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Anti-inflammatory activity of various seaweeds in LPS-PG stimulated HGF-1 cells

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Objectives: Many seaweeds are considered important natural resources since their abundant bioactive compounds. The present study tried to analyze the anti-inflammatory activity of several seaweeds in the human gingival fibroblast (HGF)-1 cells.

Methods: Lipopolysaccharide from *Porphyromonas gingivalis* (LPS-PG), one of the main causes in the progression of periodontal inflammation, was applied to induce inflammatory response in HGF-1 cells. Nitric oxide (NO) was used as one of the hallmarks for inflammation, which was analyzed by the Griess reaction. The protein expression level of inducible NO synthase (iNOS) was identified by the Western blot analysis.

Results: Inhibitory effect of NO production was investigated in the ethanol extracts of *Asparagopsis taxiformis* Trevisan de Saint-Léon (ATEE), *Distromium decumbens* (Okamura) Levring (DDEE), *Galaxaura fastigiata* (GFEE), *Hypnea japonica* Tanaka (HJEE), *Myelophycus simplex* (Harvey) Papenfuss (MSEE), and S*cytosiphon canaliculatus* (Setchell & N.L. Gardner) Kogame (SCEE) in LPS-PG stimulated HGF-1 cells. As a result, ATEE and HJEE the most potently inhibited while GFEE and SCEE slightly ameliorated NO production without any cytotoxicity. But, DDEE and MSEE did not exhibit the extraordinary NO inhibitory effect in LPS-PG induced HGF-1 cells. Western blot analysis was also conducted to identify the inhibitory effect of both extracts, ATEE and HJEE that sharply attenuated NO production, in LPS-PG induced HGF-1 cells. ATEE and HJEE mitigated iNOS expression in a dose dependent manner, which was in accordance with the result of NO production.

Conclusions: ATEE and HJEE in the algal extracts used in this study drastically reduced the LPS-PG-induced inflammatory response in HGF-1 cells. All of this ATEE and HJEE will be used as candidate substances for treatment and prevention of periodontal disease.

Keywords Human gingival fibroblast, Lipopolysaccharide, Nitric oxide synthase, *Porphyromonas gingivalis*, Seaweed

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Ⅰ. Introduction

Periodontal disease is characterized by the chronic inflammation that can be initiated from the infectious bacteria, which can be result the destruction of connective tissue and the resorption of dental bone[1]. Among various periodontal pathogens, *Porphyromonas gingivalis* can be recognized as one of the main causes in the progression of the periodontal inflammation. In addition, lipopolysaccharide, a bacterial endotoxin, has been identified as a critical factor for the induction of periodontitis[2]. These periodontal diseases are usually occurred not only in the people have natural tooth but also patients who implanted the titanium implant fixture[3]. According to the increased number of implant patients, a broad range of periodontal diseases are occuring such as redness, edema of soft tissues, gum bleeding, purulent inflammation and even bone absorption[4]. Lee et al.[5] reported that the ratio of implant fixture derived periodontitis can be up to 10% based on the analysis of 47 referenced related to implant initiated inflammation.

Human gingival fibroblast (HGF) is one of the major cell types located in human periodontal tissue. These cells can secret a variety of inflammatory mediators including interleukin (IL), nitric oxide (NO), prostaglandin E_2 (PGE_2), and their generating enzymes upon the inflammatory stimulation. *Porphyromonase gingivalis* has known that this bacteria can play a critical role for the progress of various inflammation initiated periodontal diseases. In addition, lipopolysaccharide from *P. gingivalis*

stimulation can activate the production of NO that is synthesized from the amino acid, L-arginine, by NO synthase (NOS). NO functions in numerous ways such as cerebrospinal fluid production and brain blood flow, blood pressure, and regulation of physiological functions related with inflammatory reactions in normal conditions[6]. PGE_2 can be produced by the amino acid, arachidonic acid, by cyclooxygenase-2 (COX-2). Exaggerated NO and PGE_2 productions in the result of upregulated inducible NOS (iNOS) and COX-2 expressions by LPS stimulation can promote acute inflammatory conditions to chronic one and even cancer[7].

Many seaweeds are considered as important food sources which are abundant dietary fiber, protein, minerals, and other bioactive compounds such as antioxidants, polyunsaturated fatty acids, carotenoids, tocopherols, terpenes, and phycocyanins[8][9]. Especially, ethanol extract of *Undaria pinnatifida* root has reported anti-inflammatory activity in RAW 264.7 cells[10]. Water and the fermentation liquid of *Saccharina japonica* was also exhibited antioxidative and anti-inflammatory activities[11]. Ethanol extract of *Ecklonia cava* showed anti-inflammatory activity through the inhibited nuclear factor (NF)κB and mitogen-activated protein kinase (MAPK) signal molecules in macrophage cells[12]. In addition, anti-inflammatory bioactive compounds from marine algae have reported its anti-inflammatory activity in animal models[13].

Though there are many researches of anti-inflammatory activity of seaweeds, there is little known about their bioavailability in periodontal tissue. Therefore, the present study aimed to examine the anti-inflammatory activity of several seaweeds in LPS-PG stimulated HGF-1 cells.

Ⅱ. Materials and Methods

1. Reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hyclone (Logan, UT, USA). *Porphyromonas gingivalis* lipopolysaccharide (LPS-PG) was obtained from Invivogen (San Diego, CA, USA). Dimethyl sulfoxide (DMSO) was purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies against iNOS and actin as well as horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were obtained from Cell Signaling Technology (Boston, MA, USA)

2. Preparation of seaweed extracts

Ethanol extracts of *Asparagopsis taxiformis* Trevisan de Saint-Léon (ATEE), *Distromium decumbens* (Okamura) Levring (DDEE), *Galaxaura fastigiata* (GFEE), *Hypnea japonica* Tanaka (HJEE), *Myelophycus simplex* (Harvey) Papenfuss (MSEE), and *Scytosiphon canaliculatus* (Setchell & N.L. Gardner) Kogame (SCEE) were obtained from Jeju Biodiversity Research Institute (Jeju, Korea).

3. Cell line culture

HGF-1 cell line was obtained from the American Type Culture Collection (ATCC, CRL-2014; Rockville, MD, USA). Cells were maintained in DMEM (Hyclone) containing 10% FBS supplemented with penicillin and streptomycin.

4. Cell viability assay

Cell viability was determined using WST-1 cell proliferation assay kit (Daeil Lab. Service, Seoul, Korea). HGF-1 cells were seeded in 24-well plates and attached for 24 h. Then the cells were treated with ethanol extracts of 6 kinds of seaweeds at indicated various concentrations for 18h. The cells were then treated with WTS for 1 h at 37 ℃. The absorbance was measured at 450 nm with a multi-detection reader (Bio-Rad Laboratory, Hercules, CA, USA).

5. Analysis of NOS activity

NOS activity was measured as previously described[14]. HGF-1 cells were seeded in a 6-well plate and pre-incubated with 50 ㎍/ml, 100 ㎍/ml, 250 ㎍/ml concentrations of agents for 2 hr. Then, 1 ㎍/ml of LPS-PG was treated and incubated for 10 hr to NOS induction. To measure NOS activity in cell lysates, cells were lysed by three times of freeze-thaw cycle

in 0.1 ml of 40 mM Tris buffer (pH 8.0) containing 5 μg/ml of pepstatin A, 1 μg/ml of chymostatin, 5 μg/ml of aprotinin, and 100 μM phenyl methyl sulfonyl fluoride. Bradford assay was applied to determined the protein concentration. Twenty micrograms of protein were incubated in 20 mM Tris-HCl (pH 7.9) containing 4 μM FAD, 4 μM tetrahydrobiopterin, 3 mM DTT, and 2 mM each of L-arginine and NADPH. The reaction was performed in triplicate for 3 hr at 37°C in 96-well plate. Residual NADPH was oxidized enzymatically and the Griess reaction was performed.

6. Western blot analysis

HGF-1 cells in 100-mm dishes were incubated with indicated concentrations of each extract for 2 hr and then incubated with LPS-PG (1 μg/ml) for 18 hr. Then, cells were washed twice with PBS and scraped into 0.4 ml of protein extraction solution (M-PER, Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at room temperature. The lysis buffer containing the disrupted cells was centrifuged at 13,000 ×g for 10 min. Protein samples (25 μg) from each lysate were separated on a 10% SDS-polyacrylamide gel and electrotransferred to PVDF membrane (Merck Millipore, Billerica, MI, USA). Membranes were blocked for 1 hr at room temperature with 5% nonfat dry milk in TBST solution. The reactions were then incubated at 4°C overnight with a 1:1,000 dilution of each primary antibody. After overnight incubation, the membranes were washed and further incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG for 2 hr at room temperature. The blots were developed with ECL developing solution (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and data were quantified using the Gel Doc EQ System (Bio-Rad Laboratory, Hercules, CA, USA).

7. Statistical analysis

All data are expressed as the mean ± S.D of data tested in triplicate. Statistical analysis was performed using SPSS version 25.0 (SPSS Institute, Chicago, IL, USA).

One-way batch variance analysis was performed to analyze the differences between groups, and the post analysis was performed by Duncan. In addition, the statistically significant difference was set to $p<0.05$.

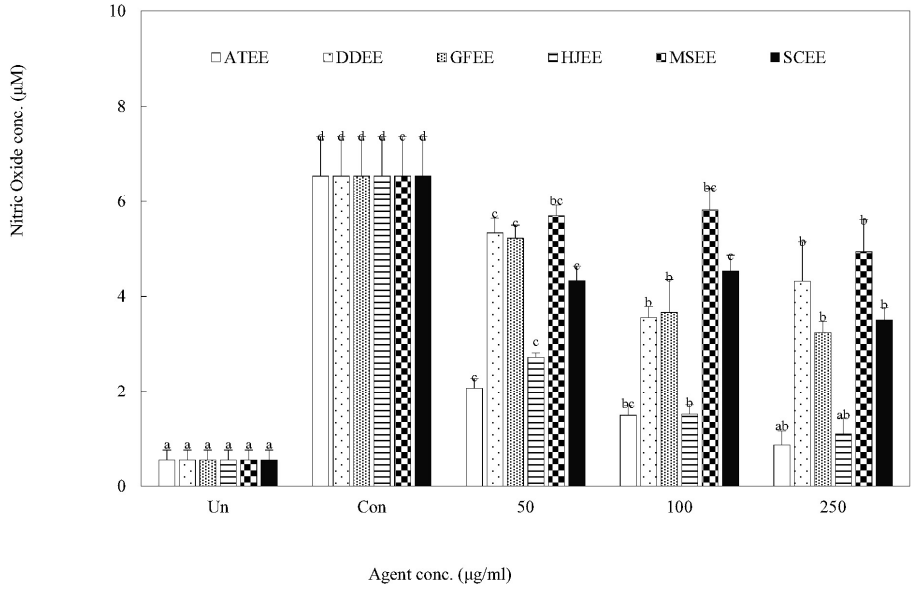
Ⅲ. Results and Discussion

Stimuli for inflammatory reactions such as cytokine, ultraviolet (UV) exposure, chemical injury, and bacterial LPS can initiate the generation of NO, PGE_2, and tumor necrosis factor (TNF)-α. Overproduced inflammatory mediators have been reported to be related to numerous physiological disorders[7]. NO, which is produced by iNOS, can react with superoxides to produce peroxynitrite that contributes to the etiology of cardiovascular disease and cancer by promoting inflammatory responses and oxidative stress[15]. Besides, aberrant iNOS expression was identified in periodontitis diseased patients than healthy people[16]. Therefore, the regulation of iNOS and its product, NO, might be an important target in the treatment for periodontal disease.

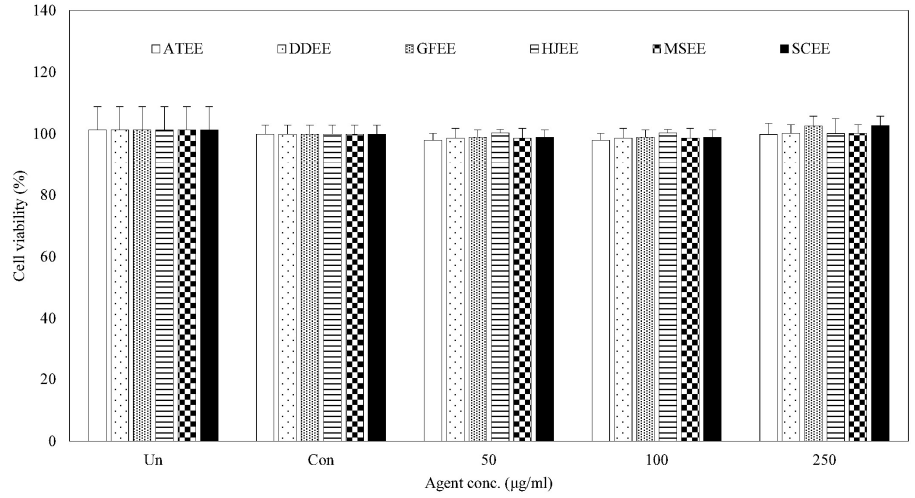
In this study, HGF-1 cell line was seeded and 6 kinds of seaweed extracts were treated to investigate the anti-inflammatory activity. As shown in Figure 1, ATEE and HJEE exhibited the most potent NO inhibitory effect while GFEE and SCEE slightly attenuated NO production in LPS-PG stimulated HGF-1 cells. On the other hand, DDEE and MSEE did not show any remarkable NO inhibitory effect compared with other extracts. All extracts did not exhibit any cytotoxic effect (Figure 2).

The protein expression level of iNOS was determined by Western blot analysis. As shown in (Figure 3), iNOS expression level was sharply induced by LPS-PG treatment, which was attenuated by ATEE and HJEE treatment in HGF-1 cells.

Marine algae have been used for a long time as dietary and medicinal purposes to Asian due to rich dietary fibers, vitamins, proteins, and bioactive compounds[17]. Among 6 kinds of seaweeds used in this study, ethanol extract of *D. decumbens* (Okamura) Levring reported that this alga strongly inhibited inflammation through the regulation of ERK1/2, Akt and nuclear factor (NF)-κB signaling pathway in LPS stimulated nasal polyp-derived fibroblast (NPDFs)[17]. *A. taxiformis* Trevisan de Saint-Léon has shown the potent antioxidative and cytototoxic

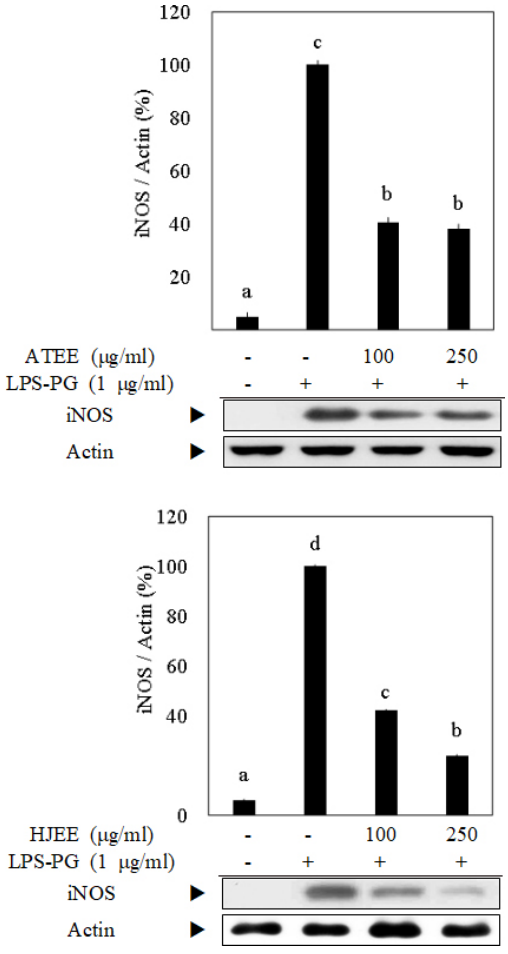


<Figure 1> The effect of ethanol extracts of various seaweeds on inhibition of NO production in LPS-PG stimulated the HGF-1 cell line. Data represent the mean±SD of triplicate experiments. Sharing the same superscript is the result of post-hoc analysis using Duncan in the analysis of variance between groups, and a statistically significant difference was explained with $p<0.05$. ATEE, *Asparagopsis taxiformis* Trevisan de Saint-Léon ethanol extract; DDEE, *Distromium decumbens* (Okamura) Levring ethanol extract; GFEE, *Galaxaura fastigiata* ethanol extract; HJEE, *Hypnea japonica* Tanaka ethanol extract; MSEE, *Myelophycus simplex* (Harvey) Papenfuss ethanol extract; SCEE, S*cytosiphon canaliculatus* (Setchell & N.L. Gardner) Kogame ethanol extract.



<Figure 2> The effect of ethanol extracts of various seaweeds on cell viability in LPS-PG stimulated the HGF-1 cell line. Data represent the mean±SD of triplicate experiments. Sharing the same superscript is the result of post-hoc analysis using Duncan in the analysis of variance between groups, and a statistically significant difference was explained with $p<0.05$. ATEE, *Asparagopsis taxiformis* Trevisan de Saint-Léon ethanol extract; DDEE, *Distromium decumbens* (Okamura) Levring ethanol extract; GFEE, *Galaxaura fastigiata* ethanol extract; HJEE, *Hypnea japonica* Tanaka ethanol extract; MSEE, *Myelophycus simplex* (Harvey) Papenfuss ethanol extract; SCEE, S*cytosiphon canaliculatus* (Setchell & N.L. Gardner) Kogame ethanol extract.

activity that suggests their possible use in the development of pharmaceutical drugs[18]. In addition, an active compound from *H. japonica* Tanaka exhibited the platelet aggregation activity and the primary structures of two hemagglutinins were identified[19][20]. But, there was no evidence for the bioavailability of *G. fastigiata, M. simplex* (Harvey) Papenfuss, and *S.*



<Figure 3> The effect of ATEE and HJEE on the protein expression level of iNOS in LPS-PG stimulated the HGF-1 cell line. The protein expression level of iNOS was measured at indicated concentrations of ATEE and HJEE (100, 250 μg/ml) by Western blot analysis. ATEE and HJEE significantly inhibited LPS-PG induced iNOS expression. The relative induction of iNOS was quantified by densitometry and actin was used as an internal control. Data represent the mean±SD of triplicate experiments. Sharing the same superscript is the result of post-hoc analysis using Duncan in the analysis of variance between groups, and a statistically significant difference was explained with $p<0.05$. ATEE, *Asparagopsis taxiformis* Trevisan de Saint-Léon ethanol extract; HJEE, *Hypnea japonica* Tanaka ethanol extract.

canaliculatus (Setchell & N.L. Gardner) Kogame, so far. Throughout these results, ATEE and HJEE strongly inhibited NO production, while GFEE and SCEE slightly ameliorated an inflammatory mediator in LPS-PG stimulated HGF-1 cells.

ATEE and HJEE markedly inhibited NO production and its corresponding enzyme, iNOS, expression in LPS-PG stimulated HGF-1 cells. These results indicate that ATEE and HJEE among marine algae might be a potential candidate for a treatment strategy against periodontal inflammatory disorders.

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