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Inhibitory Effect of *Hizikia fusiformis* Solvent-Partitioned Fractions on Invasion and MMP Activity of HT1080 Human Fibrosarcoma Cells

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ABSTRACT: Matrix metalloproteinases (MMPs) are endopeptidases that take significant roles in extracellular matrix degradation and therefore linked to several complications such as metastasis of cancer progression, oxidative stress, and hepatic fibrosis. *Hizikia fusiformis*, a brown algae, was reported to possess bioactivities, including but not limited to, antiviral, antimicrobial, and anti-inflammatory partly due to bioactive polysaccharide contents. In this study, the potential of *H. fusiformis* against cancer cell invasion was evaluated through the MMP inhibitory effect in HT1080 fibrosarcoma cells *in vitro*. *H. fusiformis* crude extract was fractionated with organic solvents, H_2O, *n*-BuOH, 85% aqueous MeOH, and *n*-hexane (*n*-Hex). The non-toxicity of the fractions was confirmed by MTT assay. All fractions inhibited the enzymatic activities of MMP-2 and MMP-9 according to the gelatin zymography assay. Cell migration was also significantly inhibited by the *n*-Hex fraction. In addition, both gene and protein expressions of MMP-2 and -9, and tissue inhibitor of MMPs (TIMPs) were evaluated by reverse transcription-polymerase chain reaction and Western blotting, respectively. The fractions suppressed the mRNA and protein levels of MMP-2, MMP-9 while elevating the TIMP-1 and TIMP-2, with the H_2O fraction being the least effective while *n*-Hex fraction the most. Collectively, the *n*-Hex fraction from brown algae *H. fusiformis* could be a potential inhibitor of MMPs, suggesting the presence of various derivatives of polysaccharides in high amounts.

Keywords: cell migration, zymography, *Hizikia fusiformis*, MMP, TIMP

INTRODUCTION

Spread and growth of malignant tumorous cells are conducted through invasive and metastatic properties of cancer cells. Evolution of a primary abnormal tissue growth from a neoplasm to invasive tumor cells that can travel to distant sites from their origin include several-step biological processes which are regulated by many different factors including but not limited to degradation of extracellular matrix with proteolytic enzymes (1). Matrix metalloproteinases (MMPs), zinc-containing calcium-dependent endopeptidases, are responsible for the proteolytic degradation of extracellular matrix and hence are closely linked to tumor invasion, angiogenesis and metastasis of cancer. In invasive tumor cells, two MMPs, MMP-2 and MMP-9, are pre-dominantly upregulated and play crucial roles in invasion and metastatic spread of tumor cells (2, 3). Regulation of MMP activities are carried out by intracellular inhibitors called tissue inhibitors of metalloproteinases (TIMPs) of which TIMP-1 and TIMP-2 are studied in detail in cancer cells. In this context, during cancer spread and growth, the balance between MMPs and TIMPs are suggested to be deteriorated promoting a new target for therapeutic action against malignant tumors. Inhibition of MMPs coupled with increases in TIMP activity and expression are also considered as a promising way in this matter (4). Therapeutic intervention in metastasis and growth of tumor cells is achieved by new or derived agents that are sourced from medicinal plants and traditional folk medicine (5-7). The scientific world witnessed the discovery and development of novel and potential bioactive substances against cancer proliferation and tumor spread from natural sources, especially both terrestrial and marine plants (8-10), suggesting that plants might exhibit anti-cancer properties through MMP/TIMP regulation of tumor cells.

Marine plants, particularly macroalgae, which are a part of the diet in China, Korea, and Japan, are documented

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to possess health beneficial properties of which anti-tumor and anti-MMP are an important part (11-13). *Hizikia fusiformis* is a brown macroalga widely distributed in Korea and Japan (14). Reports have documented the nutraceutical potential of *H. fusiformis* as it shows health benefits such as anti-oxidant, anti-inflammatory, anti-cholesterol effects (15-17). Fucoidan and laminarin are the major constituents of *H. fusiformis*, with published bioactive effects and are considered to be behind the health beneficial properties of *H. fusiformis* along with their derivative compounds that are present in different amounts (18). We already demonstrated that *H. fusiformis* crude extract had a preliminary MMP-inhibitory effect among other seaweed samples (19). However, to the best of our knowledge, there is no study that reports the MMP-inhibitory effect of *H. fusiformis* with a suggestive action mechanism, testing both regulatory pathways and enzyme activity. In the present study, the effects of *H. fusiformis* solvent-partitioned extracts (HFEs) were evaluated in regard to their ability to inhibit MMP activity and cell invasion.

MATERIALS AND METHODS

Plant materials and fractionation

H. fusiformis was purchased from Parajeju (Jeju, Korea) in 2013. The sample was air-dried outdoors under the shade, ground to powder, and extracted with EtOH 3 times. The extracts were later concentrated under reduced pressure with a rotary evaporator (80 mbar, 50°C). The crude extract was subjected to suspension in CH_2Cl_2 and water. Next, the CH_2Cl_2 layer was fractionated by 85% aqueous MeOH (85% aq. MeOH) and *n*-hexane (*n*-Hex) while the water layer was partitioned with *n*-buthanol (*n*-BuOH) and water (H_2O), respectively. Overall, the solvent-based partition yielded the *n*-Hex, 85% aq. MeOH, *n*-BuOH, and H_2O fractions that were used in further assays after dissolving in 10% dimethyl sulfoxide (DMSO) and kept at −20°C.

Cell culture and cell viability determination

HT1080 human fibrosarcoma cells were cultured in T-75 culture flasks (Nunc, Roskilde, Denmark) in an incubator at 37°C and 5% CO_2 atmosphere using Dulbecco's modified Eagle, medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 μg/mL penicillin-streptomycin (Gibco-BRL). The medium was changed twice or three times each week.

For cell viability assessment, cells were cultured in 96-well plates at a 5×10^3 cells/well density. Following 24 h incubation, the cell culture medium was removed, and the cells were washed with fresh medium and prior to treatment with the medium with or without HFEs (5 and 50 μg/mL). Cells were re-washed with fresh medium after 24 and 48 h of incubation, and 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (1 mg/mL) was introduced to the wells, followed by a 4 h incubation. Finally, 100 μL of DMSO was used for each well in order to solubilize the formazan crystals for the determination of formazan crystal amount which was measured by the absorbance at 540 nm using a GENios® microplate reader (Tecan Austria GmbH, Grödig, Austria). Cell viability was defined by the absorbance value as a way to indicate the amount of MTT converted into formazan crystal. Viability of cells was determined as a percentage in comparison to the untreated control wells against the sample treated wells and dose response curves were established.

Cell migration assay

Cells were grown on a 12-well culture dish to 90% confluence followed by forming an injury line with a width of 2 mm from scraping vertically across the cell layer with a sterile scraper. After floating cell debris was removed by washing with phosphate-buffered saline, the cell medium was serum-free medium, and cells were treated with 50 μg/mL HFEs. Cell migration was observed under an inverted microscope (Nikon Eclipse TS100, Nikon Instruments Inc., Melville, NY, USA) and photographs were taken at incubation starting time and after 24 h of incubation. Migrations were quantified by pixel colors of image where cells were absent using Adobe Photoshop CC 2017 software (Adobe Systems, San Jose, CA, USA).

MMP enzymatic activity determination by gelatin zymography

Enzymatic activities of MMP-2 and MMP-9 from HT1080 cells that were treated with or without HFEs were detected by gelatin zymography. HT1080 cells were cultured in 24-well plates with a density of 2×10^5 cells/well in a serum-free medium and were introduced to different concentrations of HFEs for 1 h. Phorbol 12-myristate 13-acetate (PMA, 10 ng/mL) (Sigma-Aldrich Co., St. Louis, MO, USA) was used to enhance the MMP expression and cells were further incubated for 24 h after PMA treatment. Total protein contents of the cells were normalized using the Bradford protein determination method. Next, cell culture medium was subjected to substrate-gel electrophoresis. Conditioned cell culture media with the same amount of protein was transferred onto 10% polyacrylamide gels under non-reducing conditions containing 1.5 mg/mL gelatin. Polyacrylamide gels were then washed with 50 mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100 to remove any remaining sodium dodecyl sulfate (SDS). After the washing process, gels were incubated for 48 h at 37°C in a developing buffer contain-

ing 10 mM $CaCl_2$, 50 mM Tris-HCl, and 150 mM NaCl to facilitate the digestion of gelatin by MMPs. Areas of gelatin hydrolyzation by MMP were observed as clear zones against the blue background of Coomassie Blue staining under a CAS-400SM Davinch- Chemi imager™ (Davinch-K, Seoul, Korea).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted by TRIzol reagent (Thermo-Fisher Scientific, Waltham, MA, USA) from HFE-treated and control wells. Any changes in the concentration of mRNA for MMP-2 and MMP-9 were determined by RT-PCR. Briefly, 2 μg of total RNA from cells was converted to single stranded cDNA using a reverse transcription system (Promega, Madison, WI, USA). The target cDNA was amplified using the following primers: forward 5'-TGA-AGG-TCG-GTG-TGA-ACG-GA-3' and reverse 5'-CAT-GTA-GCC-ATG-AGG-TCC-ACC-AC-3' for MMP-2; forward 5'-CAC-TGT-CCA-CCC-CTC-AGA-GC-3' and reverse 5'-CAC-TTG-TCG-GCG-ATA-AGG-3' for MMP-9; forward 5'-AAT-TCC-GAC-CTC-GTC-ATC-AG-3' and reverse 5'-TGC-AGT-TTT-CCA-GCA-ATG-AG-3' for TIMP-1; forward 5'-TGA-TCC-ACA-CAC-GTT-GGT-CT-3' and reverse 5'-TTT-GAG-TTG-CTT-GCA-GGA-TG-3' for TIMP-2; forward 5'-GCC-ACC-CAG-AAG-ACT-GTG-GAT-3' and reverse 5'-TGG-TCC-AGG-GTT-TCT-TAC-TCC-3' for β-actin. Cycles were 95°C for 45 s, 60°C for 1 min and 72°C for 45 s for amplification. Following the completion of 30 cycles, the final products were separated by electrophoresis on 1.5% agarose gel for 30 min at 100 V. Gel staining was carried out with 1 mg/mL EtBr and visualization by UV light using Alpha-Ease® gel image analysis software (Alpha Innotech, San Leandro, CA, USA).

Western blot analysis

Immunoblotting was performed according to common standard procedures. Briefly, HT1080 cells were agitated in radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich Co.) at 4°C for 30 min. Cell lysates (35 μg) were then subjected to separation using 12% SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK), blocking with 5% skim milk and hybridization with primary antibodies (diluted 1:1,000). After incubation with horseradish-peroxidase-conjugated secondary antibody at room temperature, immunoreactive proteins were detected using an electrochemiluminescence kit (Amersham Pharmacia Biotech Inc.) according to the manufacturer's instructions. Protein bands were observed using a CAS-400SM Davinch-Chemi imager™ (Davinch-K).

Statistical analysis

The data were presented as a mean of three different experiments±standard deviation. Differences between the calculated means of the each individual group were determined by one-way ANOVA coupled with Duncan's multiple range tests. Any difference was considered statistically significant at $P<0.05$. The statistical software SAS v9.1 (SAS Institute Inc., Cary, NC, USA) was used for analyses.

RESULTS AND DISCUSSION

MMPs are known to influence and intervene with several important pathways for metastasis, oxidative stress and fibrosis (20,21). Hence, successful inhibition of MMP activity is steadily gaining high interest as a way to produce potent pharmaceuticals against metastasis-linked complications and tumor growth. MMP inhibitors of natural origin are being recently studied, and marine organisms hold a great deal of potential in this context due to their surviving in a unique and challenging environment. Various organisms, especially marine plants contain bioactive metabolites, some of which are credited as potent MMP-inhibitors and their mechanism of actions have been proposed (22,23). In order to provide valuable insights on that matter, *H. fusiformis* was studied to evaluate its MMP-inhibition efficiency and possible MMP inhibiting constituents. In this regard, for future utilization through activity-based isolated and elucidated bioactive substances, crude extract of *H. fusiformis* was fractioned with organic solvents and solvent- partitioned extracts.

Effect of HFEs on enzymatic activity of MMP-2 and MMP-9

First, HFEs were tested for their cytotoxic presence in the human fibrosarcoma cell line HT1080 for 48 h at two different concentrations (5 and 50 μg/mL) (Fig. 1). The cytotoxicity test revealed that these concentrations were cytocompatible and any observed inhibition of MMP-2 and MMP-9 activity was not caused by any cytotoxic influence. The elevated cell viability in H_2O HFE treated wells suggested that this fraction contains compounds with proliferation enhancing properties. Studies reported that aqueous extracts of plant samples could yield proliferation enhancing effects (24) which may be the reason for the elevated proliferation observed in H_2O HFE treated cells.

HFEs were analyzed for their possible activity to inhibit MMP-2 and -9 enzymes following PMA stimulation. Gelatinolytic activity of MMP-2 and -9 secreted from fibrosarcoma cell line HT1080 were evaluated with gelatin zymography, which was carried out with PMA stimulated conditioned medium of HFE treated cells (Fig. 2). Introduction of PMA (10 ng/mL) to cells resulted in en-

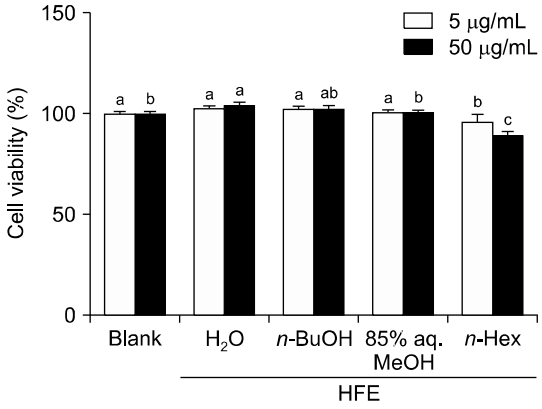

Fig. 1. Effect of solvent-partitioned *Hizikia fusiformis* extracts (HFEs) on cell viability of HT1080 human fibrosarcoma cells. HT1080 cells were treated with or without different concentrations of HFEs and incubated for 48 h. Viability of cells following incubation was measured by the absorbance at 540 nm according to their ability to form MTT formazan crystals. Values are mean±SD (n=3). Means with the different letters (a-c) are significantly different (P<0.05) by Duncan's multiple range test.

hanced activation of MMP-2 and -9, and the gelatinolytic activity in gelatin zymography was elevated. Among the tested samples, *n*-Hex decreased most of MMP-2 and -9 activities in a dose-dependent manner relevantly higher than that of other samples. The remaining HFEs were observed to inhibit MMP activity in the order of *n*-BuOH, 85% aq. MeOH, and H_2O fractions. Inhibition of MMP enzymatic activities indicated that active samples possess bioactive substances that could have inhibited the MMP-2 and -9 activities directly or by regulating the expression of MMP-2 and MMP-9 through their intracellular pathways without directly affecting enzymatic activity.

Effect of HFEs on MMP-2 and MMP-9 intracellular pathways in HT1080 cells

The possible MMP inhibitory presence of HFEs was further evaluated through their effect on cell migration using HT1080 human fibrosarcoma cells. Cells without any treatment showed signs of migration after 24 h incubation while treated cells were observed to have hindered migration patterns (Fig. 3). Treatment with 50 μg/mL HFEs inhibited the migration ability of tumor cells indicating a possible inhibition activity of MMPs, which are important enzymes for the invasive nature and migration of tumor cells (5). HFEs were ordered as *n*-Hex, 85% aq. MeOH, *n*-BuOH, and H_2O according to their level of cell migration inhibition. Their inhibition rate of migration was calculated as percentage of PMA-induced control cell group and observed to be 87.84%, 79.65%, 48.21%, and 15.89% for *n*-Hex, 85% aq. MeOH, *n*-BuOH, and H_2O, respectively. Further, RNA and total protein levels of MMP-2 and -9 were determined by RT-PCR and immunoblotting with levels of TIMP-1 and -2. TIMPs reported to elevate the activity of MMP-2 (4). RT-PCR and immunoblotting results suggested the treatment with HFEs was able to suppress the expression of MMP-2 and -9 in terms of both mRNA (Fig. 4) and protein levels (Fig. 5). The presence of TIMPs is considered to inhibit MMP activity as a part of a cellular response for extracellular stimuli (25). PMA stimulation caused TIMP levels to decrease and MMP expression to increase (Fig. 4). However, treatment with HFEs was observed to produce mixed results regarding the effect on the TIMP levels following PMA stimuli. The expected results were to inhibit MMP expression while enhancing TIMP expression in order to regulate extracellular matrix degradation. Only 85% aq.

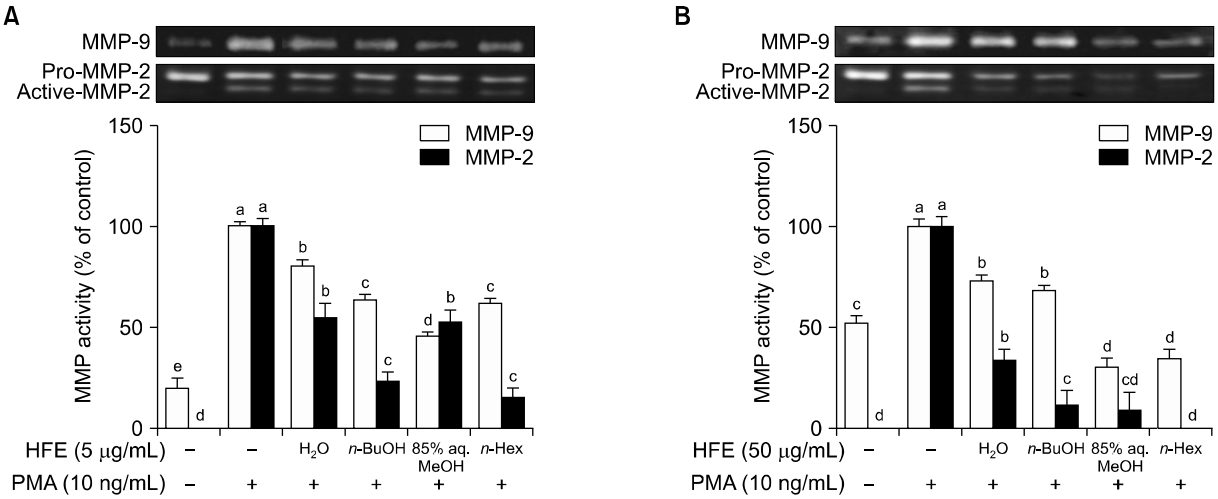

Fig. 2. Effect of 5 (A) and 50 μg/mL (B) solvent-partitioned *Hizikia fusiformis* extracts (HFEs) on enzymatic activity of matrix metalloproteinase (MMP)-2 (active) and MMP-9 tested by gelatin zymography. Phorbol 12-myristate 13-acetate (PMA)-stimulated cells were treated with or without different concentrations of HFEs and incubated for 24 h. Following incubation activity of MMP-2 and MMP-9 enzymes were observed on polyacrylamide gels containing gelation for enzymes to cleave. Band sizes of multiple assays (n=3) were quantified and depicted as percentage of activity compared to the PMA-stimulated untreated control group. Means with the different letters (a-d) are significantly different (P<0.05) by Duncan's multiple range test.

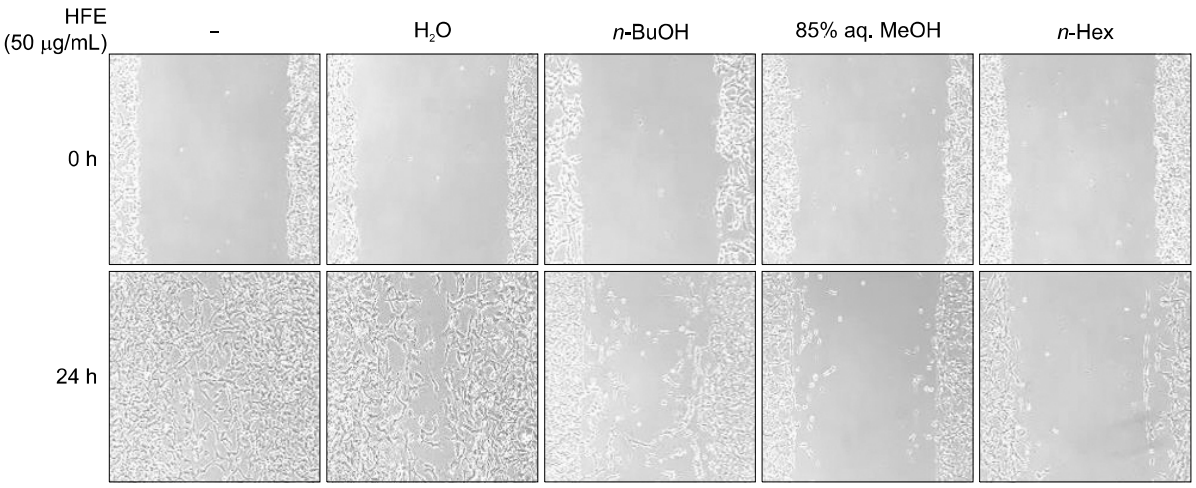

Fig. 3. Effect of solvent-partitioned *Hizikia fusiformis* extracts (HFEs) on migration ability of phorbol 12-myristate 13-acetate-stimulated HT1080 human fibrosarcoma cells. HT1080 cells were introduced an injury line of a 2 mm width and treated with or without 50 μg/mL HFEs. Following a 24 h incubation, cell images were taken to observe the ability of the cells to migrate through the injured line.

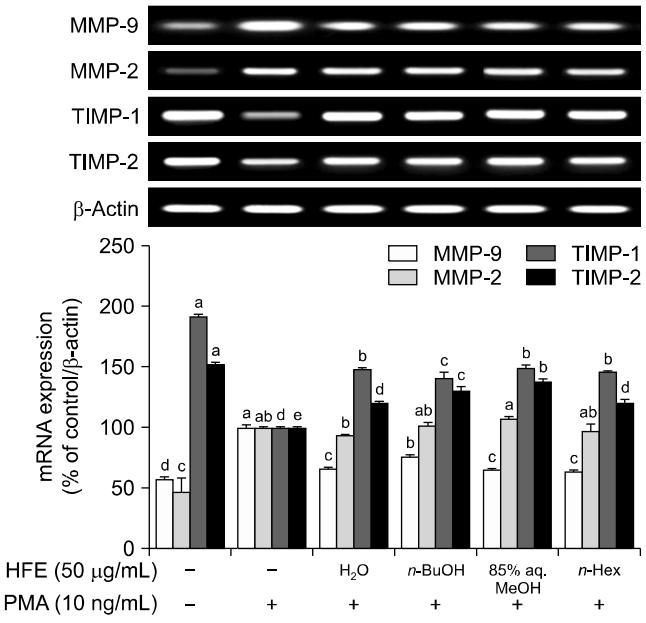

Fig. 4. Effect of solvent-partitioned *Hizikia fusiformis* extracts (HFEs) on mRNA levels of matrix metalloproteinase (MMP)-2, MMP-9, inhibitor of MMP (TIMP)-1, and TIMP-2. β-Actin was used as an internal standard. HT1080 cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and treated with or without HFEs. mRNA levels of MMP pathway proteins were measure by reverse transcription of the total cellular RNA with specific primers. mRNA levels were observed by gel electrophoresis. Band sizes of multiple assays (n=3) were calculated and depicted as percentage difference compared to the PMA-stimulated untreated control group. Values were normalized against housekeeping β-actin mRNA levels. Means with the different letters (a-e) are significantly different (P<0.05) by Duncan's multiple range test.

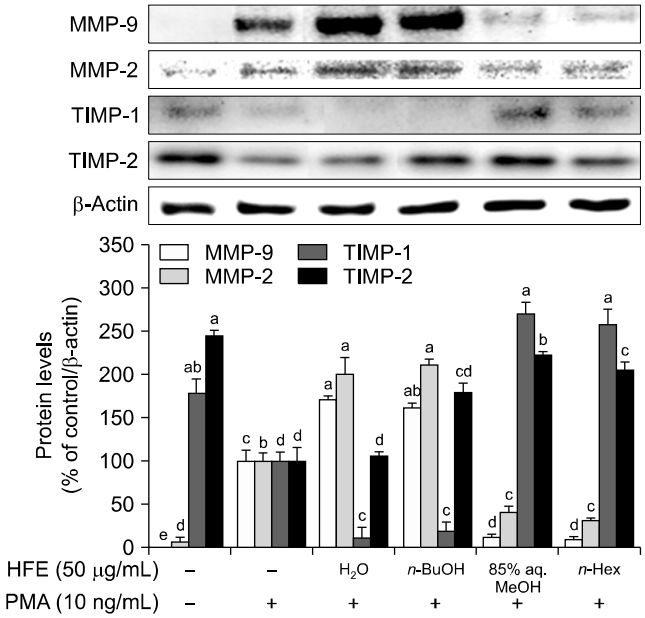

Fig. 5. Effect of solvent-partitioned *Hizikia fusiformis* extracts (HFEs) on protein levels of matrix metalloproteinase (MMP)-2, MMP-9, inhibitor of MMP (TIMP)-1, and TIMP-2. β-Actin was used as an internal standard. HT1080 cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and treated with or without HFEs. Protein levels of aforementioned proteins were observed by immunoblotting with specific antibodies from total protein content of cells. Band sizes were calculated and depicted as percentage difference compared to the PMA-stimulated untreated control group. Values were normalized against housekeeping β-actin protein levels. Means with the different letters are significantly different (P<0.05) by Duncan's multiple range test.

MeOH and *n*-Hex were able to regulate MMP-2, MMP-9, TIMP-1, and TIMP-2 levels in an expected manner where suppression of MMP expression was coupled with elevated TIMP levels. On the other hand, protein levels of MMP-2 and -9 were slightly elevated after H_2O and *n*-BuOH HFE treatments with elevated TIMP-1 and -2 levels while mRNA levels did not depict any significant changes for all tested HFE samples. Also a slight contrast was observed between the effects of HFEs on mRNA and protein levels. This might be credited to the effect of TIMP-2 protein on the expression of MMPs. The active

fractions on TIMP pathway was observed to exhibit decreased protein levels of MMPs, while the remaining HFEs were not able to show similar effects on protein levels, which was suggested to result in the contrast of mRNA and protein levels. In addition, as mentioned earlier elevated levels of TIMP-2 caused an increase in MMP-2 protein levels with the *n*-Hex HFE treatment. TIMP-1 and TIMP-2 both act as regulators for extracellular matrix composition through MMP actions. However between the two, TIMP-2 can act as an activator for MMPs (26). In addition, each TIMP has slightly different effectiveness to different types of MMPs. For instance, while TIMP-1 is more effective in inhibiting MMP-9, TIMP-2 can inhibit MMP-2 better than other TIMPs (26). Considering, a higher TIMP-2 expression might also cause elevated MMP protein translation, but this does not correlate with activated MMP activity as other TIMPs also play roles in the activation of expressed enzymes. In this context, higher levels of MMP-2 and MMP-9 in H_2O and *n*-BuOH HFE treated cells might suggest an effect on TIMP-2 regulation of MMPs. Both fractions did not show any effect on TIMP-1 expression but showed a slight increase in TIMP-2 protein levels, which further strengthen the suggestion. Nonetheless, HFEs were shown to have an effect on both activity and expression of MMP pathways but with different mechanism of actions. In the cases of H_2O and *n*-BuOH samples, the suggested action mechanism was through inhibiting the activation of MMP-2 and MMP-9 enzymes. Both fractions were unable to regulate the expression of intracellular MMP pathways and exhibited elevated protein levels of both MMPs. However, MMP-2 and MMP-9 activities were hindered, indicating an inhibitory activity directly on enzymatic presence. Failure to inhibit cell migration also suggested that both fractions were incapable of inhibiting MMP-2 and MMP-9 expression at the cellular level. In terms of the remaining HFEs, 85% aq. MeOH and *n*-Hex, it was suggested that an intracellular regulation mechanism through TIMP-linked pathways of MMP activity was key for protein and mRNA levels and enzymatic activity.

The possible chemical composition of the *n*-Hex HFE, the most active sample according to current results, was suggested to be formed mostly with polysaccharide-based compounds according to previous reports (27,28). Reports indicated that the hexane fraction usually contains less polar substances, especially sugar based compounds with long chains. Although, these compounds exhibit specific and uniform bioactivities compared to the variable effects of phenolic substances, the hexane fractions are known to possess enzyme inhibitors. A study by Zhu et al. (14) depicted that *H. fusiformis* contains various polysaccharide derivatives, including the sulfated polysaccharide fucoidan, which possess potent bioactivities and are common bioactive substances of brown algae with health beneficial effects (11,29). Considering all, it can be suggested that the *H. fusiformis* hexane fractions showed anti-MMP effects through content of polysaccharide substances, possibly derivatives of polysaccharide chains coupled with active side chains.

In conclusion, extracts from *H. fusiformis* were able to inhibit both MMP activity and intracellular MMP pathways, regulated through TIMP expression. The bioactive compounds behind the anti-MMP effect of *H. fusiformis* were suggested to be of polysaccharide-origin with the *n*-Hex solvent fraction being the most effective to inhibit MMP activity and expression. On the other hand, detailed evaluation of *H. fusiformis* and its constituents will provide valuable insights for its utilization as a functional food and future studies on its action mechanism is urged to better understand its potential. Further examinations are required to elucidate the effective substances of *H. fusiformis* and evaluate their mechanisms of action in order to facilitate the utilization in the development of nutraceuticals with MMP inhibitory properties. Nevertheless, in the current state, *H. fusiformis* was suggested as a potential nutraceutical with health benefits due to its potential anti-MMP effect and bioactive compound content.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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