

Full Length Research Paper

# Anti-inflammatory activity of brown alga *Dictyota dichotoma* in murine macrophage RAW 264.7 cells

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COX-2 (Cyclooxygenase-2) and iNOS (inducible nitric oxide synthase) are involved in various pathophysiological processes such as inflammation. In a search for inhibitors of COX-2 and iNOS, we found that extracts of *Dictyota dichotoma* inhibit Nitric oxide (NO) and PGE<sub>2</sub> generation in LPS-stimulated RAW 264.7 macrophage cells, as well as levels of iNOS and COX-2 mRNA and protein. *D. dichotoma* were extracted with 80% EtOH. The extract was then partitioned with hexane, CH<sub>2</sub>Cl<sub>2</sub>, EtOAc, BuOH, and water, successively. The results indicate that the CH<sub>2</sub>Cl<sub>2</sub> fraction of *D. dichotoma* extract is an effective inhibitor of LPS- induced NO and PGE<sub>2</sub> production in RAW 264.7 cells. These inhibitory effects of the CH<sub>2</sub>Cl<sub>2</sub> fraction of *D. dichotoma* were accompanied by decreases in the expression of iNOS and COX-2 proteins and iNOS and COX-2 mRNA in dose-dependent pattern. To test the inhibition effects of *D. dichotoma* fractions on other cytokines, we also performed RT-PCR on TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in LPS-stimulated RAW 264.7 macrophage cells. In these assays, the CH<sub>2</sub>Cl<sub>2</sub> fraction of *D. dichotoma* also showed decreases in the expression of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 mRNA. Based on these results, we suggest that *D. dichotoma* extracts may be considered possible anti-inflammatory candidates for human health.

**Key words:** COX-2, Cytokine, *Dictyota dichotoma*, inflammation, iNOS (inducible nitric oxide synthase), PGE<sub>2</sub>.

## INTRODUCTION

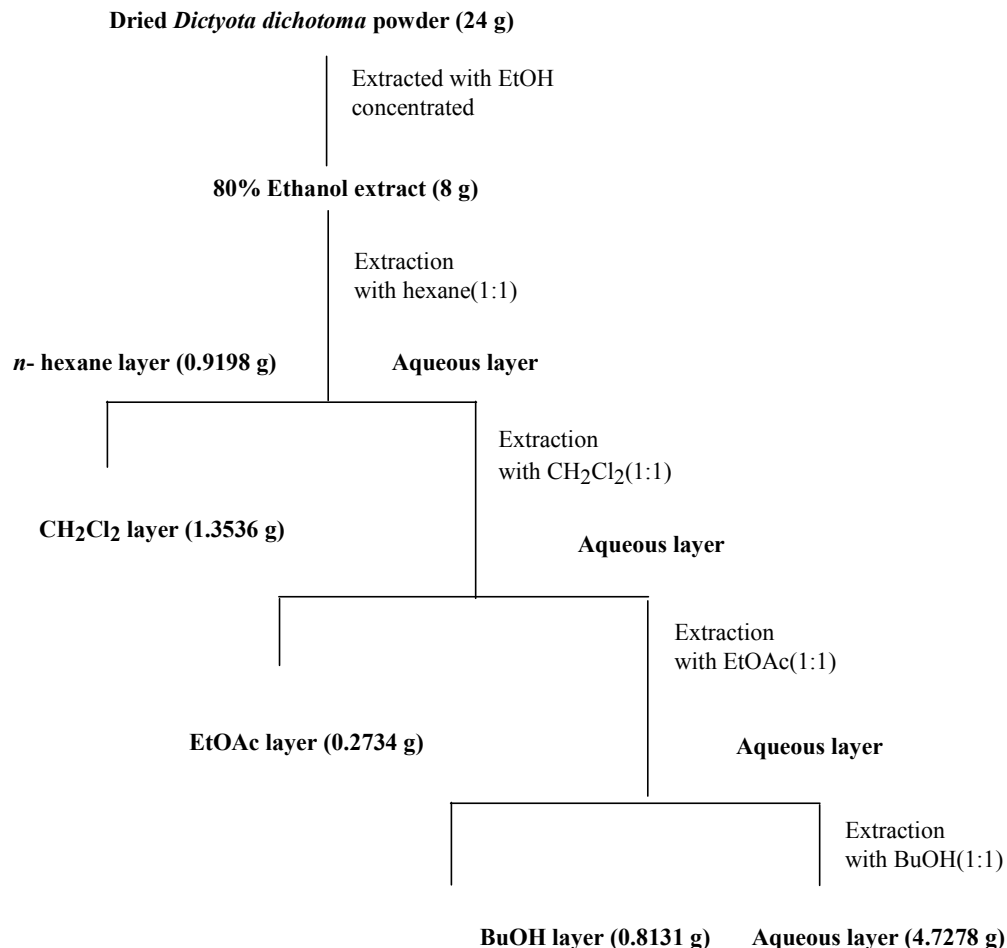
Inflammation symptoms occur when the human body attempts to counteract potentially injurious agents such as invading bacteria, viruses, and other pathogens (Henderson et al., 1996; Ulevitch and Tobias, 1995; Hersh et al., 1998). Inflammation can also be induced by biochemical and pharmacological agents from the environment, in addition to a diverse and potentially huge array of cell types and soluble mediators including cytokines (Nicod, 1993; Rouveix, 1997; Boraschi et al., 1998; Dinarello, 2000; Turcanu and Williams, 2001).

The anti-inflammatory activity of solvent extracts from organism has been measured by the activity of inflammatory mediators and pro-inflammatory cytokines on the chronic inflammation of RAW 264.7 cell (Park et al., 2005). Among pro-inflammatory cytokines, IL-1 $\beta$ , IL-6

and TNF- $\alpha$  are known to produce fever, inflammation, tissue destruction, and sometimes induce shock and consequently death as being administered to humans (Dinarello, 2000). Inflammatory mediators to test the anti-inflammatory activity of solvent extracts includes: Nitric oxide (NO) and Cyclooxygenase (COX).

NO has been known as an important molecule to regulate the biological activities in vascular, neural, and immune systems (Moncada et al., 1992). Infected macrophages produce NO to mediate host defense functions, including antimicrobial and anti-tumor activities; however its excess production causes tissue damage associated with acute and chronic inflammation (MacMicking et al., 1997). COX is the key enzyme for the conversion of arachidonic acid (AA) to prostaglandins (PGs) (Vane et al., 1998). There are two isoforms of COX; one is the COX-1, a constitutive enzyme, which is responsible for the production of PGs with general house-keeping functions such as maintenance of renal perfusion and a protective effect on the gastric mucosa against

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**Scheme. 1.** Sequential purification using solvent partitioning from *D. dichotoma*.

ulceration; and the other is the COX-2, an inducible enzyme, responsible for the production of PGs (Needleman and Isakson, 1998). COX-2 is expressed during inflammation by cytokines and bacterial products, such as lipopolysaccharide (LPS), and it produces PGs contributing to the pain and swelling of inflammation (Hla and Neilson, 1992; O'Sullivan et al., 1992).

*Dictyota dichotoma* is a marine brown alga distributed worldwide from temperate to subtropical regions. There has been continued taxonomic controversy about *D. dichotoma* due to its high morphological variability which has been attributed largely to locality. Especially the plants collected along the coasts of the West-North Pacific are identified as a different species from the Atlantic (Hwang et al., 2005). So we named the plants from this area as *D. dichotoma* (Hudson) Lamouroux (*sensus in* Okamura). There are a few reports on the biological activity of solvent extract constituents from this species except for report on acidic compound (Sasaki et al., 2005). This study has been designed to estimate the anti-inflammatory effect of solvent fraction constituents from thallus of this species by measuring the production

of pro-inflammatory factors (TNF- $\alpha$ , IL-1, IL-6, iNOS, COX-2 and PGE<sub>2</sub>) in murine macrophage RAW 264.7 cells.

## MATERIALS AND METHODS

### Materials and solvent extraction

Thallus of *D. dichotoma* were collected in April 2006, from Jeju Island, Korea. The voucher specimen with number JBR-251 is deposited at the herbarium of Jeju Biodiversity Research Institute. The materials for extraction were cleaned, dried at room temperature for 2 weeks and ground into a fine powder. The dried alga (24 g) was extracted 80% ethanol (EtOH; 2 L) at room temperature for 24 h and then evaporated under a vacuum. The evaporated EtOH extract (8 g) was suspended in water (1 L), and partitioned with four solvents, n-hexane (1 L), dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>; 1 L), ethylacetate (EtOAc; 1 L), and butanol (BuOH; 1 L), and this partition was repeated three times. Yields and ratios of these four solvent partitions were represented in parentheses for n-hexane (0.9198 g, 11.30%), CH<sub>2</sub>Cl<sub>2</sub> (1.3536 g, 16.62%), EtOAc (0.2734 g, 3.36%), BuOH (0.8131 g, 9.99%) and H<sub>2</sub>O (4.7278 g, 58.06%) fractions (Scheme 1).

**Table 1.** The sequences of primers used in RT-PCR analysis and the sizes of RT-PCR products.

Gene		Primer sequences	Fragment size(bp)
TNF-	F	5'-TTGACCTCAGCGCTGAGTTG-3'	364
	R	5'-CCTGTAGCCCACGTCGTAGC-3'	
IL-1	F	5'-CAGGATGAGGACATGAGCACC-3'	447
	R	5'-CTCTGCAGACTCAAACCTCCAC-3'	
IL-6	F	5'-GTA CTCCAGAAGACCAGAGG-3'	308
	R	5'-TGCTGGTGACAACCACGGCC-3'	
iNOS	F	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'	496
	R	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'	
COX-2	F	5'-CACTACATCCTGACCCACTT-3'	696
	R	5'-ATGCTCCTGCTTGTAGTATGT-3'	
-actin	F	5'-GTGGGCCGCCCTAGGCACCAG-3'	603
	R	5'-GGAGGAAGAGGATGCGGCAGT-3'	

### Cell culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, KOREA) and cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO Inc, NY, USA) supplemented with 100 U/ml of penicillin, 100 g/ml of streptomycin and 10% fetal bovine serum (FBS; GIBCO Inc, NY, USA). The cells were incubated in the presence of 5% CO<sub>2</sub> at 37°C and were subcultured in every three days.

### LDH cytotoxicity assay

Lactate dehydrogenase (LDH) leakage is a means of measuring membrane integrity as a function of the amount of cytoplasmic LDH released from the cytosol into the medium. LDH activity was determined following the production of NADH during the conversion of lactate to pyruvate (Fernandez et al., 2006). The release of LDH from RAW 264.7 cells was used to detect cytotoxicity and was measured at the end of each proliferation experiment. The LDH activity was determined using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA). Briefly, culture medium was centrifuged at 12,000 rpm for three min at room temperature to ensure accumulation of cells. The cell-free culture medium (50 l) was collected and then incubated with 50 l of the reaction mixture cytotoxicity detection kit for 30 min at room temperature in the dark. 1 N HCl (50 l) was added into each well to stop the enzymatic reaction. The optical density of the solution was then measured by using an ELISA plate reader at 490 nm. Percentage cytotoxicity was determined as relative to the control group. All experiments were performed in triplicate.

### Determination of NO production

After pre-incubation of RAW 264.7 cells with LPS (1 g/ml) for 24 h, the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction (Hevel and Marletta, 1997). Briefly, 100 l of cell culture medium was mixed with 100 l of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a

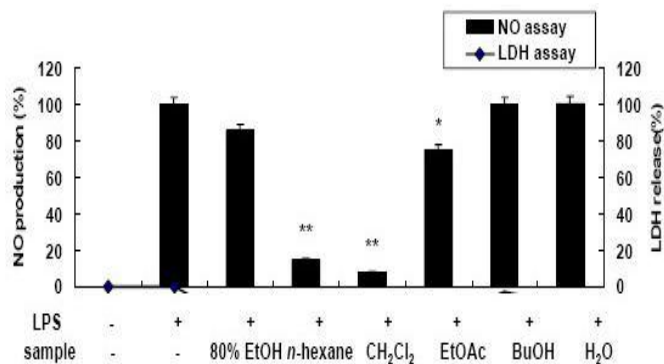
microplate reader. Fresh culture medium was used as a blank in every experiment. The quantity estimation of nitrite was based on a sodium nitrite standard calibration curve. All experiments were performed in triplicate.

### RNA isolation and RT-PCR analysis

Total RNAs from LPS treated RAW 264.7 cells were prepared by adding Tri-Reagent (MRC, Cincinnati, OH, USA), according to the manufacturer's protocol. RNA was solution was stored at -70°C until used. The reverse transcription of 1 g RNA was carried out in reaction cocktail with M-MuLV reverse transcriptase (Promega, WI, USA), oligo (dT) 18 primer, dNTP (0.5 M) and 1 U RNase inhibitor. After incubation this reaction cocktail at 70°C for 5 min, 25°C for 5 min, and 37°C for 60 min in series, M-MuLV reverse transcriptase was inactivated by heating at 70°C for 10 min. The polymerase chain reaction (PCR) was performed in a reaction buffer (cDNA, 1.25 U Taq DNA polymerase (Promega, WI, USA), 3' and 5' primer 50 M each and 200 mM dNTP in 200 mM Tris-HCl buffer, pH 8.4, containing 500 mM KCl and 1 - 4 mM MgCl<sub>2</sub>). The PCR was performed with a DNA gene cycler (BIO-RAD, HC, USA), and the amplification was followed by 30 cycles of 94°C for 45 s (denaturing), 60-65°C for 45 s (annealing) and 72°C for 1 min (primer extension). The nucleotide sequence of each primer (Lee et al., 2006) and the size of PCR product were shown in Table 1. The PCR products were electrophoresed on a 1.5% agarose gel.

### Immunoblotting

RAW 264.7 cells were pre-incubated for 18 h, and then stimulated by LPS (1 g/ml) in the presence of testing materials for 24 h. After pre-incubation, the cells were collected and washed twice with cold-PBS (phosphate buffered Saline). The lysis of cells were done in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO<sub>3</sub>, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 g/ml aprotinin, 25 g/ml leupeptin] and kept on ice for 30 min. The cell lysates were centrifuged at 12,000xg at 4°C for 15 min and then the supernatants were stored at -70°C before used in tests. Protein concentration was measured according to the Bradford method (Bradford, 1976). Aliquots of the lysates (30-50 g of protein) were



**Figure 1.** The effects of *D. dichotoma* extracts on the production of nitric oxide and cytotoxicity in RAW 264.7 cells. The production of nitric oxide was assayed from culture medium of cells stimulated with LPS (1 g/ml) in the presence of EtOH extract and solvent fractions of *D. dichotoma* (25 g/ml). NO production was determined by ELISA method. Cytotoxicity was determined using LDH method. Values are the mean  $\pm$  SEM of triplicate experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$

separated on a 8~12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking the nonspecific site with 5% nonfat dried milk, the membrane was incubated with specific primary mouse monoclonal anti-mouse iNOS Ab (1:1000, Calbiochem, La Jolla, CA, USA), and rabbit polyclonal anti-rabbit COX-2 Ab (1:1000, BD Biosciences Pharmingen, San Jose, CA, USA) at 4°C for overnight. Each membrane was further incubated for 30 min with a secondary peroxidase-conjugated goat IgG (1:5000, Amersham Pharmacia Biotech, Little Chalfont, UK) to mouse and rabbit, respectively. The immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham Biosciences, Piscataway, NJ, USA).

#### Determination of PGE<sub>2</sub> production

Each fraction (25 g/ml) from *D. dichotoma* solubilized was diluted with DMEM before treatment. Cells were treated with LPS (1 g/ml) to allow the production of cytokines for 24 h. The inhibitory effect of each fraction (25 g/ml) on the cytokines production from the LPS-treated RAW 264.7 cells was determined as described previously. Supernatants were then harvested and assayed for cytokines by ELISA. The PGE<sub>2</sub> concentration in the culture medium was quantified using a competitive enzyme immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The productivity of PGE<sub>2</sub> was measured in relative productivity to that of the control treatment (Kor and Choi, 2005). All experiments were performed in triplicate.

#### Statistical analysis

The Student's t-test and a one-way ANOVA were used to determine the statistical significant difference between the values for the various experimental and control groups. Data are expressed as means  $\pm$  standard errors (S.E.M.) and the results are taken from at least three independent experiments performed in triplicate.  $p$ -values of 0.05 or less were considered to be statistically significant.

## RESULTS AND DISCUSSION

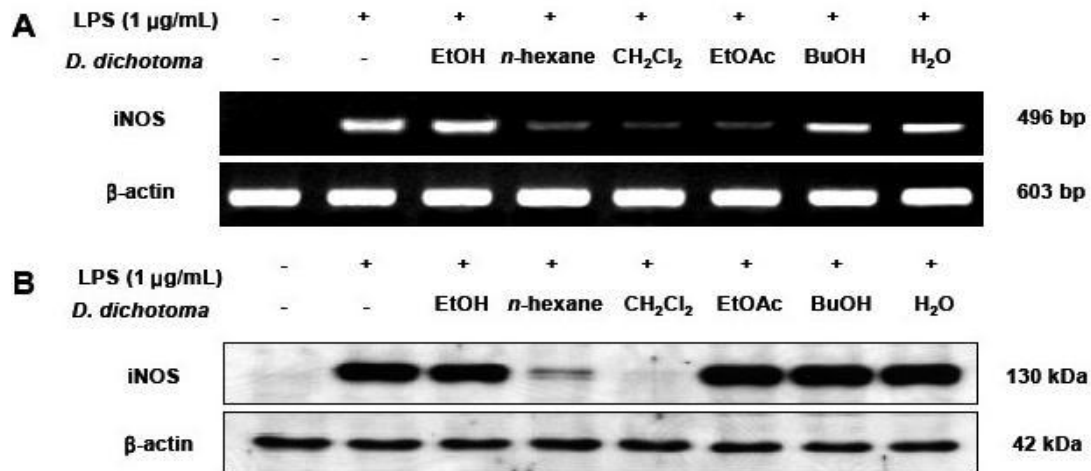
### Effect of *D. dichotoma* extracts on NO synthesis in activated macrophages

The Griess reaction, a spectrophotometric determination for nitrite, was carried out to quantify the nitrite levels in the conditioned medium of RAW 264.7 cells treated with LPS. In murine macrophage RAW 264.7 cells, LPS stimulation alone could induce iNOS transcription and its protein synthesis, and increased NO production. Furthermore, LPS stimulation was also well known to induce I B proteolysis and NF- $\kappa$ B nuclear translocation (Xie et al., 1994; Henkel et al., 1993). Therefore, this cell system was an excellent model for drug screening and the following evaluation of potential inhibitor on the pathways leading to the induction of iNOS and NO production.

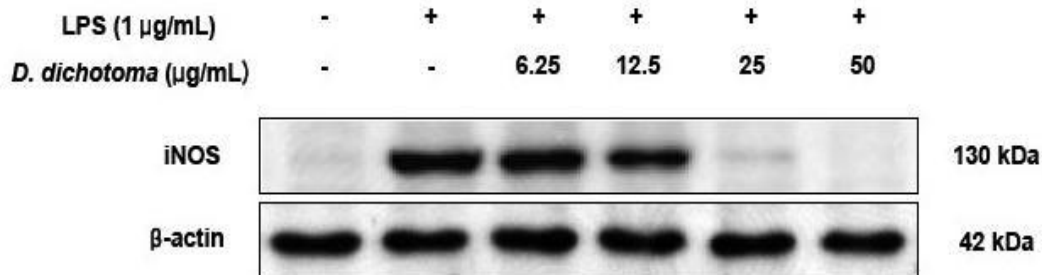
Whether the solvent extracts of *D. dichotoma* modulate NO production from murine macrophage RAW264.7 activated by LPS (1 g/ml) were examined by above nitrite assay. NO production was expressed as the ratio of the quantity of NO produced with solvent fraction and LPS to that with LPS alone (Figure 1). There was no basal NO production in the incubation with only the crude extract from *D. dichotoma* without LPS (data not shown). NO production in the cells incubated with LPS and solvent fraction of *D. dichotoma* extracts was reduced. The addition of the n-hexane and CH<sub>2</sub>Cl<sub>2</sub> fraction (25 g/ml) to medium with LPS largely inhibited production of NO with ratio of 85.09 and 92.12% respectively (Figure 1). The numbers of viable activated macrophages were not altered by the solvent fractions as determined by LDH assays, indicating that the inhibition of NO synthesis by the solvent fractions was not simply due to cytotoxic effects.

### Effect of *D. dichotoma* fractions on iNOS protein and mRNA expression in LPS-induced RAW 264.7 cells

To evaluate the anti-inflammatory effect of the different solvent fractions, the productivity of an array of iNOS, induced by LPS in RAW 264.7 cell was measured by RT-PCR and immunoblotting. After preculture of RAW 264.7 cells for 18 h, the mRNA and the protein expression of iNOS were figured in cultured cells with LPS (1 g/ml) and *D. dichotoma* fractions (25 g/ml) for the 24 h. The iNOS mRNA expression was conspicuously depressed in cells treated with n-hexane (69.52%) and CH<sub>2</sub>Cl<sub>2</sub> (81.76%) fractions of *D. dichotoma* (Figure 2A). Also, n-hexane and CH<sub>2</sub>Cl<sub>2</sub> fractions of *D. dichotoma* displayed remarkably decreased the iNOS protein levels by 30.60 and 76.00%, respectively (Figure 2B). The decrease in iNOS protein level was more pronounced in cells treated with high rather than low concentration of CH<sub>2</sub>Cl<sub>2</sub> fraction (Figure 3).



**Figure 2.** Inhibitory effects of EtOH extract and solvent fractions of *D. dichotoma* on the iNOS mRNA expression level and protein level in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells ( $1.0 \times 10^6$  cells/ml) were pre-incubated for 18 h, and the cells were stimulated with LPS (1 g/ml) in the presence of EtOH extract and solvent fractions of *D. dichotoma* (25 g/ml) for 24 h. (A) mRNA expression (B) protein level of iNOS and -Actin were determined by RT-PCR and immunoblotting.



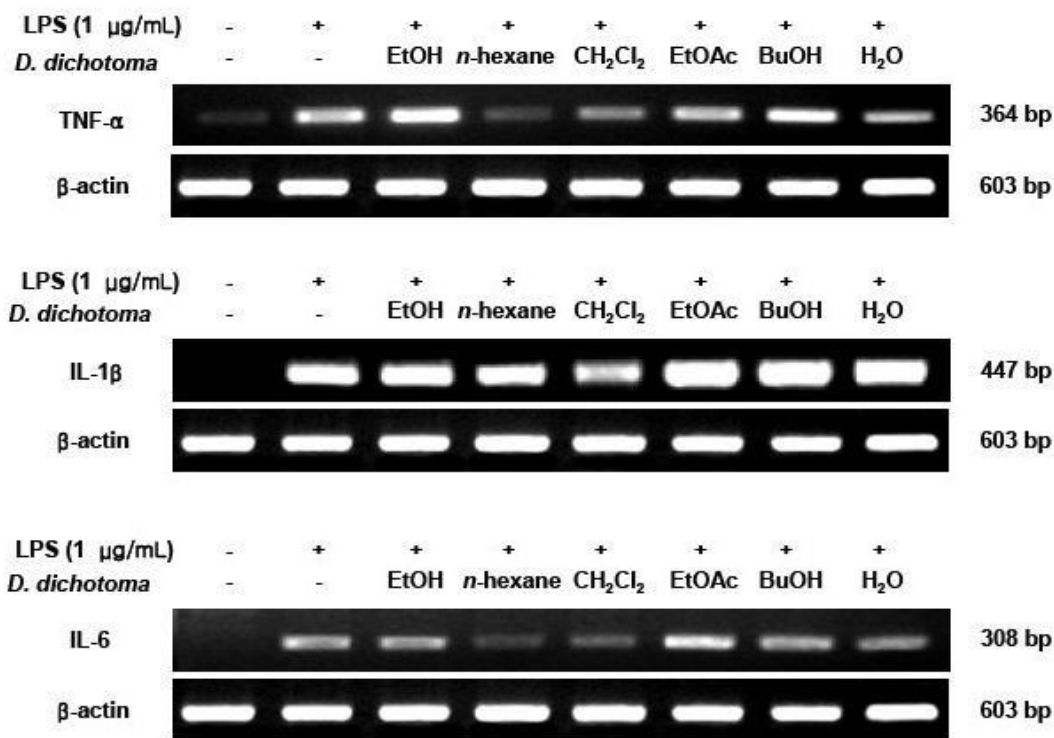
**Figure 3.** Inhibitory effects of different concentrations of CH<sub>2</sub>Cl<sub>2</sub> fraction of *D. dichotoma* on the protein level of iNOS in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells ( $1.0 \times 10^6$  cells/ml) were pre-incubated for 18 h, and the cells were stimulated with LPS (1 g/ml) in the presence of CH<sub>2</sub>Cl<sub>2</sub> fraction of *D. dichotoma* (6.25, 12.5, 25, 50 g/ml) for 24 h. iNOS protein level was determined by immunoblotting.

**Effect of *D. dichotoma* fractions on IL- 1, IL-6 and TNF- mRNA expression in LPS-induced RAW 264.7 cells**

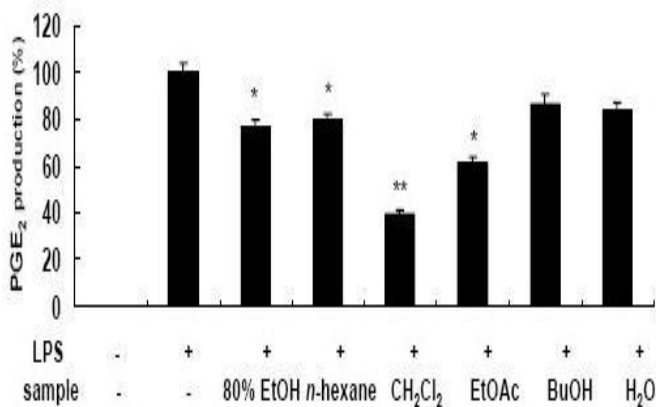
iNOS is the key enzyme that produces large amounts of NO by macrophages stimulated by bacterial endotoxin of LPS and pro-inflammatory cytokines such as interferon-(IFN - ) and TNF- (MacMicking et al., 1997; Nathan and Xie, 1994). The cytokines IL-1, IL-6 and TNF- , which are produced mainly by activated monocytes or macrophages (Dinarello, 2000). The IL-6, a potent mitogenic polypeptide, stimulated cell proliferation in a various types of cells (Stein and Sutherland, 1998).

Therefore, we determined the effect of solvent fraction from *D. dichotoma* on pro-inflammatory cytokines in murine macrophage RAW 264.7 cells using RT-PCR analyses. After preincubation for 18 h, mRNA expression of

pro-inflammatory cytokines was determined from the 8 h culture which was stimulated with LPS (1 g/ml) in the presence of *D. dichotoma* solvent fractions (25 g/ml). There were no basal change in IL-1 , IL- 6, and TNF- expression when incubated with only the crude extract from *D. dichotoma* without LPS (data not shown). The cells incubated with LPS and solvent fractions of *D. dichotoma* reduced gene expression especially in treatment with *n*-hexane and CH<sub>2</sub>Cl<sub>2</sub> fraction. After 24 h incubation with both LPS (1 g/ml) and solvent fractions of *D. dichotoma* (25 g/ml), there was remarkable inhibition of TNF- and IL-6 mRNA expression in RAW264.7 cells, especially the *n*-hexane and CH<sub>2</sub>Cl<sub>2</sub> fraction treatment. The *n*-hexane and CH<sub>2</sub>Cl<sub>2</sub> fractions (25 g/ml) clearly inhibited the mRNA expression of both TNF- and IL-6 by 61.17 and 45.82%, 75.5 and 42.75%, respectively (Figure 4). Furthermore, the CH<sub>2</sub>Cl<sub>2</sub> fraction (25 g/ml)



**Figure 4.** Effects of different solvent fractions of *D. dichotoma* extract on the mRNA expression of TNF- $\alpha$ , IL-1 and IL-6 of in RAW 264.7 cells. The mRNA expression was determined from the 24 h culture of cells stimulated with LPS (1 g/ml) in the presence of different solvent fractions of *D. dichotoma* (25 g/ml).



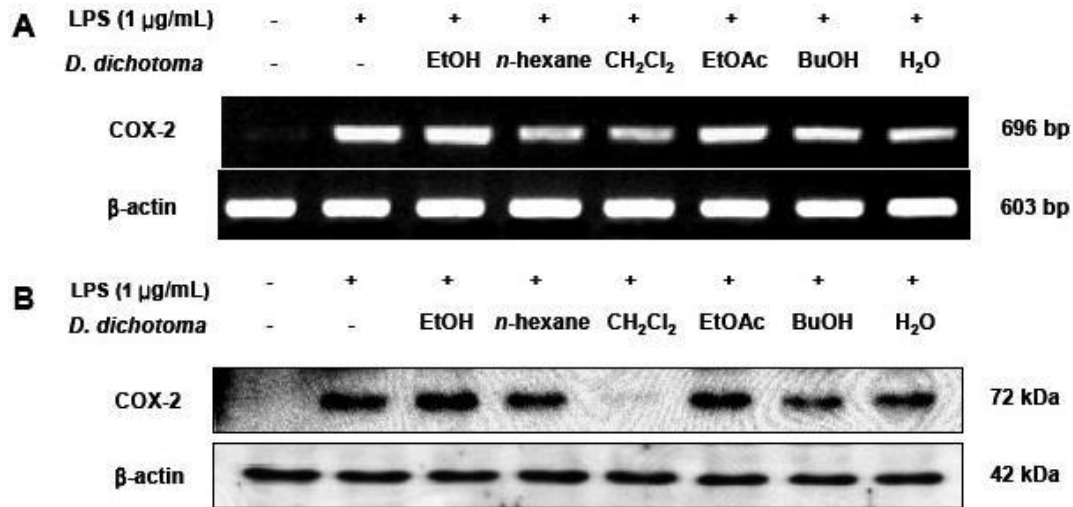
**Figure 5.** Inhibitory effects of EtOH extract and solvent fractions of *D. dichotoma* on the PGE<sub>2</sub> production in RAW 264.7 cells. RAW 264.7 cells ( $1.5 \times 10^5$  cells/ml) were stimulated by LPS (1 g/ml) with EtOH extract and solvent fractions (25 g/ml) from *D. dichotoma* for 24 h. Supernatants were then collected after 24 h and PGE<sub>2</sub> concentration from supernatants was determined by ELISA method. Values are the mean  $\pm$  SEM of triplicate experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$

**Effect of *D. dichotoma* on PGE<sub>2</sub> production, protein content and mRNA expression in LPS-induced RAW 264.7 cells**

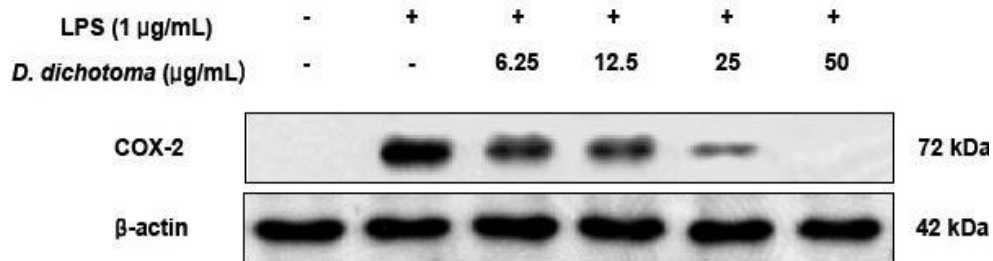
It was also reported that TNF- synergistically potentiated PGE<sub>2</sub> production stimulated by IL-1 (Lerner and Modeer, 1991). The mechanism where cytokines such as TNF- $\alpha$ , IL-6 and IL-1 act in concert to stimulate PG production is, however, not well known. The TNF- $\alpha$ , IL-6 and IL-1, are involved in bone resorption as well as the production of PGE<sub>2</sub> in various type of cells (Smith and Marnett, 1991).

To examine whether the extract and solvent fractions of *D. dichotoma* extract inhibits PGE<sub>2</sub>, cells were activated with LPS (1 g/ml) and then incubated with the extract and solvent fractions of *D. dichotoma* for 24 h. CH<sub>2</sub>Cl<sub>2</sub> (25 g/ml) fraction of *D. dichotoma* exhibited reduction in PGE<sub>2</sub> up to 39.60% (Figure 5). The mRNA expression and protein level of COX-2 were detected by RT-PCR and immunoblotting, respectively. CH<sub>2</sub>Cl<sub>2</sub> fraction of *D. dichotoma* (25 g/ml) inhibited the mRNA expression (34.05%) and protein level (64.03%) of COX-2 (Figure 6), in a dose-dependant manner

displayed strikingly decreased level of IL-1 mRNA up to 33.51%.



**Figure 6.** Inhibitory effects of EtOH extract and solvent fractions of *D. dichotoma* on the COX-2 mRNA expression and protein level in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells ( $1.0 \times 10^6$  cells/ml) were pre- incubated for 18 h, and the cells were stimulated with LPS (1 g/ml) in the presence of EtOH extract and solvent fractions of *D. dichotoma* (25 g/ml) for 24 h. (A) mRNA expression, (B) protein level of COX-2 and -Actin were determined by RT-PCR and immunoblotting.



**Figure 7.** Inhibitory effects of different concentrations CH<sub>2</sub>Cl<sub>2</sub> fraction of *D. dichotoma* on the protein level of COX-2 in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells ( $1.0 \times 10^6$  cells/ml) were pre- incubated for 18 h, and the cells were stimulated with LPS (1 g/ml) in the presence of CH<sub>2</sub>Cl<sub>2</sub> fraction of *D. dichotoma* (6.25, 12.5, 25, 50 g/mL) for 24 h. COX-2 protein level was determined by immunoblotting.

(Figure 7).

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