Antioxidant and Anti-inflammatory Activities of Butanol Extract of *Melaleuca leucadendron* L.

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Abstract

*Melaleuca leucadendron* L. has been used as a tranquillizing, sedating, evil-dispelling and pain-relieving agent. We examined the effects of *M. leucadendron* L. extracts on oxidative stress and inflammation. *M. leucadendron* L. was extracted with methanol (MeOH) and then fractionated with chloroform (CHCl₃) and butanol (BuOH). Antioxidant activity of the MeOH extract and BuOH fraction were higher than that of both α-tocopherol and butyrylated hydroxytyluene (BHT). Total phenol content in the extracts of *M. leucadendron* L., especially the BuOH fraction, well correlated with the antioxidant activity. The anti-inflammatory activity of BuOH extracts were investigated by lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E₂ (PGE₂) production, and cyclooxygenase-2 (COX-2) expression in RAW 264.7 macrophages. The BuOH fraction significantly inhibited LPS-induced NO and PGE₂ production. Furthermore, BuOH extract of *M. leucadendron* L. inhibited the expression of COX-2 and iNOS protein without an appreciable cytotoxic effect on RAW264.7 cells. The extract of *M. leucadendron* L. also suppressed the phosphorylation of inhibitor κBα (IkBα) and its degradation associated with nuclear factor-κB (NF-κB) activation. Furthermore, BuOH fraction inhibited LPS-induced NF-κB transcriptional activity in a dose-dependent manner. These results suggested that *M. leucadendron* L. could be useful as a natural antioxidant and anti-inflammatory resource.

Key words: *Melaleuca leucadendron* L., antioxidant activity, inducible nitric oxide synthase, cyclooxygenase-2, NF-κB

INTRODUCTION

Oxidative stresses strongly correlate with the aging process and certain degenerative diseases (1). The reactive oxygen species (ROSs) are involved in up-regulating inflammatory gene expressions by causing redox-based activation of nuclear factor-κB (NF-κB) and the COX-2 signaling pathways (2).

Prostaglandins (PGs) and nitric oxide (NO) are involved in various pathophysiological processes including inflammation and carcinogenesis. Prostaglandins (PGs) are lipid mediators involved in many processes, including inflammation, and are produced by many cell types. More notably, prostaglandin E₂ (PGE₂) affects cell proliferation and tumor growth and suppresses the immune response to malignant cells (3). NO plays an important role in the regulation of many physiological functions, such as host defense, neurotoxicity, and vasodilation (4). However, the excess productions of NO have been implicated with immunological and inflammatory diseases including septic shock, rheumatoid arthritis, graft rejection, and diabetes (5). The inducible isoform of cyclooxygenase, COX-2, and nitric oxide synthase (iNOS) are mainly responsible for the production of large amounts of PGE₂ and NO (6). Inhibition of PGE₂ and NO production is an important therapeutic consideration in the development of anti-inflammatory agents.

Macrophages play an important role in the host defense mechanism against bacterial and viral infections. When macrophages are activated by various stimuli, such as lipopolysaccharide (LPS) and interferon-γ (IFN-γ), they inhibit the growth of a wide variety of tumor cells and microorganisms by releasing factors such as NO, cytokines, and eicosanoid mediators of the immune response (7). PGE₂ production by COX-2 and NO production by iNOS are mainly regulated at the transcriptional level (6). NF-κB is a transcription factor that regulates the expression of multiple immune and inflammatory genes (8). LPS activates NF-κB in macrophages, which induces the expression of iNOS and COX-2 (6).

Plants have always been among the common sources of medicines, either processed as traditional preparations

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or used to extract pure active principles. Because of the large chemical diversity among natural products, many research groups screen plant extracts in their search for new promising therapeutic candidates for various diseases (9).

*Melaleuca leucadendron* L., known as the paper-bark tree (9), is widely distributed throughout Taiwan. The bark and leaves are used in folk medicine as tranquilizing, sedating, evil-dispelling, and pain-relieving agents (10,11). However, to date, no scientific data supports these activities and the mechanisms through which the extract may be functioning are still unknown. Thus, this study examined the antioxidant effects of all the methanol extract fractions of *M. leucadendron* L. and evaluated the anti-inflammatory effect of its extracts in LPS-stimulated RAW264.7 macrophages.

**MATERIALS AND METHODS**

**Plant material**

*Melaleuca lucadendron* L. was obtained from the Bio-products Research Center, Yonsei University in Seoul, Korea. The plant material was shade dried and ground to a powder. A voucher specimen is deposited at 4°C.

**Extraction and isolation**

Dried *Melaleuca lucadendron* L. (1 kg) were grounded and extracted twice with 75% methanol (4 L, v/v) for 24 hr at room temperature. The extract was concentrated, frozen, and lyophilized (22.2 g). The methanol extract was further fractionated successively with chloroform, *n*-butanol, and water. Each fraction was evaporated and dried under reduced pressure (11.2 g chloroform fraction, 2 g butanol fraction, 8.8 g water fraction).

**Total phenolic contents**

Total phenolic contents in the extract were determined according to the method of Gutfinger (12). One mL of each 1 mg/mL extract concentration was mixed with 1 mL of 2% Na2CO3 followed by standing for 3 min. Then, 0.2 mL of 50% Folin-Ciocalteau reagent was added to the mixture. After standing for 30 min, the solution was centrifuged at 13,400 × g for 5 min. The absorbance was measured at 750 nm using a spectrophotometer (Shimadzu UV-1601, Tokyo, Japan) and total phenolic contents were expressed as gallic acid equivalents (GAE).

**DPPH radical scavenging activity**

The scavenging activities of *M. leucadendron* L. extracts were determined using the stable free radical 1,1-diphenyl-2-picryl-hydrazil (DPPH), according to a modified method of Shirwaikar and Somashekar (13). DPPH solution was prepared as a 0.2 mM ethanol concentration. 2 mL of the sample was vortex-mixed with 1 mL of DPPH solution, and incubated at room temperature for 30 min. The absorbance was measured at 517 nm against a blank and the activity was expressed as DPPH scavenging activity (% inhibition) = \((A_{control} - A_{sample})/A_{control}\) × 100%, where \(A_{sample}\) is the absorbance of the sample and \(A_{control}\) is the absorbance of the control. The scavenging activity of DPPH radicals was expressed as IC50.

**Superoxide anion radical scavenging activity**

The scavenging activity for superoxide anion radical was analyzed via a hypoxanthine/xanthine oxidase generating system coupled with reducing nitroblue tetrazolium (NBT), following the method of Kirby and Schmidt (14). The reaction mixture contained 125 μL of buffer (50 mM K2HPO4/KOH, pH 7.4) and 20 μL of 15 mM Na2EDTA, 30 μL of 3 mM hypoxanthine, 50 μL of xanthine oxidase (1 unit per 10 μL buffer) and 25 μL of plant extract (a diluted sonicated solution of 10 μg per 250 μL buffer). The absorbance of the solution was measured at 540 nm.

**Antioxidant activity on linoleic acid oxidation**

The oxidation test was conducted by using the linoleic acid model system. A 0.2 mL sample solution and 0.5 mL of 0.2 M sodium phosphate buffer (pH 7.0) were mixed with 0.5mL of 2.5% linoleic acid in ethanol. The peroxidation was initiated by adding 50 μL of 0.1 M 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and carried out at 37°C for 200 min in the dark. The degree of oxidation of peroxides was examined, according to the thiocyanate method, by reading the absorbance at 500 nm after coloring with FeCl3 and ammonium thiocyanate (15). A control test was performed with linoleic acid without sample solution.

**Cell culture**

RAW264.7 murine macrophages (KCLB No. 40071) were purchased from the Korea Cell Line Bank (Seoul, Korea). The macrophages were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco/BRL, Grand Island, NY, USA) containing 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Gibco/BRL). Macrophages were then incubated in 24 well tissue plates at a density of 1 × 105 cells/mL for 24 hr at 37°C. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

**Treatment of macrophages with LPS**

Macrophages were incubated with 10 μg/mL of LPS to stimulate COX-2 and iNOS gene expression. Each sample was dissolved in dimethylsulfoxide (DMSO) and added to the incubation medium 1 hr prior to addition.
of LPS. The final concentration of DMSO was adjusted to 0.1% (v/v).

**MTT assay for cell viability**

Cell viability was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent (16). After culturing on 96 well plates for 24 hr, the cells were washed twice and incubated with 110 μL of 0.5 mg/mL MTT for 2 hr at 37°C. The medium was discarded and 100 μL DMSO was then added. After 30 min incubation, the absorbance at 570 nm was measured using a microplate reader (Molecular Devices Co., Downingtown, PA, USA).

**Determination of PGE₂ production**

Macrophages were pretreated with aspirin (250 μM) for 2 hr to inactivate the COX-1 enzyme prior to the COX-2 activity assay. The cells were first washed three times with serum-free DMEM before adding LPS (10 μg/mL) to induce COX-2 expression. The culture supernatants were collected 20 hr later for measuring PGE₂ concentrations using ELISA kits (R&D System, Minneapolis, MN, USA) according to the manufacturer’s instructions.

**Determination of nitrite production**

The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess method as described previously (17). An amount of 100 μL of each culture supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid, and 0.1% naphthylethylene dihydrochloride in water) and the absorbance of the mixture at 550 nm was measured using a microplate reader. Nitrite concentrations were calculated from a standard curve of sodium nitrite prepared in the culture medium.

**SDS-polyacrylamide gel electrophoresis and Western blot analysis**

RAW264.7 cells (1 × 10⁶ cells/mL), grown in 60 mm dishes to confluence, were incubated with or without LPS in the absence or presence of tested samples for 24 hr, respectively. Cells were washed with ice-cold phosphate-buffered saline (PBS) and stored at -70°C until further analysis. Macrophages were collected and an equal amount of protein (30 μg/lane) was loaded and electrophoresed on an 8% (for iNOS and COX-2) or 10% SDS-polyacrylamide gel (for phospho-IκBα and IκBα). Gels were then transferred to polyvinylidene difluoride (PVDF; Millipore Co., Billerica, MA, USA) membranes. Membranes were blocked and incubated for 1 hr at room temperature with 1:1000 dilutions of primary antibodies: rabbit IκBα polyclonal, mouse phospho-IκBα monoclonal, rabbit iNOS polyclonal, and goat COX-2 polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA). α-tubulin (Santa Cruz Biotechnology) was used as an internal control. The immunoreactive protein was detected using a chemiluminescent system (ECL kit; Amersham Pharmacia Co., Piscataway, NY, USA). After exposure to X-ray film, band intensities were calculated from the optical density using an image analyzer (Vilber Co., Mame-la-Vallée Cedex, France).

**NF-κB-driven reporter gene assay**

An NF-κB reporter construct, consisting of the firefly luciferase gene under the control of the consensus NF-κB site, was used to quantify NF-κB transcriptional activity. A Renilla luciferase reporter was used as an internal control to normalize the reporter gene activity. RAW264.7 cells, seeded (1 × 10⁵ cells/mL) in 60 mm dishes, were transiently transfected with the reporter construct (3 μg) using the Fugene 6 reagent (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. After 3 hr post transfection, the cultures were treated with various concentrations of compounds tested and stimulated with 10 μg/mL of LPS. After 24 hr incubation, each 60 mm culture dish was washed twice with cold PBS, harvested, and subjected to dual luciferase assays. Dual-luciferase assays were performed with the Dual-Luciferase Reporter Assays System (Promega, Madison, WI, USA), according to the manufacturer’s instructions, and luciferase activities were determined with a luminometer (FLUOSTAR; BMG Labtechnologies, Ortenberg, Germany).

**Statistical analysis**

Each experiment was performed at least in triplicate. Results are expressed as the means ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett’s t-test for multiple comparisons. Statistical significance is expressed as *p*<0.05.

**RESULTS AND DISCUSSION**

**Total phenolic contents**

Phenolic compounds have potential antioxidant activities (18). Previous studies have established phenolic compounds as major antioxidants in medicinal plants, fruits and vegetables (19,20). Total phenolic contents of methanol extracts and solvent fractions of *M. leucadenron* L. are shown in Table 1. When investigating total phenolics, the butanol (BuOH) extract showed highest levels of phenolics (508.43 ± 2.22 μg GAE/g extract) among all the four extracts (Table 1). Phenolic compounds are widely distributed throughout the plant system demonstrating variable biological activities including antioxidant, anti-inflammatory and anti-carcinogenic...
Table 1. Total phenolic contents of M. leucadendron L. extracts

| Solvent fractions | Yield (%) | Total phenolic contents (μg GAE/g extract) |
|-------------------|-----------|------------------------------------------|
| MeOH              | 2.22      | 289.23 ± 5.21                            |
| CHCl₃             | 1.12      | 107.36 ± 1.88                            |
| BuOH              | 0.20      | 508.43 ± 2.33                            |
| H₂O               | 0.88      | 122.72 ± 3.71                            |

Data representative the mean ± SD of three separate experiments.

Table 2. IC₅₀ values of radical scavenging activities and antioxidant activities of M. leucadendron L. extracts

| Solvent fractions | IC₅₀ (μg/mL) | Radical scavenging | Superoxide anion radical scavenging | Antioxidant activity on linoleic acid oxidation |
|-------------------|-------------|--------------------|-------------------------------------|-----------------------------------------------|
| MeOH              | 5.1         | 14.5               | 6.2                                 |
| CHCl₃             | 55.7        | 9.3                | 18.1                                |
| BuOH              | 4.8         | 10.1               | 3.2                                 |
| H₂O               | 60.0        | 51.0               | 20.4                                |
| α-tocopherol      | 3.9         | 5.7                | 3.7                                 |
| BHT               | 2.3         | 4.2                | 2.88                                |

BHT and α-tocopherol were used as positive controls. 

IC₅₀ value is the concentration of 50% inhibition.

Radical scavenging activities

Among the four M. leucadendron L. extracts, the BuOH fraction showed the most potent radical scavenging activity in each assay (Table 2), showing IC₅₀ at 4.8 μg/mL in the DPPH free radical scavenging assay and 10.1 μg/mL in the superoxide anion radical scavenging assay. Many studies suggested that the antioxidant activity of plants was likely related to redox properties of their phenolics’ behavior (23). In this study, positive correlations were found between total phenolic content in the M. leucadendron L. extracts and their antioxidant activities. Furthermore, the BuOH extract of M. leucadendron L. has the most potent antioxidant activity among all the four extracts.

Cell viability

The BuOH extract was used for anti-inflammatory activity because antioxidant capacity data showed that this extract has potential antioxidant activity. First, the effects of BuOH fractions on RAW264.7 cell viability were determined by an MTT assay. The BuOH fraction did not exhibit cytotoxicity at concentrations less than 1 μg/mL compared to an LPS-treated control (data not shown).

Inhibition of LPS-induced PGE₂ and nitrite production

To evaluate the anti-inflammatory activity of BuOH extract, the effects of the extract on NO and PGE₂ productions stimulated by LPS in RAW264.7 cells were investigated. As shown in Table 3, LPS increased NO production by approximately 7.6 fold; however, when the BuOH extract was treated at 0.01, 0.1 and 1 μg/mL for 24 hr, NO production decreased by 66.1, 51.4, and 45.6%, respectively. In addition, LPS increased PGE₂ production by approximately 15 fold compared to the control, and PGE₂ production decreased by 60.3, 44.1, and 33.8%, respectively.

iNOS and COX-2 have been found to be highly induced at inflammatory sites in animals as well as patients with inflammatory diseases (3,6). NO and PGE₂ are produced through the actions of iNOS and COX-2, respectively, and participate in diverse biological effects such as the regulation of vascular inflammation, neurotransmission, and apoptosis (3,4). Numerous studies have revealed that excessive NO and PGE₂ production is important in the pathogenesis of inflammation and can lead to tissue damage (5). In this study, BuOH extract significantly inhibited NO and PGE₂ production of activated macrophages, suggesting that this extract could be

Table 3. Effects on PGE₂ and nitrite production in LPS-stimulated RAW264.7 cells after in-vitro exposure to BuOH extract of M. leucadendron L.

| Treatment       | PGE₂      | % Control | Nitrite     | % Control |
|-----------------|-----------|-----------|-------------|-----------|
| Control         | 12.2 ± 0.65* | 6.83     | 1.7 ± 0.10* | 13.1  |
| LPS alone       | 178.5 ± 9.39 | 100      | 13.1 ± 1.27*| 100  |
| LPS + BuOH ex.  | 0.01 μg/mL | 107.7 ± 1.78* | 60.3     | 8.7 ± 0.73* | 66.1 |
| LPS + BuOH ex.  | 0.1 μg/mL  | 78.8 ± 3.69 | 44.1     | 6.7 ± 0.18* | 51.4 |
| LPS + BuOH ex.  | 1 μg/mL    | 69.1 ± 2.69* | 33.8     | 5.9 ± 0.16* | 45.6 |
| LPS + Indometacin³ | 0.01 μg/mL | 21.7 ± 0.90* | 21.6    | –      | – |
| LPS + Indometacin³ | 0.1 μg/mL  | 5.9 ± 1.2*   | 5.9      | –      | – |
| LPS + Indometacin³ | 1 μg/mL    | 6.1 ± 0.52*  | 6.1      | –      | – |
| LPS + L-NAME²   | 0.01 μg/mL | –         | 6.5 ± 0.19* | 49.6  |
| LPS + L-NAME²   | 0.1 μg/mL  | –         | 4.3 ± 0.17* | 32.9  |
| LPS + L-NAME²   | 1 μg/mL    | –         | 3.3 ± 0.11* | 25.5  |

*Data representative as mean ± SD of three separate experiments.

²Data representative control for PGE₂ and nitrite production, respectively.

³p<0.05, significantly different compared to LPS alone.
useful as an anti-inflammatory resource.

Inhibition of LPS-induced COX-2 and iNOS protein expression

LPS strongly upregulates iNOS and COX-2 levels in RAW264.7 cells (3). To determine whether the inhibitory effects of BuOH extract on NO and PGE$_2$ productions were related to the modulation of iNOS and COX-2 expressions, western blotting was used. As shown in Fig. 1, expression of the COX-2 and iNOS protein were barely detectable in unstimulated cells, but markedly increased after LPS treatment. Treatment with BuOH extract (0.01, 0.1 and 1 μg/mL) for LPS-stimulated macrophages caused dose-dependent inhibition of COX-2 and iNOS protein expressions, consistent with PGE$_2$ and nitrite production (Table 3). These results suggested that BuOH extract attenuates the production of NO and PGE$_2$ by inhibiting the expressions of iNOS and COX-2 in LPS-stimulated macrophage cells. Thus, the anti-inflammatory effects of the BuOH extract may be attributed to its suppressive activity on PGE$_2$ and NO production by blocking iNOS and COX-2 gene and protein expressions.

Inhibition of LPS-induced phosphorylation and degradation of iκBα

In order to explore the mechanism underlying the anti-inflammatory effect of BuOH extract, our attention focused on the NF-κB signal pathway. Recent studies have reported that NF-κB regulates the expression of iNOS and COX-2. NF-κB is one of the most ubiquitous eukaryotic transcription factors, regulating gene expression of cytokines and enzymes involved in controlling inflammatory responses (6). Functionally active NF-κB exists as a heterodimer, but is usually in an inactive sequestered complex bound to its endogenous inhibitor inhibitory kappa B-alpha (IκBα), in the cytoplasm. However, bound IκBα is rapidly phosphorylated by IκBα kinase in response to external stimuli, such as inflammatory cytokines and LPS, and is subsequently degraded by proteasomes. The free dimeric NF-κB, activated by the dissociation of IκBα, translocates into the nucleus and induces transcription of a wide variety of target genes that encode regulatory proteins, including cytokines and growth factors. The activation of these genes by NF-κB then leads to physiological responses such as inflammatory or immune responses (8).

The inhibitory effect of phosphorylation and degradation of IκBα in BuOH extracts in LPS-activated macrophages was examined (Fig. 2). The cytoplasmic levels of phospho-IκBα and IκBα were determined by western blotting. Phospho-IκBα protein concentration in RAW 264.7 cells increased after LPS treatment, but decreased after BuOH extract treatment. IκBα proteins also decreased to almost undetectable levels after LPS treatment and recovered after the BuOH extract treatment. Treatment of macrophages with 1 μg/mL of BuOH extract completely blocked LPS-induced IκBα degradation (Fig. 2). Yin et al. reported that the anti-inflammatory properties of aspirin have been linked to suppression of NF-κB activation through stabilization of IκB (24). Therefore, the phosphorylation and degradation of IκB proteins are critical in NF-κB activation.

Recently, natural compounds such as curcumin, epigallocatechin gallate, and resveratrol have been reported to ameliorate LPS-induced inflammatory response by downregulating the activities of NF-κB (6,8). Our data indicated that the BuOH extract may inhibit NF-κB activation by suppressing the phosphorylation and degradation of IκBα in LPS-induced RAW264.7 cells. Taken together, BuOH extracts of _M. leucadendron_ L. could be good candidates for the treatment of various human inflammatory disorders because its inhibitory effect on pro-inflammatory mediators is comparable to other previously characterized compounds, such as curcumin, epi-
gallicatechin gallate, and resveratrol (6).

**Blocking of NF-κB-regulated gene expression by M. leucadendron L. extract**

We performed an NF-κB-driven luciferase reporter gene assay to show that BuOH extracts were able to block NF-κB-regulated gene expressions (iNOS and COX-2). Incubation of RAW264.7 cells with LPS for 24 hr increased NF-κB transcription activity strongly, but the LPS-induction was markedly inhibited by the extracts.

The luciferase assay revealed that BuOH extracts suppress LPS-mediated gene expression mainly via inhibition of NF-κB activity. These results indicate that inhibition of COX-2 and iNOS gene expression by the BuOH extracts may be, at least in part, related to the NF-κB inhibition pathway.

*M. leucadendron* L. has been long used in traditional oriental medicine of South Asia for the treatment of inflammatory diseases; however, the activities and mechanisms through which the extract may be acting are unknown. This study supports the pharmacological basis of *M. leucadendron* L. used as a herbal medicine for the treatment of inflammation. Our study also indicates the possibility for using *M. leucadendron* L. extract in cancer chemoprevention or anticancer therapy.

In conclusion, our data suggest *M. leucadendron* L. extract exhibited noticeable antioxidant activity and mediates inhibition of COX-2 enzyme activity, which can affect related gene and protein expressions. *M. leucadendron* L. functions by a mechanism of action similar to that of NSAIDs. Together, these results add a novel aspect to the biological profile of *M. leucadendron* L.

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28 Jeonghee Surh and Jung-Mi Yun

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