Stylopine from *Chelidonium majus* Inhibits LPS-Induced Inflammatory Mediators in RAW 264.7 Cells

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Stylopine is a major component of the leaf of *Chelidonium majus* L. (Papaveraceae), which has been used for the removal of warts, papillomas and condylomas, as well as the treatment of liver disease, in oriental countries. Stylopine per se had no cytotoxic effect in unstimulated RAW 264.7 cells, but concentration-dependently reduced nitric oxide (NO), prostaglandin E₂ (PGE₂), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and the IL-6 production and cyclooxygenase-2 (COX-2) activity caused by the LPS stimulation. The levels of inducible nitric oxide synthase (iNOS) and COX-2 protein expressions were markedly suppressed by stylopine in a concentration dependent manner. These results suggest that stylopine suppress the NO and PGE₂ production in macrophages by inhibiting the iNOS and COX-2 expressions. These biological activities of stylopine may contribute to the anti-inflammatory activity of *Chelidonium majus*.

**Key words:** Chelidonium majus, Stylopine, Nitric oxide, Tumor necrosis factor-α, Interleukin-1β, Interleukin-6

INTRODUCTION

Nitric oxide (NO), prostaglandin E₂ (PGE₂) and the cytokines, such as interleukin-1 beta (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α), are well known for their involvement in the development of inflammation (Lee et al., 1992; Moncada et al., 1991; Sautebin, 2000; Wheeler and Bernard, 1999). Macrophages play significant roles in inflammatory diseases by producing these inflammatory mediators. Following exposure to immune stimulants, including bacterial toxins, such as lipopolysaccharide (LPS) and lipoteichoic acid, the production of these mediators from macrophages has been found in many inflammatory tissue along with the increased expressions of their mRNAs (Penglist et al., 2000; Yamashita et al., 2000). Although NO and pro-inflammatory cytokines are involved in the host defense mechanism, their over-production contributes to the pathogenesis of several diseases, such as sepsis, rheumatoid arthritis, atherosclerosis, pulmonary fibrosis and chronic hepatitis (Coker and Laurent, 1998; Isomaki and Punnonen, 1997). Thus, the inhibition of the production of these inflammatory mediators may prevent or suppress various inflammatory diseases.

*Chelidonium majus* L. (Papaveraceae) (*C. majus*) is a plant of great interest for its usage as an herbal medicine for various diseases in Chinese and European countries. The crude extracts from various parts of the plant, such as the roots, shoots and leaves, have been reported to contain several alkaloids, such as stylopine, sanguinarine, chelidonine, chelerythrine, berberine and coptisine (Colombo and Bosisio, 1996). Both the crude extracts of *C. majus* and purified compounds from these extracts have been reported to exhibit interesting anti-viral, anti-inflammatory, anti-tumor and anti-microbial properties both in vitro and in vivo (Colombo and Bosisio, 1996;
Stylopine is a major component of various phyto-medicinal plants, including C. majus, which has been reported to exhibit allosteric modulation of the GABAA receptor, detoxification of xenobiotics and anti-inflammatory properties (Haberlein et al., 1996; Sauto et al., 2002; Ikezawa et al., 2003). However, the effects of stylopine from C. majus on LPS-stimulated NO, PGE2 and pro-inflammatory cytokine production remain to be defined. Therefore, the effect of stylopine from C. majus on the production of NO, PGE2, and pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), and the expressions of inducible nitric oxide synthase (iNOS) and COX-2 were investigated in RAW 264.7 macrophages activated by LPS.

MATERIALS AND METHODS

Chemicals and reagents
The Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from GIBCO BRL (Grand Island, NY). The Rabbit anti-iNOS, rabbit anti-COX-1 and COX-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The LPS (phenol extracted Salmonella enteritidis), Tween 20, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS) and N,N-dimethylarginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. (ST Louis, MO). The TNF-α, IL-1β, IL-6, and PGE2 immunoassay kits (Quantikine™) were purchased from R &D System (Minneapolis, MN, USA). Ninety-six well tissue culture plates and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). All reagents were tested for their LPS content using a colorimetric Limulus amoebocyte lysate assay (detection limit, 10 pg/mL; Whitaker Bioproducts, Walkersville, MD).

Plant material
The leaves of Chelidonium majus L. (Papaveraceae) were purchased from the herbal medicine co-operative association of Jeonbuk Province, Korea, in October 2003. A voucher specimen (no. LGF777) was deposited at the Herbarium of the College of Oriental Medicine, Wonkwang University (Korea).

Extraction and isolation
The dried and powered root (2.0 kg) of C. majus was extracted three times with MeOH (6 L) for 7 days at room temperature. The combined MeOH extract was concentrated under reduced pressure to yield dark brown syrup (72 g). The MeOH extract (70 g) was suspended in H2O (1 L) and sequentially partitioned with n-hexane, CHCl3, EtOAc and n-BuOH. A portion of the CH3Cl2 soluble fraction (14 g) was subjected to chromatography on a silica gel column (400 g) using 100% n-hexane, n-hexane-EtOAc (50:1, 25: 1, 12:1, 5:1, 1:1), then 100% EtOAc to obtain 6 fractions (Fr. SM-31; 41 mg, 400 mL, Fr. SM-32; 66 mg, 800 mL, Fr. SM-33; 2250 mg, 800 mL, Fr. SM-34; 890 mg, 800 mL, Fr. SM-35; 231 mg, 800 mL, Fr. SM-36; 760 mg). A portion (150 mg) of the active fraction (Fr. SM-33) was subjected to recycling-preparative HPLC (CHCl3, flow rate; 3.5 mL/min) to yield a compound (91 mg, tr = 26.5 min). The compound was identified as stylopine (Fig. 1) by the comparison of its spectral data (MS, 1D NMR, and 2D NMR) with those of the alkaloid reported in the literature (Suau et al., 2002).

Cell culture
The murine macrophage RAW 264.7 cell line, obtained from American Type Culture Collection (ATCC, TIB 71, Maryland, USA), was maintained in 1x10^6 cells/mL cultures in DMEM supplemented with 10% heat inactivated FBS, penicillin G (100 IU/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM), and incubated at 37°C in a humidified 5% CO2 and 95% air atmosphere. On the following day, the medium was replaced with fresh DMEM, and the cells then stimulated with LPS (1 μg/mL), in the presence or absence of stylopine from Chelidonium majus, for the indicated periods. The stylopine was dissolved in DMSO and diluted with the medium to the final concentration.