Journal of Traditional and Complementary Medicine Vo1. 3, No. 1, pp. 48-52

Journal of Traditional and Complementary Medicine
Journal homepage http://www.jtcm.org

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

Chih-Chung Wu[1], Chong-Kuei Lii[2], Kai-Li Liu[3], Pei-Yin Chen[3], Shu-Ling Hsieh[4]

[1]Department of Nutrition and Health Sciences, Chang Jung Christian University, Tainan 711, Taiwan.
[2]Department of Nutrition, China Medical University, Taichung 404, Taiwan.
[3]Department of Nutrition, Chung Shan Medical University, Taichung 402, Taiwan.
[4]Department of Seafood Sciences, National Kaohsiung Marine University, Kaohsiung 811, Taiwan.

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

Correspondence to:
Dr. Shu-Ling Hsieh, Address: No. 142, Haijhuan Road., Nanzih District, Kaohsiung 81143, Taiwan. Tel: 886-7-3617141 ext. 3626; Fax: 886-7-3640634, E-mail: slhsieh@ntu.edu.tw; slhsieh@mail.nkmu.edu.tw

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],

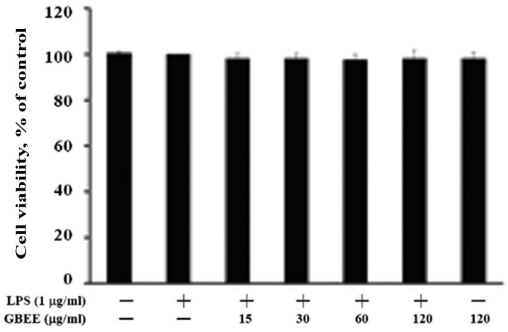

Figure 1. Effect of the *Gynura bicolor* ether extract (GBEE) on the cell viability of RAW 264.7 cells. RAW 264.7 cells were treated with 1 μg/ml lipopolysaccharide (LPS) alone or with 15–120 μg/ml of the GBEE for 24 h, and cell viability was measured by an MTT assay. Values are the mean±SD of five separate experiments and are expressed as a percentage of the DMSO vehicle control

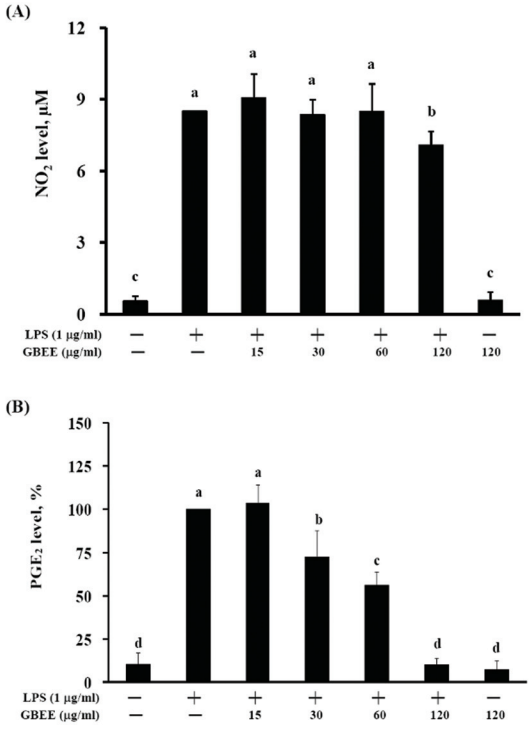

Figure 2. The *Gynura bicolor* ether extract (GBEE) suppressed lipopolysaccharide (LPS)-induced NO and PGE_2 production. RAW 264.7 cells were treated with 1 μg/ml LPS alone or with various concentrations of the GBEE for 24 h. NO production in cells treated with LPS alone was denoted as 100%. Values are the mean±SD of five separate experiments. (a–c) Values not sharing the same letter significantly differ ($P < 0.05$)

when RAW 264.7 cells were treated with 15, 30, 60, or 120 μg/ml GBEE in the absence or presence of 1 μg/ml LPS for 24 h, no changes on the growth of the cells were observed.

NO and PGE_2 production

As shown in [Figure 2A], NO production was induced by LPS compared with the vehicle control group ($P < 0.05$). When

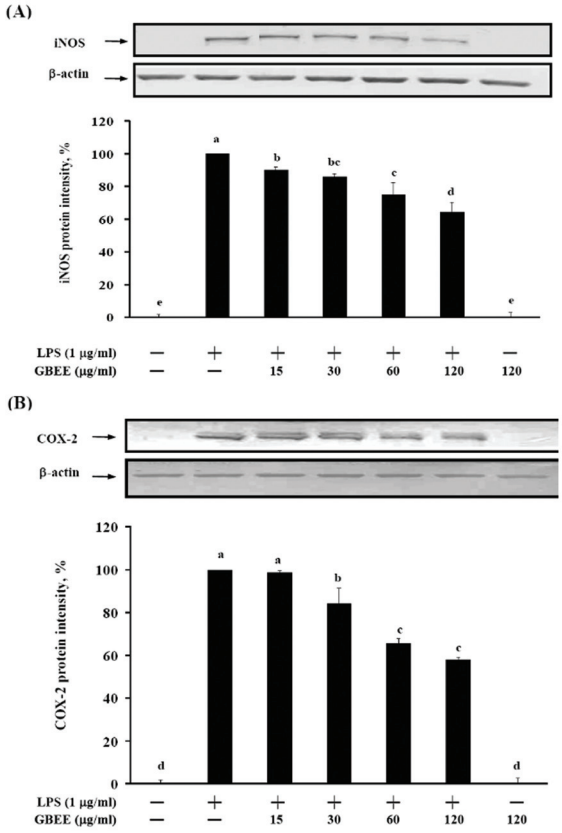

Figure 3. Suppression of lipopolysaccharide (LPS)-induced iNOS and COX-2 protein expressions by the *Gynura bicolor* ether extract (GBEE). RAW 264.7 cells were treated with 1 μg/ml LPS alone or with 15–120 μg/ml of the GBEE for 6 or 24 h, and cellular proteins were used for iNOS or COX-2 protein determinations. The iNOS and COX-2 protein levels of each sample were quantified by densitometry and are expressed as the percentage of those treated with LPS alone. Data are the mean±SD of at least five separate experiments. (a–e) Values not sharing the same letter significantly differ ($P < 0.05$)

RAW 264.7 cells were co-treated with the GBEE, LPS-induced NO production dose-dependently decreased. A 30% decrease was noted in cells treated with 120 μg/ml GBEE ($P < 0.05$). In addition, a dose-dependent decrease in LPS-induced PGE_2 production was found with GBEE treatment. In the presence of 30, 60, or 120 μg/ml GBEE treatments, PGE_2 levels were 72%, 56%, and 10%, respectively, of cells treated with LPS alone ($P < 0.05$) [Figure 2B].

iNOS and COX-2 protein expressions

Consistent with changes in NO and PGE_2 production, similar suppression of iNOS and COX-2 protein expressions was noted in cells treated with the GBEE. Immunoblots revealed that iNOS levels in RAW264.7 cells treated with 15, 30, 60, or 120 μg/ml of the GBEE were 90%, 86%, 75%, and 64% that of cells treated with LPS alone ($P < 0.05$) [Figure 3A]. A similar dose-dependent decrease in COX-2 expression by the GBEE was also noted. When RAW 264.7 cells were treated with 30, 60, or 120 μg/ml of the GBEE, COX-2 protein levels were significantly inhibited by 16%, 34%, and 41%, respectively, compared with the LPS-treated group ($P < 0.05$) [Figure 3B].

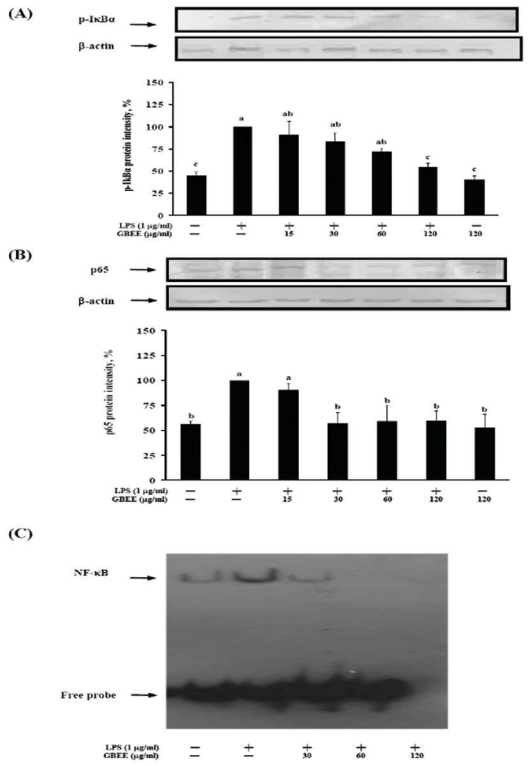

Figure 4. Suppression of lipopolysaccharide (LPS)-induced nuclear factor (NF)-κB activation by the *Gynura bicolor* ether extract (GBEE). RAW 264.7 cells were pretreated with 0–120 μg/ml of the GBEE for 3 h, followed by adding 1 μg/ml LPS for an additional 60 min. Phosphorylated (p)-IκBα (A) and nuclear p65 (B) protein levels of each sample were measured by immunoblotting. Nuclear NF-κB binding to DNA was determined by an EMSA (C) as described in "Materials and Methods" section. Proteins in each immunoblot were quantified by densitometry and are expressed as the percentage of that treated with LPS alone. Data are the mean±SD of at least five separate experiments. (A–C)^{abc} Values not sharing the same letter significantly differ ($P < 0.05$)

NF-κB activation

To examine whether suppression of iNOS and COX-2 expressions by the GBEE is dependent on inhibition of LPS-induced NF-κB activation, both western blot and EMSA assays were performed. [Figure 4A] shows that the p-IκBα protein was significantly decreased by 47% after 120 μg/ml GBEE treatment ($P < 0.05$). As shown in [Figure 4B], nucleic p-65 protein levels significantly decreased after 30, 60, and 120 μg/ml GBEE treatment by 3–41% ($P < 0.05$). NF-κB levels, however, significantly increased in nuclear extracts of LPS-stimulated cells. Results of changes in p-65 protein levels in the nucleic fraction show that the GBEE significantly decreased the translocation of NF-κB from the cytosol to nuclei in RAW 264.7 cells. Furthermore, [Figure 5C] shows that the DNA-binding activity of the NF-κB nuclear protein significantly increased in the LPS control group. However, the DNA-binding activity of the NF-κB nuclear protein was significantly inhibited when macrophages were treated with 30, 60, or 120 μg/ml of the GBEE.

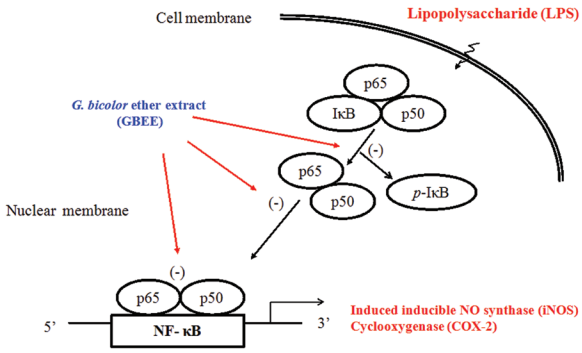

Figure 5. Proposed model for the molecular mechanisms of the regulation by the *Gynura bicolor* ether extract (GBEE) of inflammation induced by lipopolysaccharide (LPS) in RAW264.7 cells

DISCUSSION

G. bicolor is a common dietary vegetable in Taiwan and Far East. To date, *in vitro* and *in vivo* models of biological evidence of the efficacy of *G. bicolor* are still limited. Recently, Lu *et al*.[1] shows the major constituents of *G. bicolor* related to it pigment sources and physiological effects are possibly the rich flavonoids. Previous study shows that *G. bicolor* are rich in anthocyanin and there are three major anthocyanidins, peralgonidin, delphinidin, and malvidin, found in *G. bicolor*.[19,20] Recently, a higher contents of sesquiterpene compounds such as beta-caryophyllene, alpha-caryophyllene, and alpha-copaene were found in *G. bicolor* by GC-MS.[21] In addition to anthocyanin and sesquiterpene compounds, GBEE was found containing chlorophyll, quercetin, and bata-carotene in this study.

In unstimulated cells, NF-κB is sequestered in the cytoplasm by binding to the inhibitor protein, IκB.[22] Exposure to a variety of external stimuli, including inflammatory cytokines, oxidative stress, ultraviolet irradiation, or bacterial endotoxins, causes the phosphorylation of IκBα, which leads to the disassociation of NF-κB and IκBα.[23,24] Activated NF-κB is then translocated to nuclei, where it binds to the cis-acting κB enhancer element of target genes, including numerous inflammatory-, antioxidant-, and cell survival-related genes, and activates their transcription.[25,26] Previous studies showed that 0.5–200 mM β-carotene acts as an antitumor agent in colon carcinogenesis through decreasing COX-2 expression ($P < 0.05$) and PGE_2 production ($P < 0.05$) in human colon cancer cells by inhibiting IκB phosphorylation and degradation.[27] In A549 cells and Chang liver cells, 5–200 μM quercetin suppressed IκB phosphorylation by inhibiting IκB kinase activity and enhanced NF-κB nuclear translocation, which subsequently attenuated interleukin-1b, interferon-g, and tumor necrosis factor-a mixture (20 ng/ml of each)-induced iNOS and COX-2 gene transcription.[28,29] GBEE are rich in these three compounds, they may be involving antiinflammatory response of RAW264.7 cells induced by LPS.

In the present study, GBEE suppressed activation of NF-κB

downregulated NO and PGE_2 production. These findings indicate the potent antichronic inflammatory activity of *G. bicolor*.

ACKNOWLEDGEMENTS

This study was supported by a grant (NSC 95-2320-B-242-011) from the National Science Council, Taiwan.

REFERENCES

1. Lu H, Pei Y, Li W. Studies on Flavonoids from *Gynura bicolor* DC. Zhongguo xian dai ying yong yao xue 2010;27:613-4.
2. Schirrmacher G, Schnitzler WH, Grassmann J. Determination of secondary plant metabolites and antioxidative capacity as new parameter for quality evaluation: Indicated by the new Asia salad *Gynura bicolor*. J Appl Bot 2004;78:133-4.
3. Li WL, Ren BR, Min-Zhuo Y, Hu CG, Lu J, Wu L, *et al.* The anti-hyperglycemic effect of plants in genus *Gynura Cass*. Am J Chin Med 2009;37:961-6.
4. Babál P, Brozman M, Jakubovský J, Basset F, Jány Z. Cellular composition of periapical granulomas and its function. Histological, immunohistochemical and electronmicroscopic study. Czech Med 1989;12:193-215.
5. Vane JR, Bahkle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 1998;38:97-120.
6. Hibbs JB Jr, Taintor RR, Vavrin Z, Rachlin EM. Nitric oxide: A cytotoxic activated macrophage effector molecule. Biochem Biophys Res Commun 1988;157:87-94.
7. Srivastava SK, Ramana KV. Focus on molecules: nuclear factor-kappa B. Exp Eye Res 2009;88:2-3.
8. Xu F, Yuan QP, Dong HR. Determination of lycopene and β-Carotene by high-performance liquid chromatography using Sudan I as internal standard. J Chromatogr B Biomed Sci Appl 2006;838:44-9.
9. Wang H, Provan GJ, Helliwell K. HPLC determination of catechins in tea leaves and tea extracts using relative response factors. Food Chem 2003;81:307-12.
10. Wang L, Morris ME. Liquid chromatography-tandem mass spectroscopy assay for quercetin and conjugated quercetin metabolites in human plasma and urine. J Chromatogr B Biomed Sci Appl 2005;821:194-201.
11. Krizman M, Baricevic D, Prosek M. Determination of phenolic compounds in fennel by HPLC and HPLC-MS using a monolithic reversed-phase column. J Pharm Biomed Anal 2007;43:481-5.
12. Witham FA, Blaydes DF, Devlin RM. Chlorophyll absorption Spectrum and quantitative determination. In: Exercise in plant physiology. Boston: Pws Publishers; 1986. p. 128-31.
13. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986;89:271-7.
14. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}N]-nitrate in biological fluids. Anal Biochem 1992;126:131-8.
15. Lowry OH, Rosebrough NJ. Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951;193:265-75.
16. Towbin H, Staehelin T, Gordan J. Electrophoretic transfer proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci U S A 1979;76:4350-6.
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of acteriophage T4. Nature 1970;277:680-5.
18. Liu KL, Chen HW, Wang RY, Lei YP, Sheen LY, Lii CK. DATS reduces LPS-induced iNOS expression, NO production, oxidative stress, and NF-kappa B activation in RAW 264.7 macrophages. J Agric Food Chem 2006;54:3472-8.
19. Yoshitama K, Kaneshige M, Ishikura N, Araki F, Yahara S, Abe KA. Stable Reddish Purple Anthocyanin in the Leaf of Gynura aurantiaca cv, 'Purple Passion'. J Plant Res 1994;107:209-14.
20. Hayashi M, Iwashita K, Katsube N, Yamaki K, Kobori M. Kinjiso (*Gynura bicolor* DC.) colored extract induces apoptosis in HL60 leukemia cells. Nippon Shokuhin Kagaku Kogaku Kaishi 2002;49:519-26.
21. Chen J, Adams A, Mangelinckx S, Ren BR, Li ML, Wang ZT, *et al.* Investigation of the volatile constituents of different Gynura species from two Chinese origins by SPME/GC-MS. Nat Prod Commun 2012;7:655-7.
22. Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 1998;16:225-60.
23. Bours V, Bonizzi G, Bentires-Alj M, Bureau F, Piette J, Lekeux P, *et al.* NF kappa B activation in response to toxical and therapeutical agents: Role in inflammation and cancer treatment. Toxicoogy 2000;153:27-38.
24. Wang T, Zhang X, Li JJ. The role of NF-kappaB in the regulation of cell stress responses. Int Immunopharmacol 2002;2:1509-20.
25. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem 1994;269:4705-8.
26. Hawiger J. Innate immunity and inflammation: A transcriptional paradigm. Immunol Res 2001;23:99-109.
27. Palozza P, Serini S, Maggiano N, Tringali G, Navarra P, Ranelletti FO, *et al.* Beta-carotene downregulates the steady-state and heregulin-alpha-induced COX-2 pathways in colon cancer cells. J Nutr 2005;135:129-36.
28. Banerjee T, Van der Vliet A, Ziboh VA. Downregulation of COX-2 and iNOS by amentoflavone and quercetin in A549 human lung adenocarcinoma cell line. Prostaglandins Leukot Essent Fatty Acids 2002;66:485-92.
29. García-Mediavilla V, Crespo I, Collado PS, Esteller A, Sánchez-Campos S, Tuñón MJ, *et al.* The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappa B pathway in Chang liver cells. Eur J Clin Pharmacol 2007;557:221-9.