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Heme oxygenase 1-mediated novel anti-inflammatory activities of *Salvia plebeia* and its active components

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ABSTRACT

Ethnopharmacological relevance: Salvia plebeia R. Br. (SP) has been widely used as a traditional folk medicine for the treatment of infectious diseases and pain. An anti-inflammatory potential of SP has remains largely unknown.

Aim of the study: We tried to elucidate the principle mechanism and the active ingredients underlying the anti-inflammatory activities of SP.

Materials and methods: We investigated the protective activities of SP methanolic extract (SPME) and seven representative ingredients against inflammation. Quantitative analysis using HPLC-DAD-ESI/MS was conducted to determine the relative amounts of these seven active ingredients in SPME. Both *in vitro* murine macrophages and *in vivo* mouse models were employed to elucidate SP- and active ingredient-mediated anti-inflammatory effects.

Results: SPME significantly reduced inflammatory processes both *in vivo* in a TPA-induced ear edema model and *in vitro* in lipopolysaccharide (LPS)-activated macrophages. SPME decreased the release of nitric oxide (NO) and prostaglandin E_2 (PGE_2) and expression of inducible nitric oxide synthase (iNOS). Seven active components (luteoloside (C1), nepitrin (C2), homoplantagenin (C3), luteolin (C4), nepetin (C5), hispidulin (C6), and eupatorin (C7)) of SPME were analyzed and their relative concentrations were determined, demonstrating that C2, C3, C5 and C6 were present in higher amounts than were C1, C4, and C7. These major compounds inhibited NO and PGE_2 production, and iNOS and COX-II protein expression through heme oxygenase-1 (HO-1) induction *via* activation of nuclear factor erythroid 2–related factor2 (Nrf2).

Conclusion: Our data demonstrate that SPME possesses potent *in vitro* and *in vivo* anti-inflammatory activities. Nepetin and hispidulin, and their glycosides are the major active compounds in SPME, and their effects are mediated by Nrf2/HO-1 signaling. Taken together, we propose that SPME and its active ingredients may serve as novel therapeutic candidates for diseases associated with excessive inflammation.

1. Introduction

Inflammation, as the innate immune response, is a protective response to tissue injury. However, the association between chronic and excessive inflammation and a variety of disorders such as cancer, autoimmune diseases, cardiovascular problems, and degenerative neurological diseases is well-established (Lin and Tang, 2008; Scrivo et al., 2011). Modulation of inflammatory reactions is known to be essential to reduce or prevent these diseases, and also to alleviate painful conditions. Although the control of inflammation has long been a traditional therapeutic goal, the incidence of certain inflammatory diseases, such as asthma and allergies, has been significantly increased over the past few decades, and inflammation remains a major therapeutic target (Thorburn et al., 2014). Currently available anti-inflammatory treatments mainly consist of glucosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and immunosuppressive drugs. While these drug treatments are commonly used to treat pain and chronic conditions associated with inflammation, their usage is

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often hampered either by low efficacy or severe side effects. There remains a continuing need to develop novel anti-inflammatory compounds, and herbal medicinal plants, which have been traditionally used to treat or prevent chronic inflammatory diseases, may serve as good sources of candidates.

Due to the complexity of inflammatory processes, several strategies have been established to develop anti-inflammatory activities. Regulation of inflammatory mediators is one such strategy that could serve to minimize amplification of inflammatory reactions. During the process of inflammation, infiltrating cells release various mediators, including nitric oxide (NO), prostaglandins (PGs), and interleukins (ILs) (Mequanint et al., 2011). The synthesis and release of these inflammatory mediators are affected by the activity of corresponding enzymes, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-II (COX-II) (Yun et al., 2008). Among the various cellular signaling pathways responsible for modulation of these pro-inflammatory enzymes, nuclear factor erythroid 2–related factor 2 (Nrf2) has been suggested to play important roles through the regulation of expression of stress-related genes, including hemeoxygenases (HO) (Nguyen et al., 2003). Recent studies have revealed that pharmacologic upregulation of HO-1, a stress-inducible isoform of the 32 kDa heat shock protein, can attenuate inflammatory processes, and provided protective effects against inflammation in a murine model of non-autoimmune arthritis and sepsis (Benallaoua et al., 2007; Otterbein and Choi, 2000; Park et al., 2013).

Salvia Plebeia R. Br. (SP) belongs to the family Lamiaceae, and is an edible plant widely distributed in many countries including Australia, India, China, Japan, and Korea (Nugroho et al., 2012; Sales et al., 2010). Ethnopharmacological evidence indicates that this plant has been used in folk medicines to treat various inflammatory diseases, such as menorrhagia, gonorrhea, diarrhea, hemorrhoids, tumors, cough, and hepatitis (Jung et al., 2009). Extracts and pure compounds isolated from SP possess potent anti-oxidant, and radical scavenging activities (Ai-li and Chang-hai, 2006; Gu and Weng, 2001; Kang et al., 2003; Lim et al., 2007; Weng and Wang, 2000). Additional biological activities, such as sedative, gastro-protective, hepato-protective, anti-allergic and anti-cancer properties have also been reported (Bae et al., 2007; Nugroho et al., 2012; Jin et al., 2011; Jo et al., 2010; Shin and Kim, 2002). Several flavonoid compounds have been isolated from this plant, such as hispidulin, homoplantaginin, nepetin, nepitrin, luteolin, luteoloside, or eupatorin (Ai-li and Chang-hai, 2006; Gu and Weng, 2001; Jin et al., 2008).

An anti-inflammatory activity of the ethanolic extract of whole parts of SP has been demonstrated in macrophages *in vitro* and in *in vivo* models, using a chorioallantoic membrane (CAM) assay and and acetic acid-induced writhing response test (Jung et al., 2009). A recent paper by Choi et al. (2015) showed that an SP ethanolic extract significantly reduced arthritis in a murine model (Choi et al., 2015). Jo et al. (2010) also reported that SP aqueous extract inhibited the production of pro-inflammatory mediators including TNF-α, IL-6 and NO in lipopolysaccharide-activated macrophages (Jo et al., 2010). Although an anti-inflammatory potential of SP is supported by traditional and experimental evidence, the principle active ingredient and its mode of action still remain uncertain.

In this study, we examined the anti-inflammatory activity of a methanolic extract of SP (SPME) in a mouse model of ear edema. Using activated macrophages, we confirmed that SPME inhibited the release of inflammatory mediators of NO and PGE_2. To identify its principle components, we examined the relative concentrations and anti-inflammatory activities of seven representative flavonoids in SPME. The identity of the active compounds, their relative concentrations in SPME, and the underlying mechanisms were elucidated. Our study provides new insights into the anti-inflammatory activity of SP, as well as the therapeutic potential of its active ingredients as novel treatments for inflammatory diseases.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS, *Escherichia coli* O111:B4), dimethyl sulfoxide (DMSO), sodium nitrite, 2-mercaptoethanol, indomethacin, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and phorbol 12-myristate 13-acetate (TPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard compounds luteoloside, nepitrin, homoplantagenin, luteolin, nepetin, hispidulin, and eupatorin were purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Primary antibodies to iNOS, COX-II and Nrf2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against β-actin, GAPDH, HO-1, and Lamin A/C were purchased from Sigma-Aldrich, Millipore (Billerica, MA, USA), Enzo Life Sciences (Plymouth Meeting, PA, USA), and Cell Signaling Technology (Beverly, MA, USA), respectively. Goat anti-IgG horseradish peroxidase-conjugated secondary antibody was purchased either from Santa Cruz Biotechnology Inc. (anti-mouse IgG; Santa Cruz, CA, USA) or from Biorad (anti-rabbit IgG; Hercules, CA, USA).

2.2. Plant materials and preparation of extracts from Salvia plebeia

The leaves of *Salvia plebeia R. Br.* (SP) were purchased at a local herbal market in the southern area of Korea, in September 2012. The plant was authenticated and a voucher specimen has been deposited in the Herbarium of the College of Pharmacy, Hanyang University (No. HYUP-DP-002). The dried SP leaves (1 kg) were extracted with 3 L of 70% MeOH for 2 weeks at room temperature. The SP extract solution was vaporized under reduced pressure to yield a crude total methanolic extract (SPME). The methanolic extract was vaporized to dryness under reduced pressure and stored at 4 °C.

2.3. Identification and quantification of active ingredients in SPME

Seven representative active ingredients (luteoloside (C1), nepitrin (C2), homoplantagenin (C3), luteolin (C4), nepetin (C5), hispidulin (C6) and eupatorin (C7)) were selected based on previous reports (Ai-li and Chang-hai, 2006; Gu and Weng, 2001; Jin et al., 2008; Lee et al., 2010; Weng and Wang, 2000), and standard compounds of each components were purchased from Biopurify Phytochemicals Ltd. (Chengdu, China). Flavonoids in SPME were analyzed by HPLC-DAD-ESI/MS as follows: Agilent1260 HPLC system with Inno C18 column (250 mm × 4.6 mm, 5 μm, Young Jin Biochrom Co., Ltd., Seongnam, Korea) was used at a flow rate 1 mL/min of mobile phase controlled by binary pumps at 40 °C. The mobile phase was, A = acetonitrile (0.1% formic acid) B = water (0.1% formic acid), run under gradient elution of 10–90% A (0–50 min). Column was equilibrated for 10 min before the next injection and injection volume was 10 μL. The effluent was monitored at 254 nm, and the UV spectrum range was 200–400 nm. The ESI-MS data were obtained in Advion Expression CMS system (New York, USA). ESI-MS conditions were as follows: negative (C1–C6) and positive (C7) ion mode; mass range, *m/z* 100–1000; capillary temperature, 200 °C; capillary voltage, 150 V; source voltage offset, 30; Source voltage span, 10; source gas temperature, 150 °C; ESI voltage, 3500 V.

Quantitation of flavonoids in SPME was performed by calibration curve equation corresponding to their reference standard