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Inhibitory Effect of *Ulva fasciata* and *Desmarestia viridis* on the Production of Nitric Oxide, Prostaglandin E_2, and Pro-inflammatory Cytokines in RAW 264.7 Cells

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ABSTRACT

Nitric oxide (NO) and prostaglandin (PG)E_2, known inflammatory mediators, are critically involved in the pathogenesis of a large number of human inflammatory diseases. Therefore, a search of inducible nitric oxide synthases (iNOS) and cyclooxygenase 2 (COX-2) selective inhibitors is a useful strategy to find functional substances to alleviate inflammatory disease. In our search for anti-inflammatory ingredients, we found that extracts of *Ulva fasciata* (UFE) and *Desmarestia viridis* (DVE) inhibit the generation of NO and PGE_2 in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. *U. fasciata* and *D. viridis* were extracted with 80% ethanol and then partitioned successively with ethyl acetate. The ethyl acetate fractions are effective dose-dependent inhibitors of LPS-induced NO and PGE_2 synthesis in RAW 264.7 cells. To test the inhibitory effects of UFE and DVE on pro-inflammatory cytokines, we performed ELISA assays for tumor necrosis factor (TNF)-α, IL (interleukin)-1β, and IL(interleukin)-6 in LPS-stimulated RAW 264.7 macrophage cells. In these assays, the UFE and DVE showed a dose-dependent decrease in the production of TNF-α, IL-1β, and IL-6. As a preliminary study of the anti-inflammatory mechanism, we determined, using the Western blot analysis, whether or not UFE and DVE inhibit the degradation of I-kappa-B-alpha (IκB-α). Our results indicate that UFE and DVE indeed prevent the degradation of IκB-α, in a dose-dependent manner. Based on these results, we suggest that extracts of *U. fasciata* and *D. viridis* be considered candidates for anti-inflammatory agents for human use.

INTRODUCTION

Due to their enormous biodiversity and safety—as they have long been used in traditional foods and folk medicine—marine algae are considered very attractive materials to screen for medicinal natural products, Indeed, they have been consumed widely in diets and skin cures in such Far East Asia countries as Korea, China, and Japan. For this reason, seaweeds have been an extensive focus in alternative medicines, cosmetic materials, food additives, and pharmaceuticals, as well as in various industrial fields (Guinea *et al.,* 2012; Ferreres *et al.,* 2012). Indeed, many recent investigations have proven the anti-inflammatory effect of seaweeds. Yang *et al.,* (2010) reported that the anti-inflammatory effect of *Petalonia binghamiae* in LPS-induced macrophages is mediated by suppressing iNOS and COX-2; Yoon *et al.,* (2010) reported that the brown alga *Sargassum muticum* inhibits proinflammatory cytokines, iNOS, and COX-2 expression; and Yoon *et al.,* (2009) reported the inhibitory effect of the brown alga *Sargassum micracanthum* on the production of pro-inflammatory mediators and cytokines in the mouse macrophage cell line RAW 264.7. Marine algae and their organic extracts are known to contain a great number of biologically active natural products with diverse health benefits. Moreover, they are considered to be good sources of phlorotannins and have been shown to exhibit anti-inflammatory properties that act by inhibiting pro-inflammatory mediators and cytokines (Dutot *et al.,* 2012; Yang *et al.,* 2012). Kim *et al.,* (2013) recently found that an algal phlorofucofuroeckol A, isolated from *Ecklonia cava*, suppressed the expression of iNOS, COX-2, and pro-inflammatory cytokines via the inhibition of nuclear factor-κB,

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c-Jun NH_2-terminal kinases, and Akt in microglial cells. Jung *et al.,* (2013) reported the anti-inflammatory activity of the edible brown alga *Eisenia bicyclis* and its constituent phlorotannins. Also, several anti-inflammatory compounds such as apo-9'-fucoxanthinone, sargaquinoic acid, and sargachromenol have been discovered (Ham *et al.,* 2010; Yang *et al.,* 2013). These compounds, abundant in *Sargassum* sp., display anti-inflammatory, as well as various other activities. Thus, the fact that alga fractions and bioactive substances could be added to foods and cosmetics to control chronic and/or skin inflammation has already been acknowledged (Kwon *et al.,* 2013; Sarithakumari *et al.,* 2013; Yoon *et al.,* 2012).

A green alga, *Ulva fasciata* Delile, commonly known as 'sea lettuce' and called "Ttigalparae" in Korea, is widely distributed on intertidal rocks, in tide pools, and on reef flats in the Caribbean, Indian, Pacific, and eastern Atlantic Oceans. It is an important foodstuff and has been used in soups and salads (Ryu *et al.,* 2013; Chakraborty and Paulraj 2010). *U. fasciata* extracts with antimicrobial (Chakraborty *et al.,* 2010 and anti-cancer activities (Ryu *et al.,* 2013) have been reported. Furthermore, its antioxidant properties due to its sesquiterpenoids have been demonstrated with free radical-scavenging assays (Chakraborty and Paulraj 2010). However, no report of anti-inflammatory activity in dried products of *U. fasciata* has yet appeared. *Desmarestia viridis,* a species of brown alga, commonly known as stringy acid kelp, green acid kelp, sea sorrel, or Desmarest's green weed, and in Korea as Soekkorisanmal, is found in shallow intertidal areas worldwide. Neither has any report of a biological activity of *D. viridis* appeared until now.

In our search for natural anti-inflammatory agents from marine organisms, we observed that extracts of *U. fasciata* and *D. viridis* strongly inhibit NO production. The aim of the present work was to evaluate the properties of *U. fasciata* and *D. viridis* that could be harnessed for human benefit.

MATERIALS AND METHODS

Preparation of seaweed extracts

U. fasciata and *D. viridis,* collected on Jeju Island in June 2011, were identified by Dr. Wook Jae Lee at Jeju Technopark (JTP). Voucher specimens (CSC-005 and CSC-006, respectively) were deposited at the Department of Chemistry, Jeju National University, Jeju, Korea. The *U. fasciata* and *D. viridis,* washed and then dried for 2 weeks at room temperature, were ground to a fine powder. Five-gram samples of the each were extracted for 24 h at room temperature with 2 L of 80% ethanol and then evaporated under vacuum. The dried *U. fasciata* and *D. viridis* extracts were suspended in 1 L of water and partitioned successively four times with 1 L of EtOAc.

RAW 264.7 cell culture

RAW 264.7 cells, purchased from the Korean Cell Line Bank (Seoul, Korea), were maintained in an incubator at 37 °C, under 5 % CO_2, in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % heat inactivated fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). For this study the cells were mechanically passaged by dissociation every other day; they underwent fewer than 25 passages.

Lactate dehydrogenase (LDH) assay

LDH activity was determined from the nicotinamide adenine dinucleotide hydrogen (NADH) produced during the conversion of lactate to pyruvate in the RAW 264.7 cells. The release of LDH, used to detect cytotoxicity, was measured at the end of each proliferation experiment with an LDH cytotoxicity detection kit (Promega, Madison, WI, USA). RAW 264.7 cells (1.8×10^5 cells/mL) plated onto 96-well plates were pre-incubated for 18 hr and subsequently treated for 24 h at 37 °C with LPS (1 μg/mL) coupled with various concentrations of UFE and DVE. An aliquot of 50 μL of the reaction mixture from the LDH cytotoxicity detection kit was added to each sample, and the reaction was incubated for 30 min at room temperature in darkness. Then, 50 μL of the stop solution from the LDH kit was added to each sample to halt the enzymatic reaction, and the absorbance was measured at 490 nm using a microplate reader (PowerWave X340, Bio-tech Instruments, Inc., Vermont, USA). The cytotoxicity was determined as a percentage relative to the control group. All experiments were performed in triplicate.

Determination of NO Production

RAW 264.7 cells (1.8×10^5cells mL^{-1}) were plated in a 24-well plate and, after 18 h, the cells were pre-incubated, at 37 °C for 1 h, in different concentrations of UFE and DVE. They were then incubated at 37 °C for another 24 h with LPS (1 μg mL^{-1}). The nitrite that accumulated in the culture medium, measured according to the method described by Yoon *et al.,* (2010) and based on the classical Griess reaction, was taken as indicative of the NO production. The culture medium was collected and centrifuged at 750×g to precipitate any remaining cell debris, the supernatant was collected and incubated at room temperature for 10 min in an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), and the absorbance at 540 nm was measured in a microplate reader (PowerWave X340, Bio-tech Instruments, Inc., Vermont, USA). The concentration of nitrite was estimated against a sodium nitrite standard calibration curve.

Assays of PGE_2 and pro-inflammatory cytokines

RAW 264.7 cells (1.8×10^5 cells mL^{-1}) were plated in a 24-well plate containing 1 ml of DMEM medium for 18 h, followed by treatment with LPS in the presence of various concentrations of UFE and DVE. After another 24 h of incubation, the PGE_2 TNF-α, IL-6, and IL-1β in the cell culture medium were quantified using a competitive enzyme immunoassay kit (R&D Systems, MN, USA) according to the manufacturer's instructions. Values for the release of PGE_2 TNF-α, IL-6, and IL-1β were determined relative to the controls.

Immunoblotting

The IκB-α protein expression in macrophage RAW 264.7 cells was determined as described in a previous report (Yang *et al.*, 2013). The cells (2.5×10^6 cells/mL) were pre-incubated for 18 hr, and then stimulated for 24 h by 1 μg/mL of lipopolysaccharide (LPS) in the presence of the ethyl acetate fraction of *U. fasciata* (UFE) and *D. viridis* (DVE). After incubation, the cells were collected and washed twice with cold PBS. The cell pellets were lysed with RIPA lysis buffer (Santa Cruz, CA, USA) supplemented with protease inhibitors and kept on ice for 30 min. The cell lysates were then centrifuged for 20 min at 12,000×g, 4°C, to remove cell debris. The resulting supernatants were stored at -70°C before being used in experiments. The protein concentration was determined with a Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL). The protein lysates (40 μg) were denatured and separated on SDS–polyacrylamide gels and were then transferred to polyvinylidene difluoride membranes (BIO-RAD, HC, USA). Nonspecific binding sites on the membranes were blocked overnight with 5% nonfat dry milk at 4°C and the membranes then incubated with mouse monoclonal anti-mouse IκB-α antibody (1:1,000, Cell Signaling Technology, Danvers, MA, USA) and β-actin antibody clone AC-74 (1:10,000, Sigma-Aldrich (St. Louis, MO, USA) at 4°C overnight. Each membrane was further incubated for 30 min with a secondary peroxidase-conjugated goat IgG (1:5000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The immunoactive protein bands were developed with enhanced chemiluminescence kits (Amersham Life Sciences, Arlington Heights, IL, USA).

Statistical analysis

All sets of data were obtained in triplicate and are represented as the mean ± standard error (SE). The statistical significance was determined for each treated group and analyzed by Student's t-test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The unique nature of marine environments and the biological diversity among marine creatures have led to the abundant attention given natural products derived from marine creatures as candidates for medicinal or cosmetic materials that can be used to develop novel therapeutic agents (Blunt *et al.*, 2013; Blunt *et al.*, 2012; Kim and Pangestuti 2011). Indeed, the pharmacology of marine natural products with anti-melanogenic (Ko *et al.*, 2013), anti-aging (Balboa *et al.*, 2013; Choi *et al.*, 2012), anti-inflammatory (Yang *et al.*, 2013), and anti-bacterial (Lee *et al.*, 2013) activity has been reported. As a part of our on-going screening program to evaluate the potential human benefit of Korean seaweeds, we have investigated in this study the ability of *U. fasciata* and *D. viridis* to decrease inflammatory mediators and cytokines, promising therapeutic targets for a nonspecific immune response. We prepared ethyl acetate extracts from *U. fasciata* (UFE) and *D. viridis* (DVE) and examined their effects on LPS-stimulated inflammation in murine macrophage RAW 264.7 cells. Macrophages are essential for the host defense system and the secretion of various pro-inflammatory mediators such as nitric oxide (NO) and prostaglandin (PG)E_2, in response to inflammatory stimuli such as bacterial lipopolysaccharide (LPS). However, overproduction of these mediators, which can obtain from an inappropriate activation of the macrophages, leads to many chronic inflammatory conditions. Therefore, inhibiting the pro-inflammatory mediators and suppressing the macrophage mechanisms constitute a rational strategy for developing therapeutic agents, which could be useful to treat such chronic modern inflammatory diseases as atherosclerosis, diabetes, arthritis, infections, and cancer (Murakami and Ohigashi 2007).

In this study, first we evaluated the effect of UFE and DVE on NO synthesis in activated macrophages. We used the Griess reaction, a spectrophotometric determination for nitrite, to measure the accumulation of nitrite (a stable, oxidation product of NO) in the medium. NO production was determined in RAW 264.7 cells stimulated for 24 h with LPS in the presence or absence of UFE and DVE. As shown in Figure 1, compared with the control, LPS (1 μg mL^{-1})-stimulated RAW 264.7 cells generated significantly increased nitrite levels, and treatment with UFE and DVE inhibited NO synthesis. The reduction in LPS-induced nitrite was dose-dependent, with half maximal inhibitory concentration (IC_{50}) of 15.0 μg/mL and 41.6 μg/mL, respectively ($p < 0.05$). The fact that LDH assays showed that the concentration of viable activated macrophages had not been altered by the UFE and DVE indicates that the UFE and DVE inhibition of NO synthesis could not be atrributed simply to cytotoxic effects.

Cyclooxygenase (COX)-2, another pro-inflammatory mediator, plays a pivotal role in the pathogenesis of inflammation, as it catalyzes the conversion of arachidonic acid to prostaglandins (PGs). Therefore, we next examined the effects of UFE and DVE on the synthesis of PGE_2 in LPS-stimulated RAW 264.7 macrophages, measuring the PGE_2 generated from RAW 264.7 cells cultured with LPS (1 μg mL^{-1}) in both the presence and the absence of UFE and DVE. As shown in Fig. 2B, the UFE suppression of LPS-induced PGE_2 production were concentration-dependent, with IC_{50} values of 46.6 μg/mL.

Previous studies have demonstrated that the release of such inflammatory mediators as IL-1β, IL-6, NO, and PGE_2 is stimulated by TNF-α, which modulates important cellular events such as gene expression and cellular proliferation, which lead to aggravation and progression of various diseases that contribute to tissue damage and multiple organ failure (Marcus *et al.*, 2003). Similarly, Schrader *et al.*, (2007) suggested that TNF-α-induced IL-6 production is a prerequisite for increased NO production. Thus, cellular manipulation for TNF-α, IL-6, and IL-1β syntheses is very important for regulating the inflammatory responses. For this reason, we used the ELISA assay to investigate the effect of UFE and DVE on the production of pro-inflammatory cytokine (TNF-α, IL-6, and IL-1β) in LPS-stimulated RAW 264.7

macrophage cells. As shown in Fig. 3A & B, pretreatment with UFE and DVE prevented the expected LPS-induced release of TNF-α; the reaction is dose-dependent, with IC_{50} values of 48.2 μg/mL and 9.1 μg/mL, respectively.

As expected from the potential effect of UFE and DVE on NO and TNF-α production, UFE and DVE also inhibited LPS-induced IL-6 and IL-1β production, and the reaction is concentration dependent (Figs. 3C to 3F). Since NF-κB must be activated before it can allow LPS or pro-inflammatory cytokines to induce either COX-2 or iNOS, we next asked whether or not UFE and DVE were involved in the NF-κB pathway in LPS-stimulated RAW 264.7 cells. The direct correlation between activation of NF-κB and rapid proteolytic degradation of IκB-α has been well documented (Mauro *et al.*2009; Singh and Mittal 2008; Solt and May 2008). Indeed, many research works have shown that anti-inflammatory agents inhibit the activation of NF-κB by preventing the degradation of IκB-α (Yang *et al.*, 2013; Yang *et al.*, 2010; Yoon *et al.*, 2009). Thus, in this study, we undertook to determine whether or not UFE inhibits the degradation of IκB-α. As shown in Fig. 4, LPS induced the transient degradation of IκB-α in LPS-stimulated RAW 264.7 cells, whereas UFE and DVE prevented the degradation of IκB-α in a dose-dependent manner. These results suggest that the COX-2 and iNOS expressions inhibited by UFE & DVE occurred via suppression of an IκB-α degradation, thereby preventing NF-κB activation.

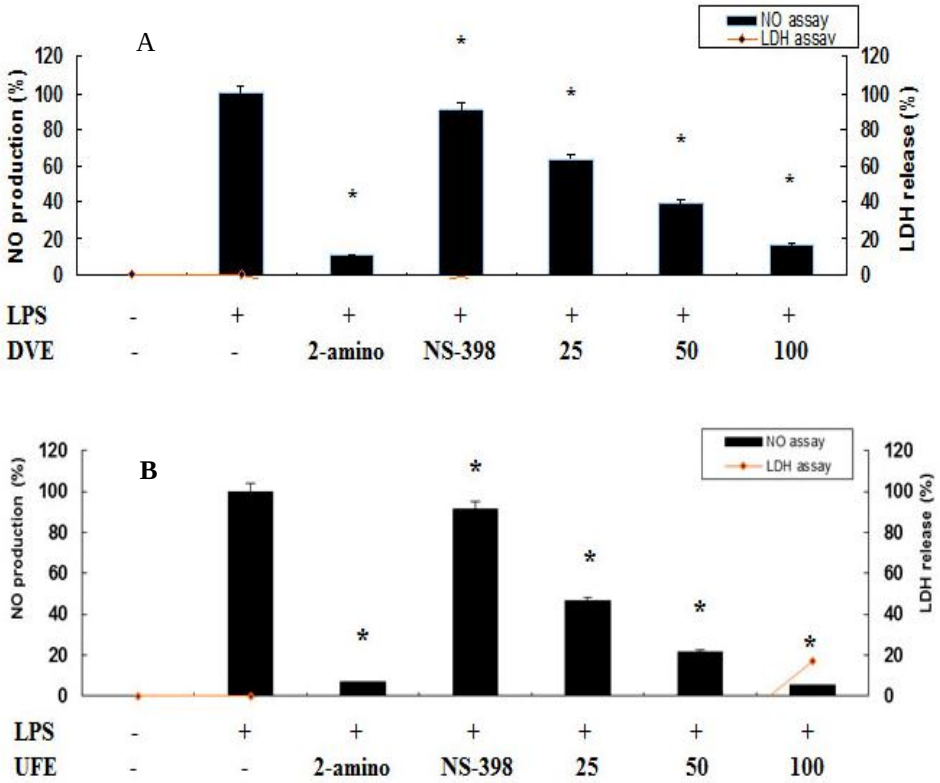


Fig. 1: Inhibitory effects of the ethyl acetate fraction of *Ulva fasciata* (UFE) and *Desmarestia viridis* (DVE) on nitric oxide (NO) production in RAW 264.7 cells. The production of nitric oxide in a culture medium of cells that had been stimulated with LPS (1 μg/mL) for 24 h in the presence of UFE and DVE (25, 50, and 100 μg/mL) was determined. NS-398 (20 μM) and 2-amino-4-methylpyridine (20 μM) were used as controls. NO production was determined by the Griess reagent method and cytotoxicity by the LDH method. The values displayed are the mean ± SEM of triplicate experiments. ** P<0.05; ** P<0.01*

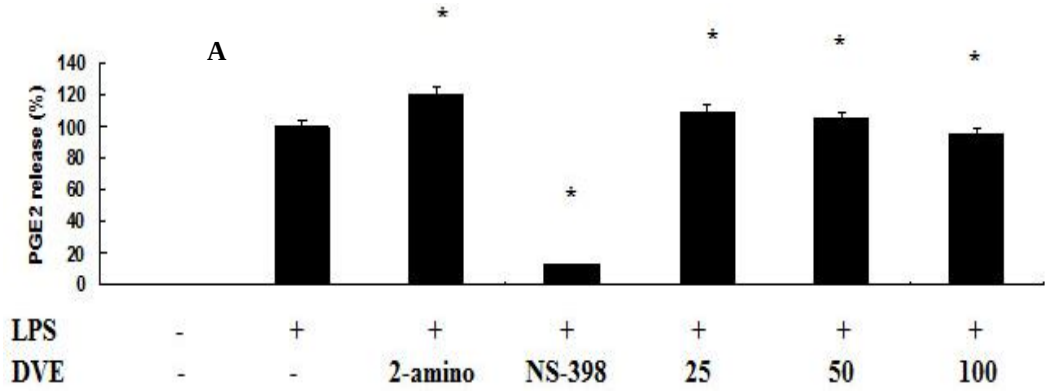


Fig. 2:

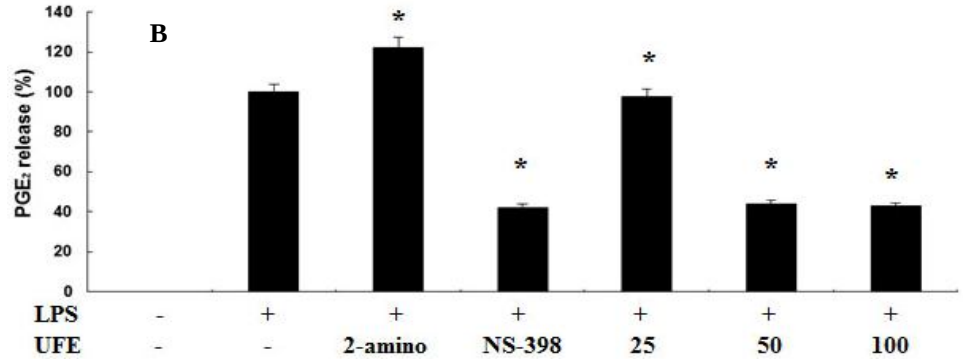
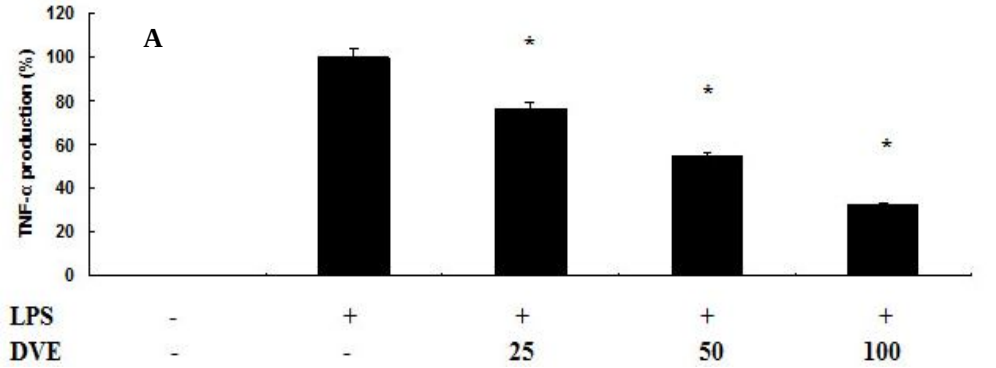
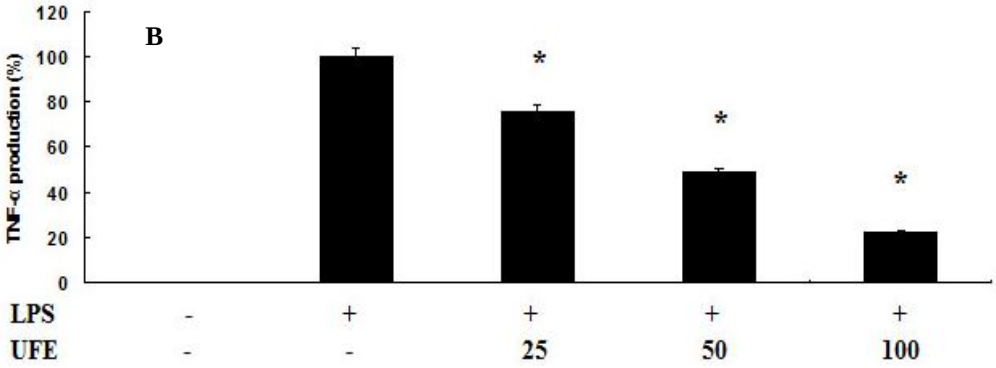
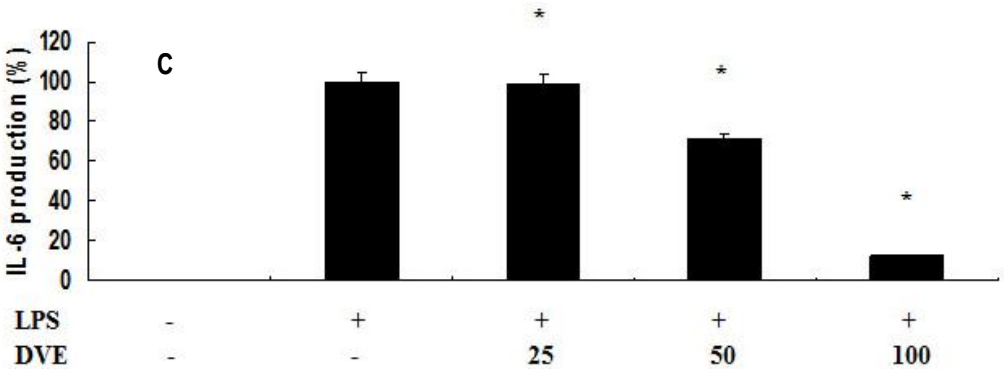


Fig. 2: Inhibitory effects of UFE and DVE on the production of PGE_2 in RAW 264.7 cells. After pre-incubation for 18 h, RAW 264.7 cells (1.8×10^5 cells/mL) were stimulated with LPS (1 μg/mL) in the presence of UFE and DVE (25, 50, and 100 μg/mL). PGE_2 from the culture supernatants was measured with an enzyme-linked immunosorbent assay (ELISA) kit. The values displayed are the mean ± SEM of triplicate experiments. ** P<0.05; ** P<0.01.*







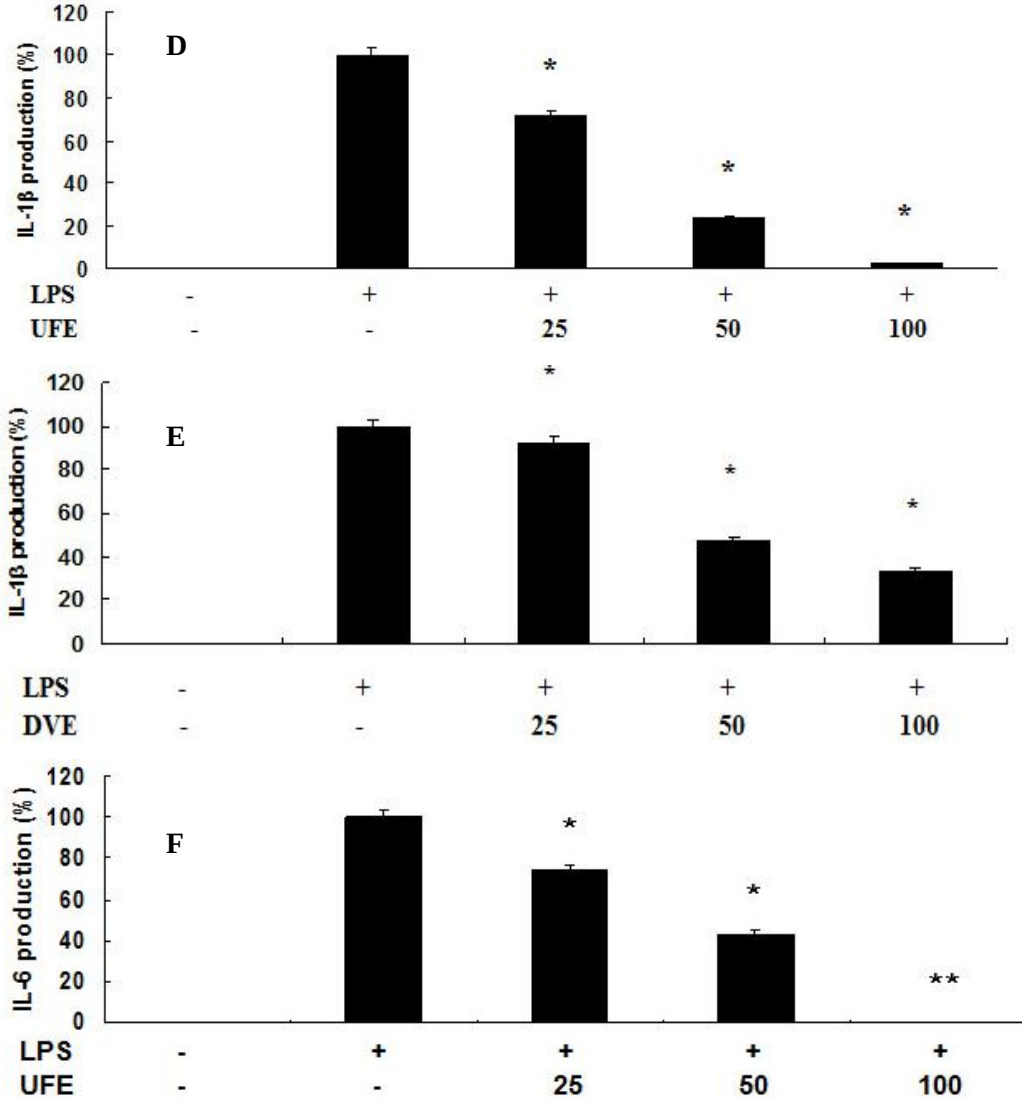


Fig. 3: Inhibitory effects of UFE and DVE on the production of TNF-α, IL-1β, and IL-6 in RAW 264.7 cells. The production of TNF-α, IL-1β, and IL-6 in culture media of RAW 264.7 cells stimulated with LPS (1 μg/mL) for 24 h in the presence of UFE and DVE (25, 50, and 100 μg/mL) was determined. The values displayed are the mean ± SEM of triplicate experiments. ** P<0.05; ** P<0.01.*

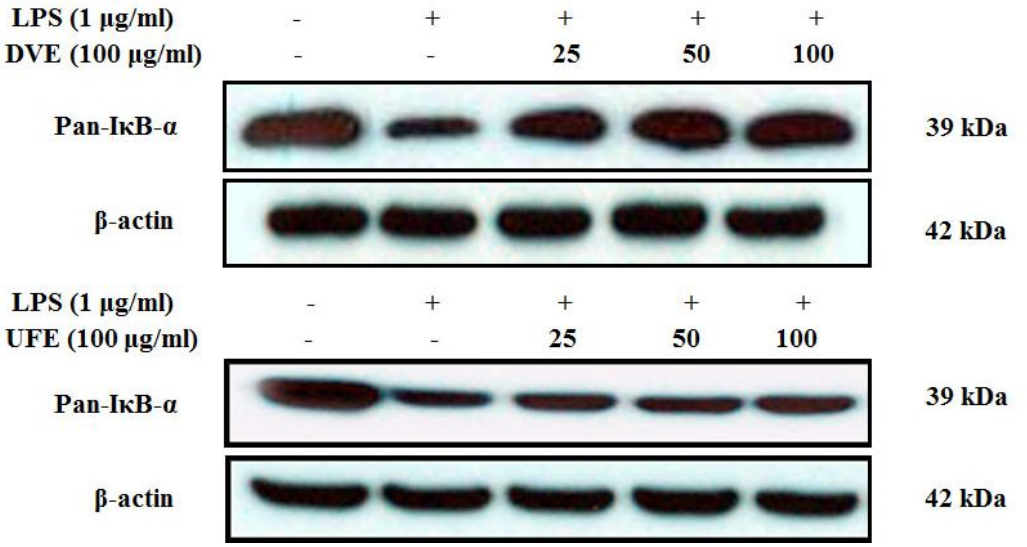


Fig. 4: Inhibitory effects of UFE and DVE on the protein level of IκB-α in RAW 264.7 cells. RAW 264.7 cells (2.5 × 106 cells/ mL) were pre-incubated for 18 h, then incubated for 2 h with UFE and DVE (25, 50, and 100 μg/mL), and finally stimulated for 15 min with LPS (1 μg/mL). The levels of pan-IκB-α were determined using an immunoblotting method. β-actin antibody was used as a loading control.

CONCLUSION

The sum of these results suggests that the inhibitory effects that UFE and DVE exert on the production of inflammatory mediators, including NO, PGE_2, IL-1β, and IL-6, by LPS-stimulated RAW264.7 macrophages are accomplished by preventing IkB-α from degrading. These mechanisms could provide scientific support for the use of UFE and DVE as clinically proven anti-inflammatory herbal medicines.

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