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Anti-inflammatory, anti-angiogenic and anti-nociceptive activities of an ethanol extract of *Salvia plebeia* R. Brown

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ABSTRACT

Ethnopharmacological relevance: Salvia plebeia R. Brown has been used for the treatment of a variety of inflammatory diseases, cold and tumors in many countries, including Korea and China.
Aim of the study: This study aimed to assess anti-inflammatory and related activities of an ethanol extract (SPEE) prepared from the dried whole parts of *Salvia plebeia*.
Materials and methods: Anti-angiogenic and anti-nociceptive activities of SPEE were analyzed using the chick chorioallantoic membrane (CAM) assay and acetic acid-induced writhing response, respectively. Anti-inflammatory activity of SPEE was evaluated using acetic acid-induced vascular permeability, carrageenan-induced inflammation in the air pouch and analyses of nitrite content and induced nitric oxide synthase (iNOS) level in the macrophage cells.
Results: SPEE gave rise to a significant inhibition in chick chorioallantoic membrane angiogenesis. SPEE exhibited anti-inflammatory activities in vascular permeability and air-pouch models. In the air-pouch model, SPEE was able to diminish exudate volume, number of polymorphonulcear leukocytes and nitrite content. SPEE also displayed anti-nociceptive activity in the writhing response model in mice. SPEE significantly decreased nitrite content and induced nitric oxide synthase (iNOS) in the lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells, while it could not modulate cyclooxygenase-2 (COX-2) and matrix metalloproteinase-9 levels in the stimulated phages. SPEE decreased reactive oxygen species (ROS) level in the stimulated macrophages.
Conclusion: The ethanol extract (SPEE) of *Salvia plebeia* possesses anti-inflammatory and related anti-angiogenic, anti-nociceptive and antioxidant activities, which offers partial support to its folkloric use.

1. Introduction

Salvia plebeia R.Br. (Labiatae), an annual, hairy herb widely distributed in many countries, such as Korea, China, India, Iran and Australia, is used as a folk medicine for the treatment of a variety of inflammatory diseases including hepatitis, cough, diarrhea, gonorrhea, menorrhagia, tumors and hemorrhoids (Chopra et al., 1986; Jin et al., 2008). Phytochemical analyses have shown that *Salvia plebeia* possesses royleanonic acid, hispidulin, eupatorin, luteolin, nepetin, coniferyl aldehyde, and 2′-hydroxy-5′-methoxybiochanin A, which contain antioxidant properties (Ai-li and Chang-hai, 2006; Jin et al., 2008; Weng and Wang, 2000).

A methanol extract of *Salvia plebeia* displayed antioxidant and nitrite scavenging activities in *in vitro* experimental models (Lim et al., 2007). Other extracts and pure components prepared from *Salvia plebeia* also showed strong antioxidant activities using the antioxidant stability instrument (Ai-li and Chang-hai, 2006; Gu and Weng, 2001; Weng and Wang, 2000). An aqueous extract of *Salvia plebeia* exhibited antiallergic activities by inhibiting passive cutaneous anaphylaxis and the histamine release form rat peritoneal mast cells in a dose-dependent manner (Shin and Kim, 2002). Extracts of *Salvia plebeia* were able to suppress the growth of tumor cells and enhance the tumoricidal activity of macrophages in combination with interferon-γ (Um et al., 1996). In this work, it is demonstrated that an ethanol extract of *Salvia plebeia* contain anti-inflammatory and related anti-angiogenic and anti-nociceptive activities in *in vivo* and *in vitro* models, which partly supports its folkloric use.

2. Materials and methods

2.1. Chemicals and fertilized eggs

Evans blue, retinoic acid, indomethacin (IND), dexamethasone (DEXA), *Escherichia coli* lipopolysaccharide (LPS), λ-carrageenan,

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carboxymethyl cellulose (CMC), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenylmethanosulfonyl fluoride, leupeptin and pepstatin and Griess reagent were obtained from Sigma Chemical Co. (St. Louis, MI, USA). Fertilized brown Leghorn eggs were purchased from Pulmuone Food Co., Seoul, Korea. All other chemicals used were of reagent grade or better. All experiments, done in this work, were independently repeated at least three times.

2.2. Plant material

The whole parts of *Salvia plebeia*, obtained at a local market, Kangwon-do, Korea, in March, 2008. The botanical identity was authenticated by Prof. Ki-Oug Yoo, Department of Biology, Kangwon National University, Chuncheon, Korea. Its voucher specimen was deposited in the herbarium of Department of Biology, College of Natural Sciences, Kangwon National University under the acquisition number KWNU56525.

2.3. Experimental animals

Male ICR mice (about 25 g) were purchased from Samtaco Animal Farm, Osan, Korea. They were housed under the condition of 23 ± 2 °C with a 12-h light/dark cycle. Food and tap water were supplied ad libitum. At least 7 mice were used in each experimental group. The ethical guidelines, described in the NIH Guide for Care and Use of Laboratory Animals, were followed throughout the animal experiments. Animal experiments performed in this work were approved by the Ethical Committee, Kangwon National University, Chuncheon, Korea.

2.4. Preparation of the ethanol extract (SPEE)

The dried whole parts of *Salvia plebeia* were ground and extracted with 70% ethanol for 1 week at room temperature. The liquid extract was evaporated *in vacuo* to generate its dried powder (SPEE) with a yield of 16.8%. In animal experiments, SPEE was dissolved in 1% CMC in saline, whereas, in other experiments, it was dissolved in 70% ethanol.

2.5. GC/MS analysis

GC/MS analysis was done on an HP-6890 GC/HP 5973 MSD system using a fused silica capillary column (30 m × 0.25 mm i.d., thickness 0.25 μm, DB-5MS 122-5532). The oven temperature, maintained at 100 °C, was programmed at 5 °C/min to 270 °C. Helium was used as a carrier gas. Identifications were performed by comparison of spectra with mass spectral databases (NIST, 2005).

2.6. Cell culture

The RAW264.7 cells, a murine macrophage cell line, were obtained from American Type Culture Collection (Manassas, VA, USA). The murine cells were grown in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES (pH 7.5), 100 μg/ml streptomycin and 100 units/ml penicillin. The cells, plated at a density of 1×10^6 and preincubated for 24 h at 37 °C, were maintained in a humidified atmosphere with 5% CO_2. The mammalian cells were grown to 80–90% confluence, and subjected to no more than 20 cell passages.

2.7. Chorioallantoic membrane (CAM) assay

An anti-angiogenic activity of SPEE was determined using CAM assay as earlier described (Song et al., 2004). After the fertilized chicken eggs were kept for 3.5 days in a humidified egg incubator at 37 °C, about 2 ml of albumen was aspirated through the small hole drilled at the narrow end of the eggs, allowing the small chorioallantoic membrane and yolk sac to drop away from the shell membrane. In the 4.5-day-old chick embryo, an SPEE-loaded Thermanox coverslip was applied onto the CAM surface. Two days after returning the chick embryo to the egg incubator, an appropriate volume of 10% fat emulsion (Intralipose®) was injected into a 6.5-day-old embryo chorioallantois. The branching pattern of each egg, observed under a microscope, was graded as 0, 1+ or 2+. Convergence of a few vessels toward the CAM surface was denoted as 1+, and 2+ reflected an increased density and length of vessels toward the CAM surface.

2.8. Acetic acid-induced vascular permeability

An acetic acid-induced vascular permeability test was carried out using a modification of the method of Whittle (1964). Fifty minutes after oral administration of vehicle (1% CMC in saline), SPEE (50, 100 or 200 mg/kg) or indomethacin (IND, 10 mg/kg), 0.1 ml/10 g body weight of 2% Evans blue was injected intravenously into each mouse. Ten minutes later, 0.1 ml/10 g body weight of 0.7% acetic acid in saline was intraperitoneally injected. Twenty minutes after the injection of acetic acid, the mice were sacrificed by cervical dislocation. After 10 ml of saline was injected into the abdominal cavity, the washings were collected into test tubes. Concentrations of Evans blue in the washings were determined by the absorbance at 590 nm. The vascular permeability was represented in terms of the absorbance (A_{590}).

2.9. Acetic acid-induced writhing response

Anti-nociceptive activity of SPEE was determined by measuring the response to an intraperitoneal injection of acetic acid solution, manifesting as a contraction of the abdominal muscles and stretching of hind limbs (Olajide et al., 2000). Each experimental group was administered orally with vehicle (1% CMC in saline), SPEE (50, 100 or 200 mg/kg) or indomethacin (IND, 10 mg/kg). One hour after the oral administration, 0.7% acetic acid at the dose of 0.1 ml/10 g body weight was injected. From 10 min later, the number of writhes was counted during the following 10 min period.

2.10. Carrageenan-induced inflammation in the air pouch

Following a modification of the procedure of Ghosh et al. (2000), λ-carrageenan-induced inflammation test in the air pouch was carried out. Six days before sample administration, the air pouches were made in the intrascapular region of mice by initial subcutaneous injection of 4 ml sterile air, and 3 days later, reinforced with additional 2 ml sterile air. On day 0, vehicle (1% CMC in saline), SPEE (0.1, 0.3 or 1.0 mg/pouch) or dexamethasone (DEXA, 0.01 mg/pouch) was administered into the pouch right after the injection of 1 ml of 2% λ-carrageenan. After 16 h, the pouch cavity was opened and the exudate was collected. The exudate volumes were measured using a graduate tube, and the polymorphonuclear leukocytes in the diluted aliquots were counted in a standard hemocytometer chamber.

2.11. Nitrite analysis

Nitrite (NO_2^-) contents in the media obtained from the cell cultures and the exudates prepared from the air pouches were determined based on the Griess reaction (Sherman et al., 1993). The samples (100 μl) were reacted with 100 μl Griess reagent (6 mg/ml) for 10 min at room temperature, and then NO_2^- concentration was