Glossogyne tenuifolia Acts to Inhibit Inflammatory Mediator Production in a Macrophage Cell Line by Downregulating LPS-Induced NF-κB

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Abstract
Glossogyne tenuifolia (hsiang-ju) (GT) is a traditional antipyretic herb used in Chinese medicine; however, no information is available to explain its action. The objective of this research was to elucidate the molecular pharmacological activity and the effective components in the ethanol extract of GT. We found that GT had potent anti-inflammatory effects on the lipopolysaccharide (LPS)-activated murine macrophages, RAW264.7. GT downregulated LPS-induced expression of inducible nitric oxide synthase (iNOS) by blocking its transcription. GT also caused a dose-dependent inhibition of the release of prostaglandin E2 by repressing the promoter activity of the inducible cyclooxygenase (COX-2) gene. Moreover, GT exerted a dose-dependent inhibition of the LPS-stimulated release of the proinflammatory cytokines, TNF-α, IL-1β, IL-6, and IL-12. To determine the mechanism by which GT inhibits LPS signaling, we focused on nuclear factor-κB (NF-κB) activation. Western blot analysis revealed that GT abolished LPS-induced inhibitor-κB phosphorylation. The electrophoretic mobility shift assay demonstrated that GT abolished LPS-mediated κB DNA binding activity. Moreover, macrophages were transfected with a vector coding for the luciferase reporter gene under the control of NF-κB cis-acting elements, and the transfected macrophages showed that the LPS-stimulated luciferase activity was GT-sensitive. These results suggest that GT attenuates inflammatory mediator synthesis of activated macrophages through an NF-κB-dependent pathway. The active components of GT were identified as oleanolic acid and luteolin-7-glucoside. Both of these compounds inhibited LPS-stimulated inflammatory mediator production and NF-κB activation. We conclude that GT inhibits NF-κB-mediated gene expression and downregulates inflammatory mediator production in murine macrophages.

Introduction
Inflammatory responses are typically present as a series of vascular and cellular reactions initiated by injury or infection. Activation of macrophages is a key event in
the initiation and propagation of these defensive reactions. When stimulated by pathologic stimuli or injury, macrophages release nitric oxide (NO), prostaglandin E₂ (PGE₂), TNF-α, interleukin (IL)-1, IL-6, IL-12, and other proinflammatory cytokines that augment the host's defense against invasion by microbes. The release of inflammatory mediators is essential for host survival from infection, and is also required for the repair of tissue injury [8, 18, 24]. These beneficial effects, however, are critically dependent on the magnitude of the immune response, because large amounts of macrophage-derived inflammatory mediators can also cause collateral damage to normal cells [8, 9, 11] and are potentially lethal when the release is sufficient to cause systemic exposure [25]. Thus, inhibiting the overproduction of inflammatory mediators is an important therapeutic goal for drug development.

Macrophage-derived cytokines and chemokines are involved at multiple steps of both innate and inflammatory responses, and the transcriptional factor, nuclear factor-κB (NF-κB), appears to play a pivotal role in their coordinated upregulation [2]. NF-κB exists within the cytoplasm in an inactive form associated with regulatory proteins, called inhibitors of κB (IκB). When stimulated by various extracellular signals, including lipopolysaccharide (LPS), signal cascades lead to phosphorylation of IκB, which is then ubiquitinated, thereby releasing NF-κB dimers from the cytoplasmic NF-κB-IκB complex, and allowing them to translocate to the nucleus [3]. The identification of NF-κB as a key factor in the pathogenesis of inflammation suggests that NF-κB-targeted therapeutics might be effective in treating human inflammatory diseases. A variety of pharmacologic agents have been described to inhibit NF-κB at one or more activation steps of the signaling pathway. These agents include proteasome inhibitors, glucocorticoids, nonsteroidal anti-inflammatory drugs, natural compounds, and anti-inflammatory cytokines [30].

**Glossogyne tenuifolia** Cassini (hsiang-ju) (GT) is a plant native to Penghu, also known as the Pescadore Islands, Taiwan, and has a long history of being used as an antipyretic, detoxication, and anti-inflammatory remedy in folk medicine among local residents. However, no information regarding its mechanism of action or the phytochemical components responsible for its activity is available.

In this study, we investigated the detailed anti-inflammatory mechanisms of the ethanol extract of GT on the LPS-stimulated murine macrophage cell line, RAW264.7. The data suggest that GT blocks NF-κB activation, thereby inhibiting the induction of inducible nitric oxide synthase (iNOS) transcription and the release of inflammatory cytokines. Furthermore, we identified oleanolic acid and luteolin-7-glucoside as its active anti-inflammatory principles.

### Materials and Methods

**Preparation of the Ethanol Extract of GT**

GT was obtained from the Kaohsiung District Agricultural Improvement Station in Penghu, Taiwan, and the voucher specimens were deposited in the herbarium of Chia-Nan University of Pharmacy and Science. The dry above-ground part (5.46 kg) was extracted with ethanol (10 liters) at room temperature overnight four times followed by filtration. The flow-through was concentrated in vacuo to yield a dark-brown syrup (623.4 g), which was diluted with ethanol.

**Extraction and Isolation**

The crude ethanol extract of GT (623.4 g) was partitioned between hexane and 95% methanol. The 95% methanol layer was concentrated and partitioned with ethyl acetate and water. The hexane layer was passed through a chromatography column (silica gel 70, 230-400 mesh; Merck, Darmstadt, Germany) and eluted with an hexane-CH₂Cl₂ (1:1) mixture as moving solvent to obtain a purple syrup. The hexane layer with acetone and methanol. The ethyl acetate mixture was chromatographed on a silica gel (silica gel 70-230 mesh, Merck) eluted with hexane-CH₂Cl₂ (1:1) to produce luteolin-7-glucoside (1.19 g). All four compounds were identified by direct comparison (1H, 13C NMR) with authentic samples.

**Cell Culture**

RAW 264.7 cells were purchased from Culture Collection and Research Center (Hsinchu, Taiwan) and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (HyClone, Logan, Utah, USA), 2 mM glutamine, 1% nonessential amino acid, 1 mM pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, Calif., USA). Cells were maintained in a humidified incubator at 37 °C in 5% CO₂.

**Nitrite Measurement**

Nitrite production, an indicator of NO synthesis, was determined by the Griess reaction. The supernatant of cell cultures was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). The optical density at 550 nm (A₅₅₀) was measured and calculated against a sodium nitrite standard curve.

**Cell Viability**

Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to purple formazan [1]. Cells were incubated with MTT (0.5%) for 4 h at 37 °C. The medium was removed by aspiration, and formazan crystals were dissolved in DMSO. The extent of the reduction of MTT was quantitated by measurement of A₅₅₀.