The PH was prepared according to the following protocol: the dried *Pyrolae herba* was chopped using a domestic mixer and 10 parts of 30% ethanol were added. After allowing the mixture to stand at 100 °C for 2 h, the ethanol extracts were filtered and concentrated in a 60–70 °C water bath under reduced pressure. The yield of the extract was approximately 2.5%. A voucher specimen (Sinil-1023) was deposited at Life Science R&D Center, Sinil Pharmaceutical Co., Ltd. The extract was dissolved in 100% DMSO to a concentration of 300 mg/ml and diluted to 30–300 μg/ml with the media (0.1% DMSO) for the *in vitro* study with RAW 264.7 macrophages, and suspended in 5% Arabic gum for the *in vivo* animal studies.

2.3. Animals

Male ICR mice (aged 6 weeks) and male Wistar rats (aged 6 weeks) were purchased from the Dae Han Experimental Animal Center (Eumseong, Korea). The animals were housed in the animal facilities at Life Science R&D Center, Sinil Pharmaceutical Co., Ltd. Ten mice or six rats were each placed in a cage in a laminar airflow cabinet at a constant temperature and relative humidity of 22 ± 1 °C and of 55 ± 10%, respectively. All the animals were fasted overnight before dosing but tap water was provided *ad libitum*. Eight animals were used in each experimental group. The animal study was carried out in accordance with the institutional guidelines of Sinil Pharmaceutical Co., Ltd.

2.4. 12-O-Tetradecanoylphorbol 13-acetate (TPA) or arachidonic acid-induced ear edema assay

The PH was administered orally 1 h before the topical application of 2.5 μg of TPA or 2% arachidonic acid dissolved in acetone (20 μl/ear) to the right ear of mice. The activity was evaluated using the following two processes: (1) the thickness of each ear was measured 4 h after inducing inflammation using gauge calipers (Ozaki Co., Tokyo, Japan), (2) the animals were sacrificed by deep ether anesthesia 4 h after the topical treatment. Six millimeter diameter disks were removed from each ear and weighed on a balance.

2.5. Carrageenan-induced paw edema assay

The carrageenan-induced edema assay was carried out using the method reported by Winter et al. (1962), using male rats. The PH (100–400 mg/kg), Joins (400 mg/kg) or the vehicle was administered orally 1 h before injecting the carrageenan (1% in saline solution) into the subplantar region of the right hind paw. The contralateral paw was injected with an equal volume of saline. The paw volumes were determined hourly using a plethysmometer (Ugo Basile, Italy) for 4 h.

2.6. Acetic acid-induced writhing test

A slight modification of the acetic acid-induced writhing method was used (Koster et al., 1959). One hour after administering PH (20–400 mg/kg), Joins (400 mg/kg) or the vehicle orally to groups of eight mice, 0.7% aqueous solution of acetic acid was intraperitoneally administered to each mouse at a dose of 10 ml/kg body weight. Each animal was placed in a transparent observation cage 10 min after the acetic acid injection and the number of writhes per mouse was counted over a 10 min period. Writhing was defined as a contraction of the abdominal muscles together with a stretching of the hind limbs.

2.7. Cell culture and stimulation

Murine macrophage RAW 264.7 cells were cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml), in a humidified atmosphere