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Antiinflammatory Activity of *Gynura bicolor* (紅鳳菜 Hóng Fèng Cài) Ether Extract Through Inhibits Nuclear Factor Kappa B Activation

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ABSTRACT

This study investigated effects of the *Gynura bicolor* (Roxb. and Willd.) DC. ether extract (GBEE) on nitric oxide (NO) and prostaglandin (PG)E_2 production on the lipopolysaccharide (LPS)-induced inflammatory response in RAW 264.7 cells. A composition analysis of GBEE showed that the major compounds were b-carotene, chlorophyll, and quercetin, respectively. Furthermore, NO and PGE_2 levels of 120 μg/ml GBEE-treated cells were 70% and 9.8%, respectively, than those of cells treated with LPS alone. Immunoblots assays showed that the GBEE dose-dependently suppressed LPS-induced inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 protein levels. The GBEE significantly decreased cytosolic phosphorylated (p)-IκBa and nuclear p65 protein expressions. Electrophoresis mobility shift assays indicated that the GBEE effectively inhibited nuclear factor kappa B (NF-κB) activation induced by LPS. These results support a role of the GBEE in suppressing activation of NF-κB to inhibit NO and PGE_2 production in the LPS-induced inflammatory response by RAW 264.7 cells.

Key words: Cyclooxygenase, Cells, *Gynura bicolor*, Inducible nitric oxide synthase, Nuclear transcription factor-κB

INTRODUCTION

Gynura bicolor (紅鳳菜 Hóng Fèng Cài; the leaves of *Gynura bicolor* [Roxb. and Willd.] DC.) is a common vegetable in Taiwan and Far Eastern. The top and bottom sides of *G. bicolor* leaves, respectively, appear dark-green and purple. The major constituents of *G. bicolor* related to it pigment sources and physiological effects are possibly the rich flavonoids.[1] *G. bicolor* has been shown as antioxidant, antiinflammatory, and antihyperglycemic effect.[2,3] To date, limited studies have examined the biological activities of *G. bicolor,* and the working mechanisms are not yet elucidated.

Inflammation is a physiological defense response of the body to various types of injurious stimuli. Chronic inflammation is characterized by a proliferation of fibroblasts and the formation of blood vessels (angiogenesis), as well as an influx of chronic inflammatory cells, namely granulocytes (neutrophils, eosinophils, and basophils), lymphocytes, plasma cells, and macrophages.[4] In the inflammatory response process, nitric oxide (NO) and prostaglandin (PG)E_2, acts as a molecular messenger for various physiologic functions and pathologic processes.[5] Expressions of iNOS and COX-2, two major regulate enzymes of NO and

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PGE_2 production, respectively,[5,6] are primarily controlled at the transcriptional stage, and nuclear factor kappa B (NF-κB) is well recognized to play a key role.[7]

In the present study, we compared the efficacy of *G. bicolor* in modulating NO and PGE_2 production in LPS-stimulated RAW 264.7 cells, and explored the possible molecular mechanism involved.

MATERIALS AND METHODS

Preparation of the *G. bicolor* ether extract (GBEE)

Gynura bicolor (Roxb. and Willd.) DC. were purchased from Yuanshan Village (Ilan, Taiwan). The same plant growing in Department of Forestry, National Chung Hsieh University (NCHU) and identified by Dr. Yen Hsueh Tseng. A voucher specimen (TCF13549) has been deposited at NCHU. Leaves of *G. bicolor* were removed, cleaned, and blended in cold water (4°C, w/w: 1/1). The homogenates were extracted with ether (v/v:1/1) for 6 h at 4°C. Finally, extracts were stirred on a stirring plate for 4 h, and then were dried in a rotary vacuum dryer. The percent yield of the ether extract was 0.3% (w/w).

Chemical composition analysis of the GBEE

In this study, the β-carotene content was analyzed as described by Xu *et al*.[8] The gallic acid content was analyzed as described by Wang *et al*.[9] The quercetin content was evaluated as described by Wang and Morris.[10] The rutin content was determined as described by Krizman *et al*.[11] The chlorophyll content was determined using a procedure described by Witham *et al*.[12]

Cell culture and treatment

The mouse macrophage-like cell line, RAW 264.7, was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). RAW 264.7 cells (at passage 8–13) were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. Cells were plated at a density of 8×10^5 per 30-mm culture dish and were incubated until 90% confluence was reached. To determine changes in cell viability, nitrite and PGE_2 concentrations, and iNOS, COX-2, p65, and IκB protein levels, cells were treated with 15, 30, 60, or 120 μg/ml of the GBEE in the presence of 1 μg/ml lipopolysacchadides (LPS; Sigma Co., St. Louis, MO) for various time intervals as indicated. For the electrophoretic mobility shift assay (EMSA), cultures were preincubated with GBEE for 3 h and then treated with 1 μg/ml LPS for 90 min. All GBEEs were dissolved in methanol, and the final concentration of methanol added to the medium was 0.1% (v/v).

Cell viability assay

The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used to measure cell respiration as an indicator of cell viability.[13] The absorbance in cultures treated with LPS alone was regarded as 100% cell viability.

Nitrite and PGE_2 determination

Nitrate in the media was measured by the Griess assay[14] and was used as an indicator of NO production in cells. The absorbance at 550 nm was measured and calibrated using a standard curve of sodium nitrite prepared in culture medium. PGE_2 secreted into the medium was measured by a competitive enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). Concentrations of the mediator in the samples were calculated according to reference calibration curves of the standards. The results are expressed in pg/μl.

Immuoblot analysis

Cells were washed twice with cold phosphate buffered saline (PBS) and then harvested in 200 μl of lysis buffer containing 10 mM Tris–HCl, 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 20 μg/mL aprotinin at pH 7.4. Cellular protein levels were determined by the method of Lowry *et al*.[15] Equal amounts of cell protein in each sample were applied to 10% sodium dodecylsulfate (SDS) polyacrylamide gels.[16] After electrophoresis, proteins separated on the gels were transferred to polyvinylidene difluoride membranes.[17] The membranes were then incubated with an anti-iNOS, COX-2, phosphorylated (p)-IκBa, or P65 antibody (Santa Cruz Biotechnology Co., Santa Cruz, CA) at 37°C for 1 h, followed by a peroxidase-conjugated secondary antibody. The immunoreactive bands were detected with an enhanced chemiluminescence kit (Amersham ECL Advance Western Blotting Detection Kit, Amersham Pharmacia Biotech, Buckinghamshire, UK). Band intensities were measured with an AlphaImager 2000 (Alpha Innoctech, San Leandro, CA).

Nuclear protein preparation and EMSA

After preincubation with GBEE or its active peinciples for 8 h then with or without LPS for 3 h, isolation of cytosolic and nuclear fraction were practiced by a Nuclear Extraction Kit (Cayman Chemical Company. Ann Arbor, Michigan, USA). The nuclear proteins was collected and stored at -70°C until the EMSA was performed. In this study, the NF-κB DNA binding activity was analyzed by EMSA as described previously.[18]

Statistical analysis

Data are expressed as the mean ±SD from at least four independent experiments. Differences among treatments were analyzed by a one-way analysis of variance (ANOVA) and Tukey's test using the Statistical Analysis System (SAS, Cary, NC). *P* values of < 0.05 were considered significant.

RESULTS

Chemical composition of the GBEE

To determine which major pigment compounds are rich in GBEE, there are three prime plant pigments that were detected: 11.5 μg chlorophyll/g GBEE, 25.3 μg quercetin/g GBEE, and 7460 μg b-carotene/g GBEE, respectively. Gallic acid and rutin were not detectable in the GBEE.

Cell viability

In this study, a MTT assay was used to test whether up to 120 μg/ml of the GBEE caused cell damage. As shown in [Figure 1],