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Anti-inflammatory effects of essential oil isolated from the buds of *Cleistocalyx operculatus* (Roxb.) Merr and Perry

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ABSTRACT

Cleistocalyx operculatus (Roxb.) Merr and Perry buds (Myrtaceae) are widely used in folk medicine for the treatment of gastric ailments as well as an antiseptic agent in China, Vietnam and some other tropical countries. However, to be clinically useful, more scientific data are needed. In the present study, we investigated the *in vitro* and *in vivo* anti-inflammatory activities of the essential oil of the *C. operculatus* buds (EO–CO). In the applied tests, EO–CO significantly inhibited lipopolysaccharide (LPS)-induced secretion of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), in RAW 264.7 cells, a mouse macrophage-like cell line. Also the mRNA expression of TNF-α and IL-1β was suppressed by treatment with EO–CO in LPS-stimulated RAW 264.7 cells. Moreover, reporter gene analysis revealed that the EO–CO significantly blocked LPS-induced transcriptional activation of NF-κB in RAW 264.7 cells. Nuclear translocation of p65 subunit was also suppressed by EO–CO treatment. In addition, EO–CO inhibited phorbol ester-induced increase in ear swelling and skin water content in BALB/c mice. These results suggest that EO–CO might exert an anti-inflammatory effect by suppressing the expression of pro-inflammatory cytokines which is mediated, at least in part, by blocking NF-κB activation.

1. Introduction

Cleistocalyx operculatus (Roxb.) Merr and Perry (Myrtaceae) is a well-known medicinal plant, widely distributed and propagated in China, Vietnam and some other tropical countries. Several species of *Cleistocalyx* have been reported to use in folk medicine. The buds of *C. operculatus* as ingredients have been used in various beverages in Southern China since ancient times. The cardiotonic action of the buds of *C. operculatus* has been reported and the inhibition of Ca^{2+}-dependent ATPase was suggested as a possible mechanism for this action (Woo et al., 2002).

Further, inflammation is a physiological response of a body to stimuli, including infections and tissue injury, and protects a body from these inflammatory stimuli. However, excessive or persistent inflammation causes a variety of pathological conditions, such as bacterial sepsis, rheumatoid arthritis and skin inflammation (Dinarello, 1997; Palladino et al., 2003). A variety of inflammatory mediators, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are involved in the development of these inflammatory diseases (Dinarello, 1997). TNF-α is a pro-inflammatory cytokine and the major source of TNF-α is the cells of monocyte/macrophage lineage although T lymphocytes, neutrophils and mast cells also produce TNF-α (Feldmann and Maini, 2001). IL-1β is another pro-inflammatory cytokine involved in inflammatory responses and produced by a variety of cell types, including monocytes, macrophages, fibroblasts and endothelial cells (Shirakawa et al., 1993). Biological functions of TNF-α and IL-1β are similar although the structures and receptors for theses cytokines are clearly distinct (Dinarello, 2000). The secretion of TNF-α and IL-1β in response to inflammatory stimuli is regulated at both transcriptional and post-transcriptional level. The transcriptional regulation of TNF-α and IL-1β is a tightly controlled event, and NF-κB is the major transcriptional regulator of TNF-α and IL-1β gene (Ghosh et al., 1998; Hiscott et al., 1993). NF-κB is a key regulator of a various genes involved in immune and inflammatory responses (Xie et al., 1994). In resting cells, NF-κB exists in an inactive state, in the cytoplasm, complexes with an inhibitory protein, called IκB. Upon activation, IκB undergoes phosphorylation and degradation, and NF-κB is translocated into the nucleus, where it binds to DNA and activates transcription of various genes (Rice and Ernst, 1993).

Previously, we reported the chemical composition, antimicrobial and antioxidant activities of the essential oil and ethanol extract of *C. operculatus* buds against a diverse range of bacterial

Abbreviation: AOO, acetone:olive oil; ELISA, enzyme-linked immunosorbent assay; EO–CO, essential oil of the *Cleistocalyx operculatus* buds; HC, hydrocortisone; IL, interleukin; LPS, lipopolysaccharide; NF, nuclear factor; SEAP, secreted alkaline phosphates; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; RT–PCR, reverse transcription polymerase chain reaction.

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strains including food-spoilage, food-borne pathogenic and multiantibiotic-resistant bacteria (Dung et al., 2008). However, there is no any report available on anti-inflammatory activities of the essential oil of *C. operculatus* buds (EO–CO). Hence, this study was undertaken to assess the *in vitro* and *in vivo* anti-inflammatory effects of EO–CO, as well as its possible mechanism of anti-inflammatory effect.

2. Materials and methods

2.1. Plant materials, chemicals and experimental animals

The *C. operculatus* buds used in this experiment were obtained from a local herb supplier in Hanoi, Vietnam and were identified by comparing its morphological features with the specimen deposited at the Plant Laboratory, Institute of Biological Ecology and Biological Resources, Vietnamese Academy of Science and Technology, Hanoi, Vietnam.

All reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated. RAW 264.7 cells (ATCC TIB71) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in 5% CO_2 humidified air. Female BALB/c mice were purchased from Orient Bio, Inc., Seoul, Korea, and maintained as described previously (Kang et al., 2005). All animals were allowed to acclimatize to the local environment for at least 1 week before use.

2.2. Preparation of essential oil

Essential oil of *C. operculatus* buds was prepared as previously described (Dung et al., 2008). The oil was dried over anhydrous Na_2SO_4 and preserved in a sealed vial at 4 °C in the dark until further analysis (yield 0.68%, w/w).

2.3. Effect of EO–CO on the viability of LPS-stimulated RAW 264.7 cells by XTT assay

RAW 264.7 cells were plated at 5×10^5 cells/ml, and treated with LPS (200 ng/ml) for 24 h in the presence or absence of EO–CO (2.5, 5, 10 or 20 μg/ml). Cell viability was determined using a Cell Proliferation Kit II (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The XTT labeling mixture was prepared by mixing 50 volumes of 1 mg/ml sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate with 1 volume of 0.383 mg/ml of *N*-methyldibenzopurazine methyl sulfate. This XTT labeling mixture was added to the cultures and incubated for 24 h at 37 °C. Absorbance was measured at 490 nm with a reference wavelength at 650 nm.

2.4. Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells were plated at 5×10^5 cells/ml, and stimulated with LPS (200 ng/ml) for 24 h in the presence or absence of EO–CO (2.5, 5, 10 or 20 μg/ml). Culture supernatants were collected and the amount of TNF-α and IL-1β was determined by sandwich immunoassays using a protocol supplied by R&D Systems (Minneapolis, MN, USA).

2.5. Reverse transcription polymerase chain reaction (RT–PCR)

The expression of mRNA transcripts of TNF-α (forward: 5′-cctgtagcccacgtcgtagc-3′, reverse: 5′-ttgacctcagcgctgagttg-3′), IL-1β (forward: 5′-tgcagagttcctacatggtcaaccc-3′, reverse: 5′-gtgctgcctaatgtccccttgaatc-3') and β-actin (forward: 5'-tggaatcctgtggcatccatgaaac-3′, reverse: 5′-taaaacgcagctcagtaacagtccg-3′) was determined by real-time RT–PCR. Total RNA was isolated using TRIzol® Reagent (Invitrogen Life Technologies) as described previously (Lin et al., 2008). Equal amounts of RNA were reverse transcribed into cDNA using oligo$(dT)_{15}$ primers. iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and iCycler iQ™ Real-time PCR Detection System (Bio-Rad Laboratories, Inc.) were used for real-time PCR analysis. For amplification, samples were heated to 94 °C for 8 min and cycled 45 times at, 94 °C for 30 s, and 56 °C for 30 s, and 72 °C for 45 s. Using standards, the amount of TNF-α and IL-1β cDNA was determined and normalized by the amount of β-actin cDNA.

2.6. Transient transfection and secreted alkaline phosphatase (SEAP) reporter gene assay

pNF-κB-secreted alkaline phosphatase (SEAP), which contains four tandem copies of the NF-κB consensus sequence fused to a TATA-like promoter region from the Herpes simplex virus thymidine kinase promoter (Han et al., 2007) was used as a reporter plasmid and the plasmid expressing luciferase was constitutively cotransfected. Transient transfection was performed using Lipofectin reagent (Invitrogen, Carlsbad, CA, USA). Great EscAPe Chemiluminescence Detection Kit (Clontech) was used to measure the amount of secreted SEAP from culture medium. Chemiluminescence was measured using VICTOR™ Light (Perkin Elmer, Wellesely, MA, USA). The measured SEAP activity was normalized by luciferase activity.

2.7. Phorbol ester-induced skin inflammation

12-O-tetradecanoylphorbol-13-acetate (TPA) was dissolved in AOO (acetone:olive oil = 4:1) and used as an inducer of skin inflammation. The thickness of both ears of mice was measured before the start of experiment using dial thickness gauge (Mitutoyo Corporation, Kanakawa, Japan) as a reference. EO–CO was treated 30 min before TPA application and 20 μl of TPA (150 μg/ml) was topically applied to the dorsal surface of both ears to induce skin inflammation. After 4 h, ear thickness was measured again and changes in ear thickness were calculated by subtracting the ear thickness measured before the start of experiment from the ear thickness measured 4 h after TPA treatment. After measuring ear thickness, ears were removed and weighed and the weight was defined as Wet Weight. The ears were dried for 24 h in dry oven, weighed again and the weight was defined as Dry Weight. Skin water content was calculated by subtracting Dry Weight from Wet Weight and dividing this by Dry Weight again, and expressed as mg H_2O/mg Dry Weight.

2.8. Statistical analysis

The results are expressed as mean ± SD. One-way ANOVA and Dunnett's *t*-test was used for multiple comparisons using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). The criterion for statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Effect of EO–CO on the viability and pro-inflammatory cytokines TNF-α and IL-1β in LPS-stimulated RAW 264.7 cells

As described previously, TNF-α and IL-1β are important inflammatory mediators involved in a variety of inflammatory diseases (Feldmann and Maini, 2001; Shirakawa et al., 1993). These pro-inflammatory cytokines are produced in response to inflammatory stimuli at the early stage of inflammation and induce the production of various inflammatory mediators, such as nitric oxide and prostaglandins, resulting in amplification of inflammation (Dinarello, 2000). Synergistic action of TNF-α and IL-1β is also observed in most of the *in vitro* and *in vivo* models of inflammation (Dinarello, 1997). Various essential oils isolated from the plant origin have been reported to have anti-inflammatory activities. Medeiros and coworkers demonstrated that the essential oil of *Cordia verbenacea* exerts an anti-inflammatory effect by inhibiting TNF-α and IL-1β levels (Medeiros et al., 2007). Besides, anti-inflammatory activity of essential oil from leaves of *Cinnamomum osmophloeum* was reported to be mediated by blocking IL-1β and IL-6 production (Chao et al., 2005). Therefore, suppressing one of these cytokines or both might be a good strategy for the treatment of various inflammatory diseases.

In this study, we examined the effect of EO–CO on LPS-induced production of TNF-α and IL-1β, in RAW 264.7 cells to investigate the anti-inflammatory effect of EO–CO. The cytotoxic activity of EO–CO on the RAW 264.7 cells was evaluated using XTT cell proliferation kit. The 5×10^5 cells/ml treated with LPS (200 ng/ml) were incubated for 24 h in the presence or absence of EO–CO (2.5, 5, 10 or 20 μg/ml). The concentration of EO–CO and the duration of EO–CO treatment used in this study had no significant effect on the viability of RAW 264.7 cells (Fig. 1A).

As shown in Fig. 1B and C, LPS substantially increased the production of TNF-α and IL-1β in RAW 264.7 cells. However, EO–CO inhibited LPS-induced production of TNF-α and IL-1β in a concentration-dependent manner in RAW 264.7 cells. Treatment with 10 μg/ml and 20 μg/ml of EO–CO caused 33% and 45% inhibition of TNF-α production, respectively, in LPS-stimulated RAW 264.7 cells (Fig. 1B). IL-1β production was also reduced to 73% and 84% of vehicle-treated control by treatment with 10 μg/ml and 20 μg/ml of EO–CO, respectively (Fig. 1C).