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Anti-inflammatory effects of *Gracilaria vermiculophylla* Papenfuss extract on *Porphyromonas gingivalis* stimulated RAW 264.7 cells

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Seaweed *Gracilaria vermiculophylla* Papenfuss, commonly called as 'Worm wart weed', is a red alga widely distributed in the coastal areas of several countries. Though *G. vermiculophylla* has been reported to have antioxidant and anti-inflammatory effects, such effects on periodontal diseases remain unclear. In this study, we investigated the anti-inflammatory effects of *G. vermiculophylla* on the production of inflammatory cytokines in *Porphyromonas gingivalis* induced RAW 264.7 cells. *Gracilaria vermiculophylla* on that RAW 264.7 cells had no cytotoxic effect on cell viability compared with untreated controls. In *P. gingivalis* stimulated RAW 264.7 cells, *G. vermiculophylla* treatment reduced nitric oxide (NO) levels in a concentration-dependent manner by downregulating inducible nitric oxide synthase (iNOS) proteins. Reverse transcription-quantitative (RT-q) PCR inhibited interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α mRNA. Western blot analysis found that both inhibitor of kappa B alpha (IκBα) kinase (IKK) phosphorylation and IκBα degradation in *P. gingivalis* stimulated RAW 264.7 cells was inhibited by *G. vermiculophylla* in a dose-dependent manner. In addition, *G. vermiculophylla* treatment reduced the nuclear translocation of nuclear factor (NF)-κB p65, suggesting that the anti-inflammatory effect of *G. vermiculophylla* is associated with the inhibition of NF-κB signaling pathways. Overall, the findings indicate that the red alga *Gracilaria vermiculophylla* extract may have anti-inflammatory effects on periodontitis and can serve as a potent therapeutic agent to prevent periodontal disease.

Keywords: Anti-inflammation, Gum disease, Macrophage, NF-κB, Periodontitis, Nitric oxide, Seaweeds, Worm wart weed

Inflammation is a defense mechanism against tissue damage or infection that causes edema, fever, and erythema due to the activation of inflammatory mediators[1]. The inflammatory response is essential for biological defense, but the excessive secretion of inflammatory agents in this process causes chronic inflammation or immune hypersensitivity. This can cause chronic inflammatory diseases such as periodontal inflammation[2]. Periodontitis, also known as gum disease, is an inflammatory disease of dental support tissues, including the alveolar bone, and is associated with pathogenic substances, such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*[3]. *P. gingivalis* is a Gram-negative anaerobic bacteria associated with periodontal disease, including viral factors, such as endotoxins and gingipains[4]. Lipopolysaccharide (LPS) endotoxins or metabolites formed by these microorganisms increase the secretion of pro-inflammatory cytokines from tissues and immune cells[5].

Macrophages, immune cells present in all tissues, first recognize inflammation introduced into the body by the toll-like receptor 4 (TLR4) and release inflammatory mediators such as nitric oxide (NO), interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α[6-8]. Macrophages activated by periodontal pathogens induce inflammatory reactions via the intracellular signaling pathway of nuclear factor (NF)-κB. NF-κB is a transcriptional regulator of iNOS expression, which is inactivated by binding to IκB, an inhibitory protein in the cytoplasm[9]. Periodontal pathogens activate IκBα kinase (IKK)α and IKKβ through phosphorylation, thereby phosphorylating IκB[10,11]. When the phosphorylated IκB kinase complex is stimulated by periodontal pathogens, IκB collapses and NF-κB is translocated into the nucleus. Activated NF-κB moves into the nucleus to promote cytokine expression and accelerates the inflammatory

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response through increased NO and iNOS expression[12,13]. Therefore, the inhibition of NF-κB activation is an important therapeutic target for inflammatory diseases[14].

Inflammation is involved in the pathogenesis of many diseases[15-17]. In addition to oral health problems, periodontal pathogens are closely associated with systemic diseases, including Alzheimer's disease, cardiovascular disease, rheumatoid arthritis, and diabetes[18,19]. Therefore, the search for candidate substances that can regulate the expression of inflammatory mediators in response to periodontal pathogens is important for the prevention and treatment of systemic diseases and periodontitis. Antibiotics, such as chlorhexidine, doxycycline and minocycline have been used to treat periodontitis[20,21]. However, these drugs can cause various side effects, such as antibiotic resistance and drug hypersensitivity. Alternative treatments are being developed[22,23].

Studies have shown that seaweeds native to the coast are rich in dietary fiber, vitamins, and minerals and contain a large number of bioactive substances, which have antibacterial, antioxidant, anti-inflammatory, and anticancer effects[24-26]. Studies on algae largely consist of mainstream brown algae. Studies on red algae are relatively few. *Gracilaria vermiculophylla* Papenfuss, commonly called warm wart weed, is a red alga widely distributed in the coastal areas of several countries. *G. vermiculophylla* has been reported to have antioxidant and anti-inflammatory properties, but the mechanisms that inhibit inflammation by periodontal pathogens remain unidentified[27,28]. In this study, we have investigated the anti-inflammatory effects of *Gracilaria vermiculophylla* extract on the production of inflammatory cytokines in RAW 264.7 cells induced by *Porphyromonas gingivalis*, a typical oral bacterium that causes periodontal disease.

Materials and Methods

Sample collection

Gracilaria vermiculophylla was collected from Ihoijin Jangheung (34°27'N, 126°56'E), on the southern coast of the Republic of Korea. The sample was removed from foreign substances using tap water and dried at room temperature (20℃). The dried samples were broken with a mixer, added 10 times the sample volume (94% ethanol), distilled for two days, and extracted thrice. The extract was concentrated in a decompression rotary concentrator (Eyela N-1000, Tokyo, Japan), dried in a freeze dryer (Ilshin TFD5505, Gyeonggi, Republic of Korea), and powdered in a crusher (Hanil HMF1000A, Gangwon, Republic of Korea). The freeze-dried extract of *G. vermiculophylla* were dissolved in dimethyl sulfoxide (DMSO) and stored at –20℃.

Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotic-antimycotic solution were obtained from Gibco BRL (Life Technologies, Carlsbad, CA, USA). The CellTiter 96 AQueous One Solution Cell Proliferation Assay and Griess Reagent System were obtained from Promega (Madison, WI, USA). IL-1β, IL-6, TNF-α and β-actin oligonucleotide primers were purchased from Bioneer (Daejeon, Republic of Korea). Antibodies targeting iNOS, phosphorylated IKKα/β (p-IKKα/β), IKKα, IKKβ, phosphorylated IκBα (p-IκBα), IκBα, and NF-κB (p65 subunit) were obtained from Cell Signaling Technologies (Danvers, MA, USA). The antibody targeting β-actin was obtained from Sigma-Aldrich (St. Louis, MO, USA) and proliferating cell nuclear antigen (PCNA) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Horseradish peroxidase (HRP)-linked secondary antibodies targeting Anti-rabbit IgG were obtained from Cell Signaling Technologies, and m-IgGκ were obtained from Santa Cruz Biotechnology.

Cell culture

RAW 264.7 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM containing 10% FBS and 1% antibiotic-antimycotic. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Cells were cultured in 96-well plates (1×10^5 cells/mL) and incubated overnight. RAW 264.7 cells were treated with *G. vermiculophylla* extract at different concentrations (15-500 μg/mL) and incubated at 37℃ with CO_2 5% for 24 h. MTS solutions were subsequently added to each well at a ratio of 1:5 and incubated for 2 h at 37℃. Optical density (OD) at 490 nm was measured using a microplate reader (TECAN, Männedorf, Switzerland).

NO assay

RAW 264.7 cells were plated at 5×10^5 cells/mL in a 24-well cell culture plated and incubated overnight. The cells were pre-treated with *G. vermiculophylla* at concentrations of 125, 250 or 500 µg/mL for 2 h. The cells were further stimulated with *P. gingivalis* and incubated for 24 h. The supernatant (50 µL) was mixed with an equal volume of Griess reagent and incubated at 20°C for 10 min. Absorbance was measured at 540 nm and a standard curve was obtained using sodium nitrite ($NaNO_2$).

Reverse transcription-quantitative (RT-q) PCR

Total RNA was separated from the cultured cells using TRIzol reagent (Ambion, Carlsbad, CA, USA), according to the manufacturer's instructions. The total RNA concentration was measured using Biospec-Nanodrop (Shimadzu, Nakagyo-ku, Kyoto, Japan). cDNA was synthesized using the PrimeScript RT Reagent kit (TaKaRa, Shiga, Japan). The mRNA expression levels of IL-1β, IL-6, TNF-α, and β-actin were determined using PowerSYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The cycle threshold (Ct) value was calculated using the produced PCR curve. All target mRNA levels were expressed as normalized β-actin. Primer sequences used for RT-q PCR are listed in Table 1.

Western blot analysis

RAW 264.7 cells were seeded in 60 mm dishes at a density of 1×10^6 cells/mL for 16 h. The cells were pretreated with 125, 250 or 500 µg/ml *G. vermiculophylla* for 2 h and incubated with *P. gingivalis* (1×10^7 CFU/mL) for the indicated time. After incubation, cells were collected and washed twice with PBS (pH 7.4). Cytosolic and nuclear isolates were prepared on ice using a nuclear extraction kit (Cayman, Michigan, USA), according to the manufacturer's instructions. Extracted proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Boston, MA, USA). Membranes were blocked for 1 h at room temperature with 5% skim milk (Difco, Detroit, MI, USA), in 0.1% Tris-buffered saline with Tween 20 (TBST) buffer. Subsequently, the membranes were incubated with 1:1000 diluted primary antibodies at 4°C overnight. The sections were washed four times with TBST and incubated with 1:2500 diluted secondary antibodies for 1 h at room temperature. Protein bands were determined using chemiluminescent HRP substrate reagent (Millipore, Billerica, MA, USA) and cSeries Capture Software (Azure Biosystems, Dublin, CA, USA).

Table 1 — Primer sequences and conditions for RT-qPCR

Gene Name	GenBank Locus Number	Primer sequence (5'-3')	PCR product length (bp)
IL-1β	NM_ 008361	F: GAAAGACGGCACACCCACCCT R:GCTCTGCTTGTGAGGTGCTGATGTA	166
IL-6	NM_ 031168	F: GATGGATGCTACCAAACTGGA R: TCTGAAGGACTCTGGCTTTG	142
TNF-α	NM_ 001278601	F:CCACCACGCTCTTCTGTCTAC R:AGGGTCTGGGCCATAGAACT	103
β-actin	NM_ 007393	F: CATCACTATTGGCAACGAGC R: GACAGCACTGTGTTGGCATA	159

Statistical analysis

All experiments were carried out thrice and expressed as mean ± standard deviation (SD) based on the average value and analyzed using the Student's t-test. The data analyzed were considered significant when the *P* value <0.05 was performed. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 25.0 Software (SPSS, Chicago, Illinois, USA).

Results

Effect of *G. vermiculophylla* on viability assay of RAW 264.7 cells

Gracilaria vermiculophylla viability in RAW 264.7 cells was confirmed by the MTS assay (Fig. 1). The survival of RAW 264.7 cells was treated with different concentrations of *G. vermiculophylla* (15, 30, 60, 125,

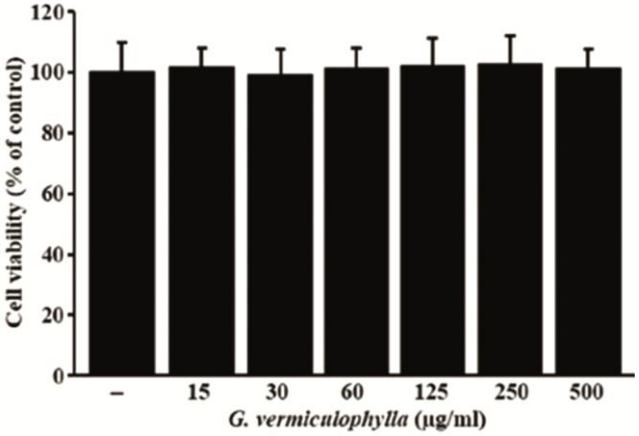

Fig. 1 — Cytotoxicity of *G.racilaria vermiculophylla* extract in RAW 264.7 cells. Untreated cells were used as a positive control. Cells were treated with *G. vermiculophylla* extract used at various concentrations of 15-500 µg/mL. The cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2- (4-sulfophenyl)-2H-tetrazolium (MTS) assay

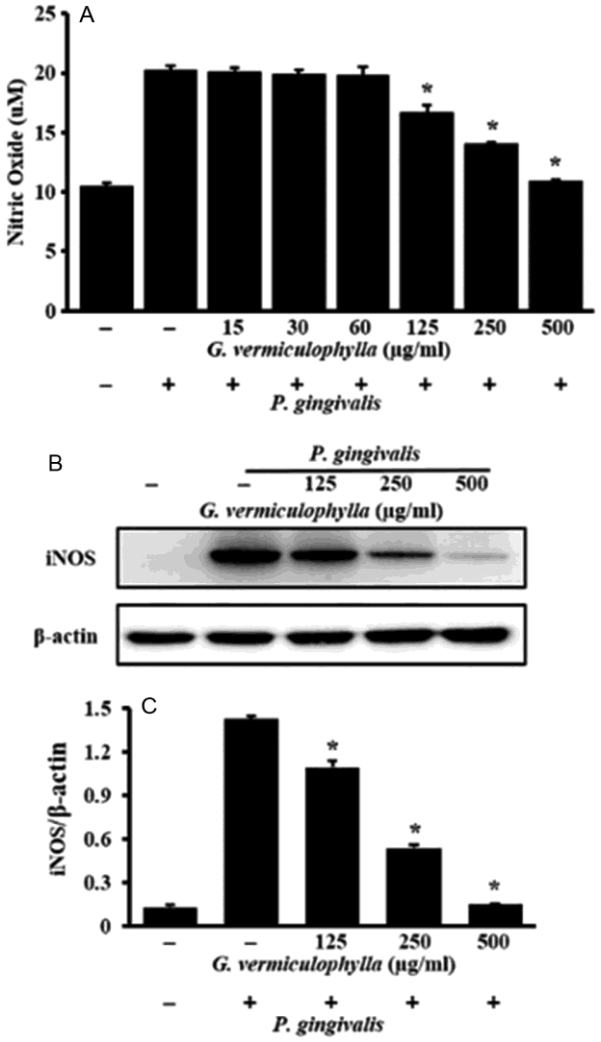

Fig. 2 — Inhibition of NO production and iNOS expression in activated RAW 264.7 cells by *G. vermiculophylla* extract. Cells were activated with *Porphyromonas gingivalis* (1×10^7 CFU/mL) for 24 h. (A) NO levels; (B) iNOS expression levels in *P. gingivalis* induced RAW 264.7 cells; and (C) Relative quantification of iNOS levels normalized to β-actin. [Data are expressed as the mean ± standard deviation (SD) of triplicate experiments. *P <0.05; compared with the *P. gingivalis*-treated group]

250 and 500 μg/mL) and incubated for 24 h. The results did not affect RAW 264.7 cell viability at 500 μg/mL concentrations compared with untreated cells. Therefore, *G. vermiculophylla* was used at concentrations of up to 500 μg/mL, the highest concentration that showed no cytotoxicity.

Gracilaria vermiculophylla inhibits effects of _Pophyromonas gingivalis_ induced NO and iNOS in RAW 264.7 cells

RAW 264.7 cells were pre-treated with *G. vermiculophylla* concentrations of 0, 15, 30, 60, 125, 250 and 500 μg/mL for 2 h, stimulated with *P. gingivalis*, and incubated at 37°C for 24 h. After 24 h, the amount of NO released into the culture medium was measured. In *P. gingivalis* stimulated RAW 264.7, the nitrite oxide (NO) levels of *G. vermiculophylla* (125-500 μg/mL) decreased in a dose-dependent manner (Fig. 2A). *G. vermiculophylla* were pre-treated with RAW 264.7 cells at 0, 125, 250 and 500 μg/mL for 2 h and stimulated with *P. gingivalis* to analyze the inhibitory effect of *G. vermiculophylla* on iNOS expression. *G. vermiculophylla* inhibited iNOS expression in RAW 264.7 cells stimulated by *P. gingivalis* (Fig. 2B). NO production was suppressed by inhibiting iNOS expression.

Cytokine expression of _G. vermiculophylla_ stimulated with _P. gingivalis_ in RAW 264.7 cells

The expression level of inflammatory cytokines of *G. vermiculophylla* was investigated in RAW 264.7 cells stimulated with *P. gingivalis*. *G. vermiculophylla* concentrations of 0, 125, 250 and 500 μg/mL in RAW 264.7 cells were pre-treated for 2 h, stimulated with *P. gingivalis*, and incubated at 37°C for 24 h. The mRNA levels of IL-1β, IL-6, and TNF-α were determined using RT-q PCR. The expression levels of inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, induced by *P. gingivalis* stimulation were inhibited in a dose-dependent manner with higher *G. vermiculophylla* concentrations (Fig. 3 A-C). *G. vermiculophylla*

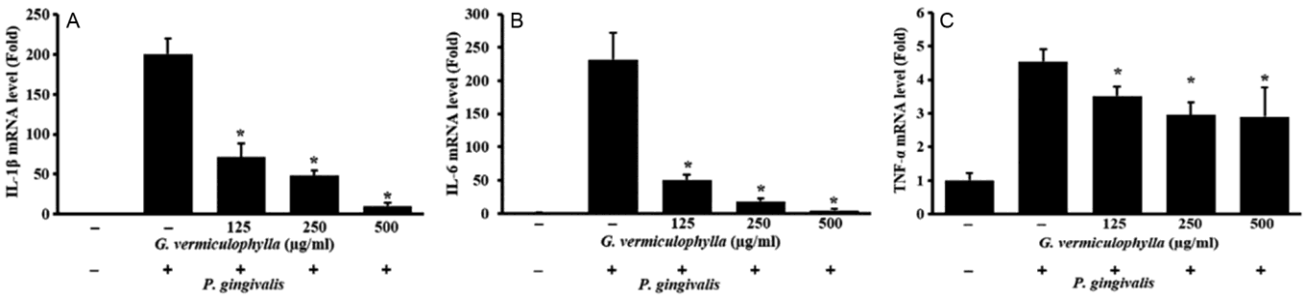

Fig. 3 — Effect *of G. vermiculophylla* extract on *P. gingivalis* stimulated cytokine expression in RAW 264.7 cells. RAW 264.7 cells were stimulated with *P. gingivalis* (1×10^7 CFU/mL) for 24 h. Expression levels of (A) IL-1β; (B) IL-6; and (C) TNF-α mRNA, normalized to β-actin. Total RNA analysis was performed to detect IL-1β, IL-6, and TNF-α mRNA expression using specific primers. [Data are expressed as mean ± SD of triplicate experiments. * P < 0.05; compared with the *P. gingivalis*-treated group]

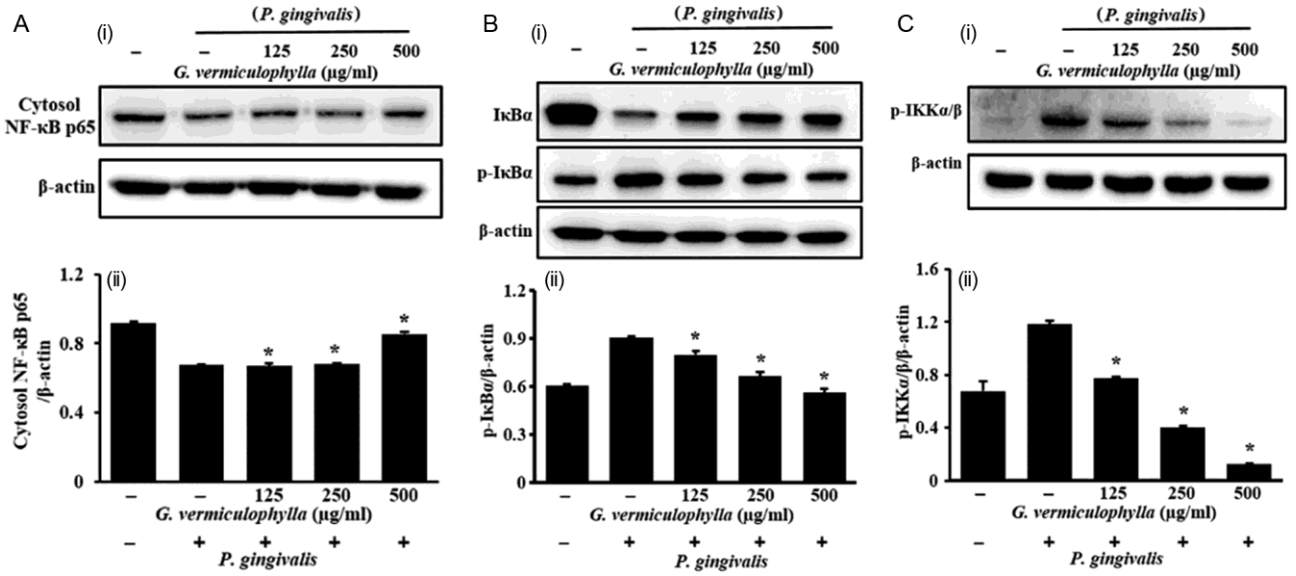

Fig. 4 — Effect of inhibiting (A) NF-κB p65 nuclear translocation; (B) IκBα phosphorylation; and (C) IKKα/β phosphorylation of *Gracilaria vermiculophylla* in RAW 264.7 cells stimulated with *Porphyromonas gingivalis* (1×10^7 CFU/mL). (i) Expression levels of NF-κB p65/p-IκBα and IκBα/p-IKKα/β in the cytosol. B-actin was used as a cytosolic loading control; and (ii) Relative quantification of cytosol NF-κB p65/IκBα phosphorylation/p-IKKα/β. [Data are expressed as mean ± SD of triplicate experiments. *P <0.05; compared with the *P. gingivalis* treated group]

inhibited the inflammatory cytokines induced by *P. gingivalis* stimulation.

Effect of *G. vermiculophylla* on NF-κB pathway activation in *P. gingivalis*-stimulated RAW 264.7 cells

In RAW 264.7, *G. vermiculophylla* was pre-treated at concentrations of 0, 125, 250 and 500 μg/mL for 2 h, and stimulated with *P. gingivalis* to confirm protein expression through Western blot analysis. Cells treated with *G. vermiculophylla* were separated into cytoplasm and nucleus to analyze the degree of phosphorylation and transcription of IKK, IκBα and NF-κB. The amount of protein expression decreased dose-dependent as *G. vermiculophylla* inhibited phosphorrylation of IKKα, IKKβ, and IκBα in *P. gingivalis* stimulated RAW cells (Fig. 4 B and C). *G. vermiculophylla* treatment of *P. gingivalis* stimulated RAW 264.7, inhibited the translocation of NF-κB from the cytoplasm to the nucleus (Fig. 4A). This inhibited the phosphorylation of IKKα, IKKβ and IκBα when treated with *G. vermiculophylla*, indicating that the concentration of NF-κB in the cytoplasm increased in a concentration-dependent manner.

Discussion

Studies on various bioactive substances contained in seaweeds have attracted attention. Research on their anti-inflammatory, anticancer, and antioxidant activities has been conducted and has attracted attention to the development of natural functional

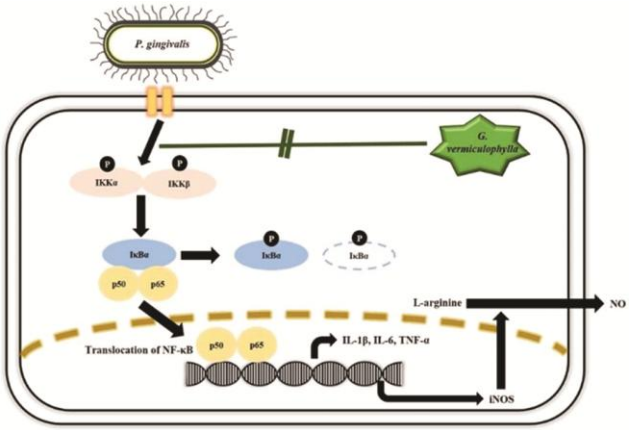

Fig. 5 — Mechanisms underlying the anti-inflammatory effects of *G. vermiculophylla* in *P. gingivalis* stimulated RAW 264.7 cells. The results suggest that *G. vermiculophylla* carried out inhibition of IKKα and IKKβ phosphorylation, IκBα phosphorylation, NF-κB p65 nuclear translocation, IL-1β, IL-6, and TNF-α mRNA expression, iNOS expression, and NO production.

foods and medicines[29,30]. *G. vermiculophylla* has been reported to have antioxidative and anti-inflammatory properties similar to those of red algae[31]. However, its anti-inflammatory effects on periodontal disease have not yet been reported. This study describes the mechanism by which *G. vermiculophylla* inhibits the expression of inflammatory cytokines after infection with periodontal pathogens (Fig. 5).

In this study, we evaluated the inhibitory effects of *G. vermiculophylla* extract on *P. gingivalis* activated

RAW 264.7 cells. Inflammatory responses induced by *P. gingivalis* increase NO production and the expression of iNOS, IL-1β, IL-6 and TNF-α. The cell viability of *G. vermiculophylla* was increased to 500 µg/mL, the highest concentration to exclude the possibility that the cytotoxicity of *G. vermiculophylla* is associated with the inhibition of inflammatory mediators.

Nitric oxide (NO) plays an important role in antibacterial activity and tumor removal. However, excessive NO production by iNOS upregulates other inflammatory cytokines and deepens inflammation[32]. Since NO is produced via NOS from l-arginine[33], the amount of iNOS protein expression in the cytoplasm was checked to confirm the correlation between the inhibition of NO production and iNOS. The expression of iNOS was significantly increased by *P. gingivalis* treatment. This increase was substantially decreased by pretreatment with *G. vermiculophylla* extract at 125, 250 and 500 µg/mL. The expression of iNOS was suppressed by *G. vermiculophylla* extract, which inhibited NO production.

Macrophages play an important role in the early stages of infection by producing cytokines, such as IL-1β, IL-6, and TNF-α during inflammatory reactions[34]. IL-1β induces osteogenic bone loss and is involved in the progression of chronic inflammatory diseases such as periodontitis[35]. The response to IL-6 can be upregulated, suggesting that the synergy between IL-1β and IL-6 results in the progression of periodontal inflammation. TNF-α also cause immune dysfunction, promotes inflammatory responses, and affects periodontitis[36]. In this study, the expression of IL-1β, IL-6 and TNF-α was increased by *P. gingivalis* treatment and significantly decreased by *G. vermiculophylla* extract at 125, 250 and 500 µg/mL pre-treatment.

NF-κB is inactivated in the cytoplasm by the inhibitory protein IκBα. When external stimulation is applied, IκBα is degraded by the IκB kinase. When IκBα is degraded, cytoplasmic NF-κB translocates to the nucleus[37]. In this study, IκBα was reduced by *P. gingivalis* stimulation of the cytoplasm. IκBα was increased by *G. vermiculophylla* extract (125, 250 and 500 µg/mL) pre-treatment. P-IκBα was increased by *P. gingivalis* treatment and significantly decreased by pre-treatment with *G. vermiculophylla* extract at 125, 250 and 500 µg/mL. In cytoplasm, NF-κB was reduced by *P. gingivalis* treatment, but its expression was increased by pretreatment with *G. vermiculophylla* extract at 125, 250 and 500 µg/mL. These results suggest that *G. vermiculophylla* may inhibit NF-κB translocation to the nucleus in the presence of IκBα in the cell by inhibiting IκBα phosphorylation in the cytoplasm. IKKα/β phosphorylation was also inhibited in a dose-dependent manner, suggesting that *G. vermiculophylla* inhibited the expression of inflammatory mediators by inhibiting the NF-κB pathway.

Periodontal disease is a chronic inflammatory disease that causes alveolar bone loss, adult tooth loss, and various complications. Periodontitis was not limited to oral diseases but affected various chronic diseases[38-40]. In particular, it is closely related to dementia[41,42]. Gram-negative bacteria, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans* are mostly found in patients with periodontal disease. Drugs such as chlorhexidine are used for periodontal diseases, but interest in natural extracts continues to increase due to the occurrence of various side effects, such as colouring, burning sensation, and pain. Therefore, studies have been conducted on natural extracts that can help prevent and treat periodontitis[43].

Conclusion

On the basis of our results, the extract of *Gracilaria vermiculophylla* was found to possess anti-inflammatory potential *in vitro* against *Porphyromonas gingivalis*-induced RAW 264.7 cells. The extract of *G. vermiculophylla* has no cytotoxicity and been shown to induce NO inhibition and iNOS suppression via the downregulation of NF-κB signaling pathways. In addition, the extract of *G. vermiculophylla* dose-dependently decreased the values of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) as compared to the corresponding value of untreated group in *P. gingivalis* induced RAW 264.7 cells. Thus, it proposes that *Gracilaria vermiculophylla* could be considered as a potent marine source for isolating therapeutic molecules against periodontitis.

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Conflict of interest

Authors declare no competing interests.

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