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**Short Communication**

# Green Alga *Ulva pertusa* Inhibits Nitric Oxide and Prostaglandin-$E_2$ Formation in Murine Macrophage RAW 264.7 Cells

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Macrophages play an important role in inflammatory disease through the release of factors such as nitric oxide (NO), prostaglandins, and cytokines involved in the immune response (Hibbs *et al.*, 1987; Palmer *et al.*, 1988; Hseu *et al.*, 2005). Activated macrophages transcriptionally express inducible nitric oxide synthase (iNOS), which catalyzes the oxidative deamination of L-arginine to produce NO, and is responsible for the prolonged and profound production of NO. Overproduction of NO by iNOS can provoke deleterious consequences such as septic shock and inflammatory diseases (Xie and Nathan, 1994; Titheradge, 1999; Zamora *et al.*, 2000; Zhou *et al.*, 2008). Prostaglandins also play a major role as mediators of the inflammatory response. The rate-limiting enzyme in the synthesis of prostaglandins is cyclooxygenase (COX). Two isoforms of COX have been identified: COX-1 and COX-2. COX-2 is induced by several stimuli, and is responsible for the production of large amounts of pro-inflammatory prostaglandins at the inflammatory site. On the basis of these observations, it has been hypothesized that inhibiting NO and prostaglandin production in macrophages could serve as the basis for the potential development of anti-inflammatory drugs.

*Ulva pertusa*, dominant seaweed in the coastal zone of Jeju Island, Korea, is distributed in various coastal waters including the brackish waters of the inner bays. It often contributes to the formation of 'green tides', which cause ecological and indirect economic damages. Recently, there have been an increasing number of reports on the formation of huge masses of algae due to eutrophication (Hiraoka *et al.*, 2004; McAvoy and Klug, 2005; Lin *et al.*, 2008). These excessive masses largely consist of various species of green algae, and *U. pertusa* has been a representative case in Jeju Island since 2007. As a result, in some areas, the excessive growth of the algae impeded the growth of aquatic crops and affected the scenic beauty of the beaches. Therefore, it is worthwhile to determine how to make use of the 'green tides', especially *U. pertusa*, in an economically productive manner. This study was designed to estimate the anti-inflammatory effect of the solvent fraction constituents of *U. pertusa* by measuring the production of NO and prostaglandin $E_2$ ($PGE_2$) in murine macrophage RAW 264.7 cells. In April 2008, specimens of *U. pertusa* were collected from Jeju Island. A voucher specimen (JBRI-08025) has been 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 (50 g) was extracted with 80% ethanol (EtOH; 2 L) at room temperature for 24 h and then vacuum-evaporated. The evaporated EtOH extract (16 g) was suspended in water (2 L) and partitioned three times with solvents -*n*-hexane (2 L), dichloromethane ($CH_2Cl_2$; 2 L), ethyl acetate (EtOAc; 2 L), and butanol (BuOH; 2 L). The yields of these solvent partitions were as follows: *n*-hexane, 1.6 g; $CH_2Cl_2$, 2.6 g; EtOAc, 0.5 g; BuOH, 1.5 g; and $H_2O$, 9.6 g. NO is an endogenous free radical species and, in small amounts, is an important regulator of the physical homeostasis, whereas large amounts of NO has been closely correlated with the pathophysiology of a variety of diseases and inflammation. After exposure to inducers, such as lipopolysaccharide (LPS) from Gram-negative bacteria, inducible NOS (iNOS) can be induced in various cells, including macrophages, smooth muscle cells, and hepatocytes, to trigger cytotoxicity, tissue damage, inflammation sepsis, and stroke (Marletta, 1993; Jiang *et al.*, 2006; Tung *et al.*, 2008). Thus, measuring NO production could be a possible method for assessing the anti-inflammatory effects of seaweed extracts. The nitrite assay was performed to determine whether the solvent extracts of *U. pertusa* modulated the NO production from murine macrophage RAW264.7 activated by LPS (1 μg/mL). NO production was expressed as the ratio of the amount of NO produced from the solvent fraction with LPS to that from LPS alone (Fig. 1). No basal NO production was observed in the incubation with only the crude extract from *U. pertusa* without LPS (data not shown). On the other hand, NO production in the cells incubated with LPS and

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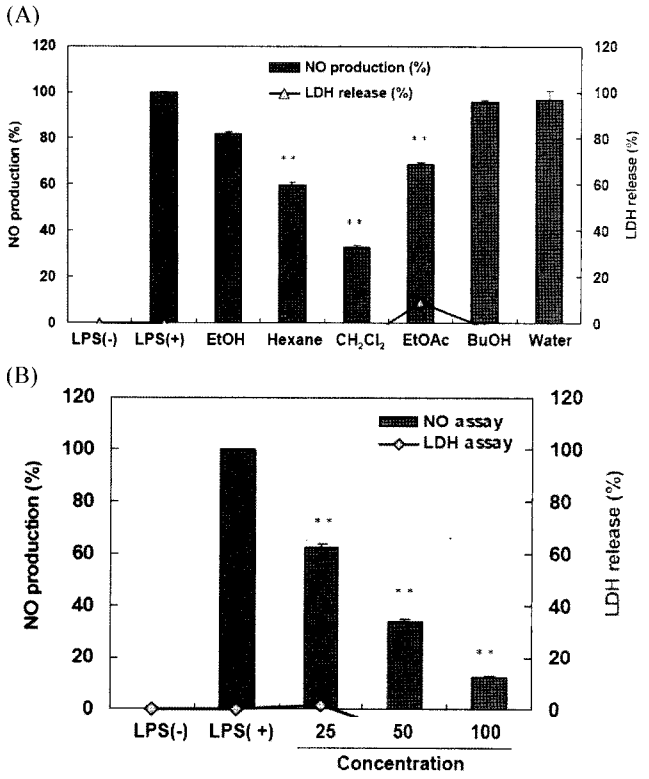


**Fig. 1. Effects of *U. pertusa* extract and solvent fractions on the production of nitric oxide and cytotoxicity in RAW 264.7 cells.** Production of nitric oxide was assayed from the culture medium of cells stimulated with LPS (1 μg/mL) in the presence of ethanol extract and solvent fractions of *U. pertusa* (A). NO production was determined using ELISA method. After the cells were treated with dichloromethane (B) fractions (25 to 100 μg/mL), nitrite production by RAW 264.7 mouse macrophage cells was inhibited in a dose-dependent manner. Cytotoxicity was determined using LDH method. Values are means±SEM of triplicate experiments. $*p<0.05$; $**p<0.01$.

the solvent fraction of the *U. pertusa* extracts decreased. The addition of the $CH_2Cl_2$ fraction (50 μg/mL) to the medium with LPS largely inhibited the production of NO with ratio of 62.45 (Fig. 1). The number of viable activated macrophages was not altered by the solvent fractions as determined by lactate dehydrogenase (LDH) assays, indicating that the inhibition of NO synthesis by the solvent fractions was not simply due to cytotoxic effects. The $CH_2Cl_2$ fraction of *U. pertusa* (25 to 100 μg/mL) inhibited NO production in a dose-dependent manner.

The activation of macrophages plays a critical role in the inflammatory process by releasing a variety of inflammatory mediators. In addition to NO production, $PGE_2$ is also an important inflammatory mediator involved in pathogenesis. Thus, determining the inhibitory effects on the abnormal accumulation of $PGE_2$ is another method by which the anti-inflammatory effects of seaweed extracts could be assessed. To examine whether the extract and the solvent fractions of the *U. pertusa* extract inhibit $PGE_2$, the cells were activated with LPS (1 μg/mL) and then incubated with the extract and the solvent

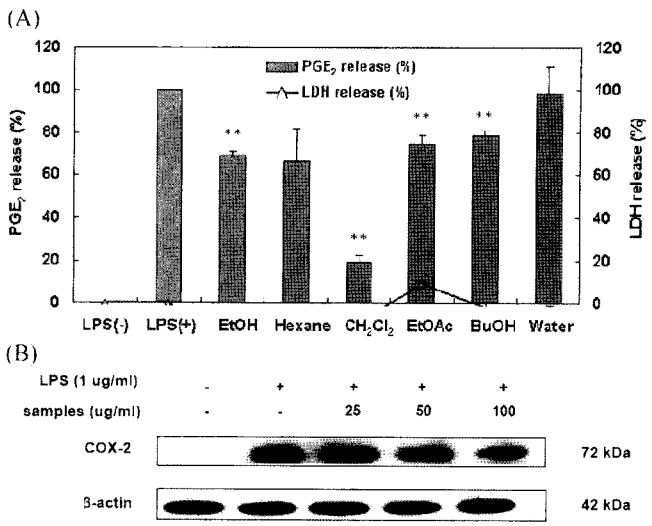


**Fig. 2. Inhibitory effects of ethanol extract and solvent fractions of *U. pertusa* on $PGE_2$ production and COX-2 expression in RAW 264.7 cells.** RAW 264.7 cells ($1.5\times10^5$ cells/mL) were stimulated by LPS (1 μg/mL) with ethanol extract and solvent fractions (50 μg/mL) of *U. pertusa* for 24 h. Supernatants were collected after 24 h, and $PGE_2$ concentration of supernatants was determined using ELISA method (A). The COX-2 levels were determined by immunoblotting. After the cells were treated with dichloromethane fraction (25 to 100 μg/mL), COX-2 expression by RAW 264.7 mouse macrophage cells was inhibited in a dose-dependent manner (B). Values are means± SEM of triplicate experiments. $*p<0.05$; $**p<0.01$.

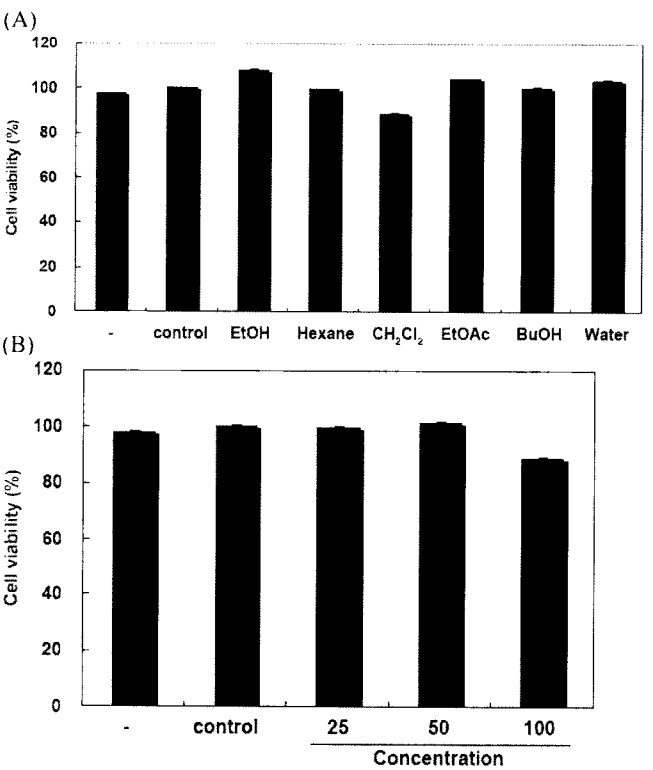


**Fig. 3. Cell viability of HaCaT Cells treated with ethanol extract and solvent fractions.** Ethanol extract and solvent fractions exhibited relatively low cytotoxicity, with cell viability almost 100% at 100 μg/mL (A). MTT assay was performed after incubation of HaCaT cells treated with various concentrations of $CH_2Cl_2$ fraction for 24 h at 37°C in a 5% $CO_2$ atmosphere (B). Absorbance was measured at 570 nm with a spectrophotometer (Power Wave, Bio-tek, Winooski, VT).

**Table 1. Results of human skin primary irritation test** ($n$=32)

| Test Materials | No. of responders | 48 h | | | | | 72 h | | | | Reaction grade[a] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1+ | 2+ | 3+ | 4+ | ± | 1+ | 2+ | 3+ | 4+ | 48 h | 72 h | Mean |
| Squalane | | [b]- | - | - | - | - | - | - | - | - | 0.0 | 0.0 | 0.0 |
| *U. pertusa* extract (0.5%) | 0 | - | - | - | - | - | - | - | - | - | 0.0 | 0.0 | 0.0 |
| *U. pertusa* extract (1%) | 0 | - | - | - | - | - | - | - | - | - | 0.0 | 0.0 | 0.0 |

[a]Reaction grade=Σ[{Grade×no. of responders}/{4 (maximum grade)×32 (total subjects)}]×100×(1/2).
[b]No reaction.
Thirty-two healthy female Korean subjects were selected based on inclusion and exclusion criteria, and written consent was obtained in each case. The average age was 39.94±6.81 years (range: 20 to 49). The subjects had no history of allergic contact dermatitis and had not used topical or systemic irritant preparations within the previous 1 month. The 80% ethanol extract of *U. pertusa* formulated with squalane (negative control) was prepared and applied at 0.5 and 1%. The patches (chambers) remained in place for 48 h. Once the patches were removed, the site was read 30 min and 24 h later; the readings were scored according to the Cosmetic, Toiletry, and Fragrance Association (CTFA) guidelines.

fractions of *U. pertusa* for 24 h. The $CH_2Cl_2$ (50 μg/mL) fraction of *U. pertusa* exhibited reduction in $PGE_2$ up to 79.02% (Fig. 2A). The level of COX-2 was detected by immunoblotting. The $CH_2Cl_2$ fraction of *U. pertusa* (25 to 100 μg/mL) inhibited the level of COX-2, in a dose-dependent manner (Fig. 2B). We next examined the cytotoxic effects of the ethanol extract and the solvent fractions of *U. pertusa* on human keratinocyte HaCaT cells (Fig. 3). Whereas the ethanol extract and the solvent fractions of *U. pertusa* at concentrations lower than 100 μg/mL showed almost 100% cell viability except for the $CH_2Cl_2$ fraction, which at 100 μg/mL showed only about 80% cell viability. In the human dermal fibroblast cells, such as HaCaT cells, the ethanol extract and the solvent fractions of *U. pertusa* oil did not show significant cytotoxic effect (data not shown). These data suggest that *U. pertusa* has a low cytotoxicity against the mammalian cells. Finally, to clinically evaluate the irritation effect of the *U. pertusa* extracts for applications on the human skin, a patch test invoving topical treatment of the *U. pertusa* extracts was performed. None of the 32 subjects experienced adverse reactions such as erythema, burning or pruritus based on the 48 and 72 h readings (Table 1). In conclusion, results of the present study demonstrated that *U. pertusa* extracts can reduce NO and $PGE_2$ production and are safe for use as a possible anti-inflammatory agent for the management of the human skin health.

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