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Effect of Siam weed extract and its bioactive component scutellarein tetramethyl ether on anti-inflammatory activity through NF-κB pathway

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ABSTRACT

Ethnopharmacological relevance: Siam weed (*Chromolaena odorata* (L.) King and Robinson) is a medicinal herb used for wound healing and inflammation-related diseases.
Aim of the study: In this study, we evaluated the molecular mechanism by which Siam weed extract (SWE) and its bioactive components, scutellarein tetramethyl ether (scu), stigmasterol, and isosakuranetin affect anti-inflammatory activity.
Materials and methods: The expression of several inflammatory proteins in RAW 264.7 (murine) macrophages was assessed by Western blot and reverse transcription-polymerase chain reaction (RT-PCR). Biochemical assays including prostaglandin E_2 (PGE_2) and nitric-oxide (NO) quantification were performed. Luciferase promoter activity and immunocytochemistry of Nuclear factor-κB (NF-κB) were investigated.
Results: Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are critical pro-inflammatory proteins. The level of protein and mRNA expression of these enzymes induced by lipopolysaccharide (LPS) was dramatically suppressed by treatment with SWE, scu, or stigmasterol compounds in a dose-dependent manner. They also reduced PGE_2 and NO release. We further analyzed the NF-κB pathway and found that the scu compound suppressed IκB kinase complex alpha/beta (IKKα/β) and Inhibitory-kappa-B-alpha (IκBα), thereby suppressing COX-2 and iNOS expression.
Conclusion: This is the first report of the anti-inflammatory molecular mechanism in SWE and/or its bioactive component scu, indicating alteration NF-κB pathway and further providing potential uses in the treatment of inflammatory-related diseases.

1. Introduction

Siam weed (*Chromolaena odorata* (L.) King and Robinson), formerly known as *Eupatorium odoratum* Linn. is a perennial scandent or semi-woody shrub in the Asteraceae family (Pandith and Gritsanapan, 2012). It is native to Central and South America; however, it is also found in Florida and Texas and spreads throughout the tropical and subtropical areas of the world, including Thailand. Siam weed has been used as a medicinal herb in many countries of tropical Africa and Asia for a variety of ailments such as fever, influenza, cold, cough, diabetes, malaria, bleeding, inflammation, and wound healing (Pisutthanan et al., 2005). Recent studies demonstrated that Siam weed extract (SWE) could enhance many pharmacological activities (Akah, 1990; Khengraeng, 2007; Thongpraditchot et al., 1994; Triratana et al., 1991; Wongkrajang et al., 1990; Wongkrajang et al., 1994); however, the molecular mechanism by which SWE affects anti-inflammation has not been elucidated.

Inflammation is a response to any tissue injury in the body caused by infection, trauma, chemicals, heat or unrecognized particles (Parimala et al., 2010). Inflammation leads to the up-regulation of several kinds of pro-inflammatory cytokines. Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase

Abbreviations: COX-2, Cyclooxygenase-2; IκBα, Inhibitory-kappa-B-alpha; IKKα/β, IκB kinase complex alpha/beta; iNOS, Inducible nitro oxide synthase; LPS, Lipopolysaccharide; NF-κB, Nuclear factor-kappaB; NO, Nitric-oxide; PGE_2, Prostaglandin E_2; RT-PCR, Reverse transcription-polymerase chain reaction; Scu, Scutellarein tetramethyl ether; SWE, Siam weed extract.

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(iNOS) are the pro-inflammatory enzymes that play a critical role in inflammation. They produce pro-inflammatory mediators, prostaglandin E_2 (PGE_2) and nitric oxide (NO), respectively, thereby enhancing expression of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β). Lipopolysaccharide (LPS) is a well-known endotoxin found in the outer membrane of Gram-negative bacteria and triggers the secretion of pro-inflammatory cytokines in many cell types, through the nuclear factor-kappaB (NF-κB)/COX-2 pathway in macrophages. The iNOS makes NO from L-arginine and is highly expressed in macrophages in response to inflammatory mediators, such as LPS, interferon-γ (IFN-γ), or pro-inflammatory cytokines. Transcription of COX-2 and iNOS are activated via the NF-κB pathway (Kim et al., 2006; Pan et al., 2008). Therefore, NF-κB is multipotent transcription factor and plays a key role in regulating the immune response to inflammatory cancer and autoimmune diseases. NF-κB is presented in the cytoplasm as inactive heterodimers composed of two subunits p50 and p65, and are bound to inhibitor proteins called IκB. Activation of the NF-κB pathway results from the phosphorylation of the IκB kinase complex (IKK). IKK-α is the predominant IκB kinase, which subsequently phosphorylates IκB, followed by the ubiquitination and proteasome-mediated degradation of IκBs. Finally, the unbound NF-κB translocates to the nucleus, binds to *cis*-acting NF-κB element, and literally turns on the particular genes, including inflammatory cytokines, COX-2, iNOS, TNF-α, and IL-1β (Pan et al., 2008).

It is known that SWE is capable of anti-inflammatory activity both *in vitro* and *in vivo*. When the methanolic extract from the aerial parts was partitioned with different solvents, only the butanolic fraction moderately inhibited COX-1 and COX-2 (Pisutthanan et al., 2005). The ethyl acetate fraction from leaf extract inhibited NO production as determined by using the Griess assay, and also inhibited the NF-κB activity in RAW 264.7 cells (Hanh et al., 2011; Lessio et al., 2005; Park et al., 2012; Sun et al., 2003). Ethanolic leaf extract exhibited high inhibitory activity against TNF-α production from *Propionibacterium acnes*, a pus-forming bacteria that triggers inflammation acne (> 70% inhibition) (Chomnawang et al., 2007). The dichloromethane extract inhibited the TNF-α and IL-1β induced by LPS of human gingival fibroblast (HGF) and monocyte (U937) cell lines, respectively (Rodanant et al., 2012). Moreover, ethanolic extract exhibited strong inhibition in tetradecanoyl phorbol acetate-induced ear edema model (Mustapha et al., 2000). Thus, it has been reported that SWE and its extract exhibit anti-inflammatory activity as described above, the molecular mechanism behind the effects of SWE and its bioactive pure compounds on anti-inflammation has not been determined in details.

The scutellarein tetramethyl ether (Scu) (4′,5,6,7-tetramethoxyflavone) (Fig. 1A) and isosakuranetin (Fig. 1C) were isolated from SWE and found as a bioactive components for hemostatic and anti-inflammatory activities, respectively (Ling et al., 2007c; Triratana et al., 1991). From our chemical screening of 70% ethanolic leaf extract by thin-layer chromatography comparing with authentic samples, we found that the scu and also the stigmasterol (Fig. 1B) are the major components of this plant extract. The stigmasterol has also been reported that possess the anti-inflammatory activity (Gabay et al., 2010; Lee et al., 2006). In this study, we examined the effect of SWE and its major bioactive components (scu, stigmasterol and isosakuranetin) on the expression of COX-2 and iNOS protein/ mRNA induced by LPS in RAW 264.7 (murine) macrophages. We also confirmed the inhibition of NO and PGE_2 production by SWE and its bioactive compounds. Finally, to clarify the mechanism for anti-inflammatory activities of SWE and its bioactive components, we examined the expression of proteins involved in the NF-κB pathway, particularly p65 translocation in the presence of scu.

2. Materials and methods

2.1. Preparation of leaf SWE

From October 2009-January 2010, Siam weed leaves were collected from 10 different locations in Thailand: northern part: N1 (Chiang Rai) and N2 (Chiang Mai); central part: C1 (Samut Sakhon) and C2 (Saraburi); northeastern parts: NE1 (Nakhon Ratchasima) and NE2 (Yasothon); eastern part E1 (Chanthaburi) and E2 (Chon Buri) and southern part: S1 (Phattalung) and S2 (Surat Thani). The plant samples were identified by Dr. Wandee Gritsanapan and the voucher specimen (CO100901-CO100910) was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The leaves were dried in a hot air oven at 60 °C for 24 h. The dried samples were ground into moderate powder. Then, 100 g each was extracted with 70% v/v ethanol (EtOH) (1:10 w/v) while shaking at 25 °C, 120 rpm for 12 h. The mixture was filtered through Whatman filter paper No.1, and the filtrate was concentrated using a rotary evaporator at 60 °C. The dark green viscous extracts were separately kept in tightly closed brown vials at 4 °C until used.

2.2. Reagents and kits

COX-2, iNOS, and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, *CA*, USA). LPS (*Escherichia coli* 0127:E8) were purchased from Sigma Aldrich (St. Louis, MO, USA). CellTiter 96 Aqueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI, USA). NO colorimetric assay kit was purchased from Oxford Biomedical Research (Rochester Hills, MI, USA). PGE_2-EIA kit-Monoclonal was purchased from Cayman Chemical (Ann Arbor, MI, USA). Scutellarein tetramethyl ether (scu), stigmasterol, and isosakuranetin were purchased from ChromaDex Inc. (Irvine, *CA*, USA), Sigma Aldrich, and Indofine Chemical Company, Inc. (Hillsborough, NJ, USA), respectively. All chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA), unless otherwise specified.

2.3. Cell cultures

RAW 264.7 (murine) macrophages (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) at 37 °C and 5% CO_2 in a humidified incubator.

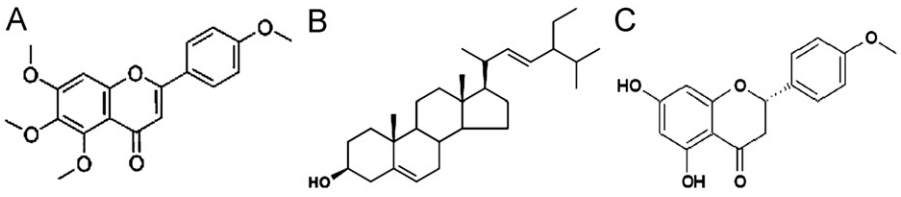

Fig. 1. Structure of bioactive components in SWE. (A) Scutellarein tetramethyl ether (Scu) (B) Stigmasterol (C) Isosakuranetin.