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Suppressive effects of acetone extract from the stem bark of three *Acacia* species on nitric oxide production in lipopolysaccharide-stimulated RAW 264.7 macrophage cells

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ABSTRACT

Objective: To compare the inhibitory effects of acetone extracts from the stem bark of three *Acacia* species (*Acacia dealbata*, *Acacia ferruginea* and *Acacia leucophloea*) on nitric oxide production.

Methods: The lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells were used to investigate the regulatory effect of acetone extracts of three *Acacia* stem barks on nitric oxide production and the expression of inducible nitric oxide synthase, cyclooxygenase-2 and tumor necrosis factor-α. Further, the phenolic profile of acetone extracts from the *Acacia* barks was determined by liquid chromatography-mass spectrometry/mass spectrometry analysis.

Results: All the three extracts significantly decreased LPS-induced NO production as well as the expression of inducible nitric oxide synthase, cyclooxygenase-2 and tumor necrosis factor-α in a concentration dependent manner (25, 50 and 75 μg/mL). In the liquid chromatography-mass spectrometry/mass spectrometry analysis, acetone extract of *Acacia ferruginea* bark revealed the presence of 12 different phenolic components including quercetin, catechin, ellagic acid and rosmanol. However, *Acacia dealbata* and *Acacia leucophloea* barks each contained 6 different phenolic components.

Conclusions: The acetone extracts of three *Acacia* species effectively inhibited the NO production in LPS-stimulated RAW 264.7 cells and the presence of different phenolic components in the bark extracts might be responsible for reducing the NO level in cells.

1. Introduction

Inflammation is the normal physiological and beneficial host response to tissue injury caused by foreign agents (physical or noxious chemical stimuli or microbiological toxins) that eventually leads to the restoration of normal tissue structure and function [1]. Macrophages play a key role in the immune system by releasing various pro-inflammatory cytokines and mediators such as interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and prostaglandin E2 (PGE2) in response to various harmful stimuli [2,3]. Among them, NO is produced enormously in macrophages by the iNOS. Prostaglandin is also an important pro-inflammatory mediator, and produced from arachidonic acid by COX-1 and 2 enzymes [4]. Under normal physiological conditions, the short-lived biomolecule, NO mediates many biological functions such as host defense, platelet aggregation, vasoregulation, and neurotransmission [5]. However, excessive production of NO and other mediators has been concerned in the development of many diseases, such as arthritis, asthma, multiple sclerosis, inflammatory bowel disease, and atherosclerosis [6,7].

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Accordingly, the regulation of these pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated macrophage cell line is an effective therapeutic strategy for the development of novel anti-inflammatory agents.

In recent times, phenolic components from plants have attracted much attention because of their large distribution with a variety of biological properties [8]. The genus *Acacia* belongs to the family of Mimosaceae, comprises more than 1 350 species, and mainly occurs in tropical and some temperate countries including Australia, Africa, India and America. In the traditional systems of medicine, different parts of *Acacia* plants are widely used as indigenous drugs to treat various ailments [9]. The acetone and methanol extracts from the bark of *Acacia leucophloea* (*A. leucophloea*), *Acacia ferruginea* (*A. ferruginea*), *Acacia dealbata* (*A. dealbata*), and *Acacia pennata* showed remarkable antioxidant properties [10]. Sowndhararajan *et al.* [11] reported that the acetone extract from the stem bark of *A. leucophloea*, *A. ferruginea* and *A. dealbata* significantly up-regulated the expression of antioxidant enzymes (superoxide dismutase, glutathione peroxidase and catalase) in hydrogen peroxide-induced human hepatoma (HepG2) cells. In Indian traditional systems of medicine, the stem bark of *A. leucophloea* is used to treat a variety of disorders including inflammation, bronchitis, wounds, ulcers, diarrhea, intermittent fevers, leprosy, toothache, *etc*. The bark of *A. ferruginea* is used traditionally for the treatments of itching, leucoderma, ulcers, stomatitis, and diseases of the blood. The bark of *A. dealbata* produces a gum, resembling gum arabic, which is used to treat bronchial disorders [10,11]. Sowndhararajan and Kang investigated the effect of ethyl acetate fraction from the acetone extract of *A. ferruginea* stem bark on ethanol-induced gastric ulcer in rats [12]. With this background, the present study was carried out to investigate anti-inflammatory potential of acetone extract from the stem bark of *A. leucophloea*, *A. ferruginea* and *A. dealbata* by measuring its ability to inhibit NO production and expression of iNOS, COX-2 and TNF-α in LPS-simulated RAW 264.7 cells.

2. Materials and methods

2.1. Chemicals

3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), penicillin–streptomycin solution and lipopolysaccharide (LPS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate buffered saline were from HyClone Laboratories, Inc. (South Logan, UT, USA). RNeasy Mini kit and SYBR green master mix were purchased from Qiagen-GmbH (Hilden, Germany). SuperScript III First-Strand synthesis system was purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were of the highest available purity analytical grade.

2.2. Plant materials and preparation of extracts

Fresh stem barks of *A. leucophloea*, *A. ferruginea* and *A. dealbata* were collected from Coimbatore, Tamil Nadu state, India. The authenticated plant specimens were deposited in the Botany Herbarium, Bharathiar University with voucher numbers: BUBH-6140, BUBH-6141, and BUBH-6142, respectively [10]. The air-dried and powdered bark samples were extracted with petroleum ether (for disposing lipid and pigments), followed by acetone using Soxhlet apparatus. Each extract was then filtered before being dried by rotary evaporation (RE300, Yamato, Tokyo, Japan) at 50 °C and the remaining water was removed by lyophilization (4KBTXL-75, VirTis Benchtop K, NY, USA).

2.3. Cell culture

The murine macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS, 100 g/L streptomycin, and 100 IU/mL penicillin at 37 °C in a 5% CO_2 atmosphere (HERAcell 150, Thermo Electron Corp. Waltham, MA, USA).

2.4. MTT cell viability assay

Cell viability of RAW 264.7 cells was determined by MTT assay. Briefly, RAW 264.7 cells were seeded at the density of 5×10^4 cells/well in 96-well culture plates and incubated for 24 h (at 37 °C and 5% CO_2), followed by the pre-treatment with different concentrations of the extracts (0–100 μg/mL). Twenty four hours later, after changing the medium, MTT was added to a final concentration of 0.5 mg/mL, and the cells were incubated for 4 h. The medium was then removed and the formazan precipitate was solubilized in dimethyl sulfoxide. The absorbance was measured at 550 nm on a microplate reader (Biotek, Winooski, VT, USA).

2.5. Inhibition of NO production

NO production was determined by measuring the level of nitrite in the culture supernatant of RAW 264.7 cells. The RAW 264.7 cells were seeded at a density of 5×10^5 cells/well in 24 well plates for 12 h at 37 °C and 5% CO_2. Then, the cells were treated with different concentrations of acetone extracts of *Acacia* barks (12.5–75.0 μg/mL, prepared in FBS-free DMEM). After 1 h treatment, cells were stimulated with 1 μg/mL of LPS for 24 h. The presence of nitrite was determined in cell culture media using commercial NO detection kit (iNtRON, Sungnam, South Korea). Briefly, 100 μL of cell culture medium with an equal volume of Griess reagent in a 96-well plate was incubated at room temperature for 10 min. Then the absorbance was measured at 540 nm in a microplate reader (Biotek, Winooski, VT, USA). The amount of nitrite in the media was calculated from sodium nitrite ($NaNO_2$) standard curve.

2.6. RNA isolation and first-strand cDNA synthesis

Total cellular RNA was isolated from cells by using a commercial kit (RNeasy Mini kit, Qiagen) as described by the manufacturer. An amount of 1 μg of total RNA was reverse-transcribed using oligo (dT) and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA)