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 tranquilizing, 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 ($CHCl_3$) and butanol (BuOH). Antioxidant activity of the MeOH extract and BuOH fraction were higher than that of both α-tocopherol and butyrated hydroxytoluene (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 E_2 (PGE_2) production, and cyclo-oxygenase-2 (COX-2) expression in RAW 264.7 macrophages. The BuOH fraction significantly inhibited LPS-induced NO and PGE_2 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 (IκBα) 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 suggestively correlate strongly 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 E_2 (PGE_2) 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 re-

jection, and diabetes (5). The inducible isoform of cyclo-oxygenase, COX-2, and nitric oxide synthase (iNOS) are mainly responsible for the production of large amounts of PGE_2 and NO (6). Inhibition of PGE_2 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). PGE_2 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 activites and the mechanims 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 grounded to a powder. A voucher specimen is deposited at $4^{\circ}C$.

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% Na_2CO_3 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 $13,400 \times g$ 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) = $\{(A_{control} - A_{sample})/A_{control}\} \times 100\%$, 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 IC_{50}.

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 K_2HPO_4/KOH, pH 7.4) and 20 μL of 15 mM Na_2EDTA, 30 μL of 3 mM hypoxanthine, 50 μL of xanthine oxidase (1unit per 10 mL 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 $37^{\circ}C$ 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 $FeCl_2$ and ammonium thiocyanate (15). A control test was performed with linoleic acid without sample solution.

Cell culture

RAW264.7 murine marcrophages (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 1×10^6 cells/mL for 24 hr at $37^{\circ}C$. Cells were maintained at $37^{\circ}C$ in a humidified atmosphere containing 5% CO_2.

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% phosporic acid, and 0.1% naphthylethylenediamine 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 (1×10^6 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 Pharmarcia 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-Vallee 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 (1×10^5 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. leucadendron* 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
$CHCl_3$	1.12	107.36 ± 1.88
BuOH	0.20	508.43 ± 2.33
H_2O	0.88	122.72 ± 3.71

Data representative the mean $\pm$ SD of three separate experiments.

Table 2. IC_{50}[1] values of radical scavenging activities and antioxidant activities of *M. leucadendron* L. extracts

Solvent fractions	IC_{50} (μg/mL)		
	DPPH radical scavenging	Superoxide anion radical scavenging	Antioxidant activity on linoleic acid oxidation
MeOH	5.1	14.5	6.2
$CHCl_3$	55.7	50.3	18.1
BuOH	4.8	10.1	3.2
H_2O	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.
[1]IC_{50} value is the concentration of 50% inhibition.

(21,22).

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 IC_{50} 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. leuca-*

dendron 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 PGE_2 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 PGE_2 production by approximately 15 fold compared to the control, and PGE_2 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 PGE_2 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 revelaed that excessive NO and PGE_2 production is improtant in the pathogenesis of inflammation and can lead to tissue damage (5). In this study, BuOH extract significantly inhibited NO and PGE_2 production of activated macrophages, suggesting that this extract could be

Table 3. Effects on PGE_2 and nitrite production in LPS-stimulated RAW264.7 cells after *in-vitro* exposure to BuOH extract of *M. leucadendron* L.

Treatment		PGE_2	% Control	Nitrite	% Control
Control		12.2 ± 0.65[3]*	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
	0.1 μg/mL	78.8 ± 3.69*	44.1	6.7 ± 0.18*	51.4
	1 μg/mL	69.1 ± 2.69*	33.8	5.9 ± 0.16*	45.6
LPS + Indometacin[1]	0.01 μg/mL	21.7 ± 0.90*	21.6	−	−
	0.1 μg/mL	5.9 ± 1.2*	5.9	−	−
	1 μg/mL	6.1 ± 0.52*	6.1	−	−
LPS + L-NAME[2]	0.01 μg/mL	−	−	6.5 ± 0.19*	49.6
	0.1 μg/mL	−	−	4.3 ± 0.17*	32.9
	1 μg/mL	−	−	3.3 ± 0.11*	25.5

[1,2]Positive control for PGE_2 and nitrite production, respectively.
[3]Data representative as mean $\pm$ SD of three separate experiments.
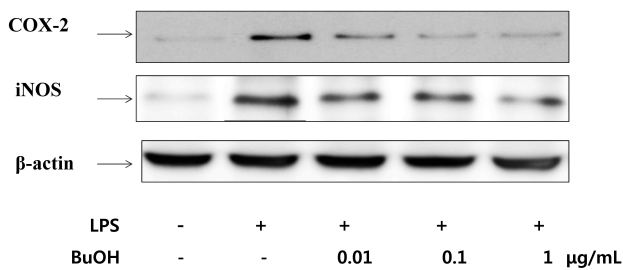*$p < 0.05$, significantly different compared to LPS alone.

Fig. 1. Inhibition of COX-2 and iNOS protein expression by BuOH extract of *M. leucadendron* L. in LPS-stimulated RAW264.7 cells. Cells were pretreated with the indicated concentrations of BuOH extract for 1 hr before being incubated with LPS (10 μg/mL) for 24 hr. Cell lysates were then prepared and subjected to western blotting using an antibody specific for COX-2 and iNOS. β-actin was used as an internal control.

Fig. 2. Effect of BuOH extract of *M. leucadendron* L. on the LPS-induced phosphorylation and degradation of IκBα in RAW264.7 cells. Cells were pretreated with the indicated concentrations of BuOH extract of *M. leucadendron* L. for 1 hr before being incubated with LPS (10 μg/mL) for 24 hr. Cell lysates were then prepared and subjected to western blotting using an antibody specific for phospho-IκBα and IκBα. α-tubulin was used as an internal control.

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 effcts 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). Fuctionally active NF-κB exists as a heterodimer, but is usually in an inactive seqesterd 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 cyto-
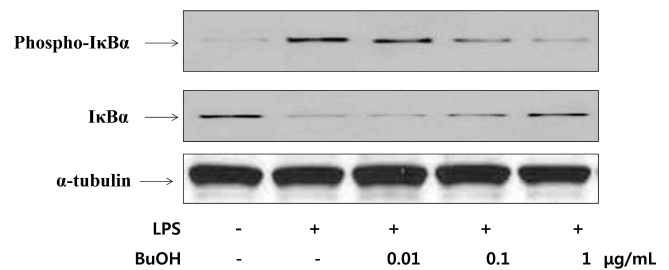
kines and LPS, and is subsequently degraded by proteosomes. The free dimeric NF-κB, activated by the dissociation of IκBα, translocates into the nucleus and induces transciption of a wide variety of target genes that encode regulatory proteins, including cytokines and growth factors. The activation of 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-
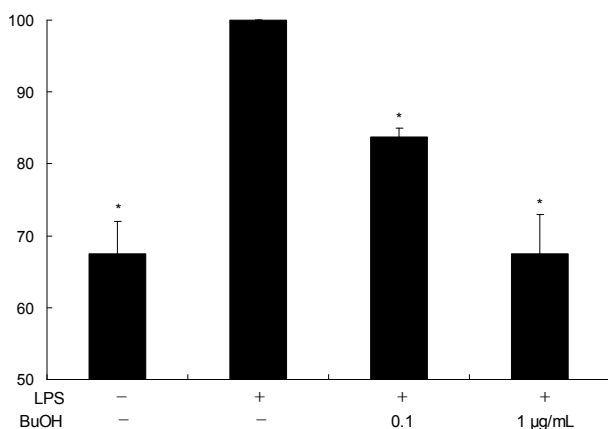
Fig. 3. Inhibition of NF-κB transcriptional activity by BuOH extract of *M. leucadendron* L. on LPS-stimulated RAW264.7 cells. The RAW264.7 cells transfected with an NF-κB reporter plasmid were treated with the indicated concentrations of BuOH extract of *M. leucadendron* L. and then stimulated with LPS (10 μg/mL). After 24 hr incubation, the activity of NF-κB was estimated by the luciferase assay. The relative NF-κB activity levels were expressed as the ratio of the activity of firefly luciferase over that of renilla luciferase. Data represent mean ±SD of three independent experiments performed in triplicate; *p<0.05 versus LPS alone.

gallocatechin 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-reglulated 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 activites 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. leucadenddron* 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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