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Pseuderanthemum palatiferum leaf extract inhibits the proinflammatory cytokines, TNF-α and IL-6 expression in LPS-activated macrophages

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ABSTRACT

The anti-inflammatory potential and underlying mechanisms of an ethanol extract of *Pseuderanthemum palatiferum* (EEP) leaves was investigated using LPS-activated macrophages. Our results show EEP produced a concentration-dependent suppression of TNF-α and IL-6 secretion by LPS-activated mouse peritoneal macrophages. EEP also suppressed LPS-induced TNF-α and IL-6 protein and mRNA levels in mouse-derived myeloid cell line RAW264.7. To further elucidate the molecular mechanisms responsible for impaired TNF-α and IL-6 regulation by EEP, the activation of transcription factors, NF-κB, C/EBP, and AP-1, was monitored using electrophoretic mobility shift assays. EEP suppressed LPS-induced NF-κB DNA binding activity within both the TNF-α and IL-6 promoters in RAW264.7 cells with impairment being more pronounced in the IL-6 promoter. In addition, EEP exhibited a concentration-dependent suppression of C/EBP and AP-1 DNA binding activity within the IL-6 promoter. Concordantly, IL-6 luciferase promoter reporter activity was also suppressed by EEP in transiently transfected RAW264.7 cells, upon LPS activation. EEP analysis by GC-MS and HPLC DAD-MSD revealed the presence of β-sitosterol and various polyphenols, respectively, which are known to possess anti-inflammatory activity. Collectively, these results suggest that the anti-inflammatory effects of EEP are mediated, at least in part, by modulating TNF-α and IL-6 expression through impairment of NF-κB, C/EBP, and AP-1 activity.

1. Introduction

Pseuderanthemum palatiferum (Nees) Radlk. (PP), known as Hoan-Ngoc, is a native plant of Vietnam. PP leaves have long been traditionally used by Thais and Vietnamese for the prevention or treatment of hypertension, diabetes, cancer, as well as inflammation associated with wound healing, general trauma, colitis and nephritis (Dieu et al., 2006; Padee et al., 2010). Khumpook et al. (2013) also demonstrated the acute and chronic anti-inflammatory property of ethanol extract from PP leaves in albino rats by using the ethyl phenylpropiolate-induced ear edema test and the cotton pellet-induced granuloma model, respectively. Giang et al. (2003) found that fractional extracts of PP leaves contain several compounds, such as phytosterols, lipids, saponins and flavonoids. PP leaf extracts display both in *vitro* (Giang et al., 2005) and in *vivo* (Khumpook et al., 2013) antioxidant activity. Likewise, we have also found that an ethanol extract of PP leaves containing phenols and flavonoids exhibited potent antioxidant activity as assessed by various *in vitro* models. In addition, the extract also displayed *in vitro* anti-inflammatory properties as suggested by suppression of NO production concomitant with iNOS and COX-2 expression in LPS plus IFN-γ-stimulated RAW264.7 cells (Sittisart and Chitsomboon, 2014). Many investigators have also demonstrated that numerous antioxidant medicinal plants possess anti-inflammatory property (de las Heras et al., 1998; Dufour et al., 2007; Sheeja et al., 2006).

LPS is an endotoxin that activates a variety of mammalian cell-types including macrophages to produce proinflammatory cytokines (Guha and Mackman, 2001). TNF-α and IL-6 are among the most important cytokines released by activated macrophages. TNF

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Abbreviation

AP-1	activator protein-1
AP	*Andrographis paniculata*
BSA	bovine serum albumin
C/EBPs	CCAAT/enhancer binding proteins
DMSO	dimethyl sulfoxide
EEP	ethanol extract of *Pseuderanthemum palatiferum*
ELISA	Enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
IL-6	interleukin-6
LPS	lipopolysaccharide
NA	naïve
NF-κB	nuclear factor-kappaB
PP	*Pseuderanthemum palatiferum* (Nees) Radlk.
qRT-PCR	Quantitative reverse transcription PCR
RLU	relative light units
TNF-α	tumor necrosis factor alpha
VH	vehicle

is a pleiotropic and multifunctional proinflammatory cytokine. It can mediate both growth promotion and inhibitory activities in many cell-types (Liu and Han, 2001) and exerts a wide spectrum of biological effects, including inflammation, lipid metabolism, coagulation, insulin resistance and endothelial functions (Founds et al., 2008). TNF-α is a regulatory factor involved in many inflammatory mediated diseases, such as septic shock, cancer, multiple sclerosis, diabetes and rheumatoid arthritis (Vassalli, 1992). Conversely, blocking elevation of TNF-α or interfering with binding to its cognate receptor can alleviate the severity of the inflammatory response (Colon et al., 2001; Mohler et al., 1993; Scallon et al., 2002). Similarly, IL-6 is a cytokine that is also induced by a variety of stimuli, including LPS and is also a major initiating stimulus of the acute phase response. In addition, IL-6 plays important roles in the immune response during chronic inflammation. Elevated IL-6 levels have also been identified in many inflammatory diseases, including rheumatoid arthritis (RA), systemic juvenile idiopathic arthritis, systemic lupus erythematosus, ankylosing spondylitis, psoriasis and Crohn's disease (Gabay, 2006; Kishimoto, 2010).

LPS induces the expression of numerous genes involved in the inflammatory process by activating several types of transcription factors (Van Miert, 2002). NF-κB is an important transcription factor playing crucial roles in the inflammatory response by regulating the gene expression of proinflammatory cytokines (e.g., IL-1, IL-2, IL-6, TNF-α, etc), chemokines, adhesion molecules, inducible enzymes (COX-2 and iNOS), growth factors, some acute phase proteins and immune receptors (Calixto et al., 2003). The mouse TNF promoter contains several LPS-inducible NF-κB binding sites (Kuprash et al., 1999). Recently, numerous plant-derived substances have been investigated for their potential to impair NF-κB binding with the intent to identify their therapeutic benefits in treating various inflammatory diseases (Calixto et al., 2003). Also involved in the regulation of inflammatory responses are CCAAT/enhancer binding proteins (C/EBPs) which are a family of six proteins containing basic leucine Zipper (bzip) motifs. The C/EBPs are critical regulators of cellular differentiation and function in multiple tissues. Among these proteins, C/EBP β and C/EBP δ are involved in the regulation of gene expression during inflammation (Poli, 1998). A third transcription factor also critically involved in regulating the inflammatory response is activator protein-1 (AP-1). AP-1 consists of either Jun homodimers or Fos/Jun heterodimeric complexes. This transcription factor binds to the TPA DNA response element (TRE) appearing in various mammalian promoters, including those of acute phase proteins and cytokines involved in mediating inflammation (Koj, 1996). Furthermore, NF-κB, C/EBP, and AP-1 are critical for transcriptional regulation of the mouse IL-6 promoter (Baccam et al., 2003). The mouse IL-6 promoter also contained the same DNA binding sequences for NF-κB and AP-1 as the human IL-6 promoter (Allen et al., 2010).

Currently, natural substances derived from plants are of potential interest for therapeutic intervention in the treatment of a variety of inflammatory diseases. Though the anti-inflammatory activities of the ethanol extract of PP leaves were demonstrated both *in vitro* and *in vivo* (Khumpook et al., 2013; Sittisart and Chitsomboon, 2014) to date, the molecular mechanisms responsible for the anti-inflammatory properties of the extract remain unknown. The objective of the present study was to investigate the anti-inflammatory effects of PP by examining its modulation of macrophage-derived TNF-α and IL-6. Here we demonstrated for the first time that EEP decreases the levels of TNF-α and IL-6 at the protein and mRNA level in LPS-activated macrophages as well as provide a partial mechanism for the immunomodulatory activity.

2. Materials and methods

2.1. Reagents

Autoradiography film was purchased from Denville Scientific Inc. (Metuchen, NJ). DNA binding sequences of NF-κB, C/EBP, and AP-1 were purchased from Integrated DNA Technologies (Coralville, IA). pGL2-basic luciferase reporter gene vector and the pmIL-6.Luc(-231) promoter/luciferase reporter gene were gifts from Dr. Gail Bishop from the University of Iowa. All cell culture media and supplements were purchased from Gibco-BRL (Grand Island, NY). All other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise indicated.

2.2. Plant material

Fresh leaves of PP were purchased from Yasothon province, Thailand. The plant was identified and authenticated by Dr. Kongkanda Chayamarit, Forest Herbarium, Royal Forest Department, Bangkok, Thailand. A voucher specimen (BKF 174009) was deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

2.3. Plant extract preparation

One and a half kilograms of fresh PP leaves were cut into small pieces and blended in 6 L of 95% ethanol. The extract was centrifuged for 10 min at 3500*g* at 4 °C and the supernatant was filtered through Whatman No. 1 filter paper. The ethanolic filtrate was concentrated using a vacuum rotary evaporator and dried by lyophilization to obtain ethanol extract of *Pseuderanthemum palatiferum* leaves (EEP; 60.41 g). The EEP was stored at −80 °C and dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) for use in experiments.

2.4. Determination of proximate compositions

Protein (991.20) was analyzed by in-house method based on Association of Analytic Chemists AOAC (2010). Total fat (922.06), ash (942.05), and moisture (934.01), were determined according to the method of AOAC International (2005). Carbohydrate content