Inhibition of MMP-2 and MMP-9 Activities by Limonium tetragonum Extract

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ABSTRACT: Matrix metalloproteinases (MMPs) are crucial extracellular matrices degrading enzymes that take important roles in metastasis of cancer progression as well as other significant conditions such as oxidative stress and hepatic fibrosis. Natural products are on the rise for their potential to provide remarkable health benefits. In this context, halophytes have been of interest in the nutraceutical field with reported instances of isolation of bioactive compounds. In this study, Limonium tetragonum, an edible halophyte, was studied for its ability to inhibit MMP-2 and -9 using HT1080 fibrosarcoma cells. Results showed that L. tetragonum extract was able to inhibit the enzymatic activity and mRNA expression of MMP-2 and -9 according to gelatin zymography and RT-PCR assays, respectively, but it was not able to significantly change the MMP pathway related factors such as tissue inhibitors of metalloproteinases. Also, Mitogen-activated protein kinases pathway-related protein levels and their phosphorylation were assayed. While the phosphorylated p38 levels were decreased, extracellular signal-regulated kinase and c-Jun N-terminal kinase were not affected by L. tetragonum treatment. In conclusion, it was suggested that L. tetragonum contains substances acting as MMP inhibitors on enzymatic activity rather than intracellular pathway intervention, which could be useful for further utilization of L. tetragonum as a source for anti-MMP agents.

Keywords: HT1080, Limonium tetragonum, MMP, TIMP, MAPK

INTRODUCTION

Cancer has been one of the most difficult diseases of the modern world in terms of prevention and treatment. The studies towards the discovery and development of compounds of natural origin have been the crucial steps of anti-tumor agent research, especially in the pharmaceutical field. Nature contains a broad range of organisms, an important source of unique chemical substances with immense potential to be regarded as therapeutic agents. Current trends credit treatments derived from natural sources to have notable effect on antitumor agent discovery (1). Natural products are of much interest to researchers mainly due to their bioavailability, specific and strong binding to drug targets, and their ability to bind targeted proteins. Among these natural products of interest, halophytes have been studied recently in order to elucidate novel compounds with efficient bioactivities of halophytes to withstand harsh environmental conditions. The metabolites produced under these conditions have already been reported to show health benefit effects in vitro (2,3).

Salt marshes and muddy seashores of South Korea contain a commonly known edible halophyte species, Limonium tetragonum, which grows in places that come into contact with waters of high salinity (4). Recent studies also reinforced the notion that L. tetragonum possesses strong beneficial effects towards several health threatening complications. Yang et al. (5) demonstrated that the methanolic extract of L. tetragonum was able to prevent liver satellite cells from undergoing proliferation, an indication for a suggested hepatoprotective effect against hepatic fibrosis. However, apart from antioxidant compounds, which are known to be present in abundance in plants of harsh environments, current studies do not contain any detailed reports on the health benefit effects of L. tetragonum-derived isolated bioactive substances (6). Matrix metalloproteinases (MMPs) play a significant...
role in the digestion of extra cellular matrix components, which are directly associated with chronic inflammation, wrinkle formation, arthritis, osteoporosis, periodontal diseases, tumor invasion, and metastasis in pathological conditions. Ongoing cancer research has acknowledged the MMPs as significant enzymes involved in several steps of cancer-related conditions. Especially in terms of invasive tumor cells, MMP expression and activity are deteriorated and closely linked with the onset of cancer symptoms. Among all the different types of MMPs, MMP-2 and -9 have been revealed to be highly active and play key roles during metastasis, essentially with elevated malignant tumor presence (7,8). Regulation of MMP-2 and -9 activities are suggested to possess a therapeutic potential against cancer. In this decade, a detailed presentation of MMP inhibitory effects of compounds derived from halophytes have been reported (9,10).

Considering the potential of halophytes, as part of a prominent research trend to develop novel substances from natural plants for nutraceutical purposes, L. tetragonum has been tested for its effect on MMP-linked pathways in vitro using a human fibrosarcoma cell model.

**MATERIALS AND METHODS**

**Extraction of L. tetragonum**
The shade-dried material (500 g) of L. tetragonum was ground and sequentially extracted twice with CH$_2$Cl$_2$ (3 L) and MeOH (3 L). The combined crude extracts were dried in vacuo to obtain a residue of 24.41 g and partitioned between CH$_2$Cl$_2$ and H$_2$O. The organic CH$_2$Cl$_2$ layer was further dried in vacuo, and the final extract (2.04 g) was dissolved in distilled water to be used in further assays.

**Cell culture and cytotoxicity determination**

Human fibrosarcoma HT1080 cells were grown as monolayers in T-75 tissue culture flasks (Nunc, Roskilde, Denmark) at 5% CO$_2$ and 37°C humidified atmosphere using Dulbecco’s modified Eagle’s medium (DMEM, 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.

In order to determine non-toxic concentrations of brown algae samples, the cytotoxic effects were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay in HT1080 cells. Briefly, the cells were cultured in 96-well plates at a density of 5×10$^4$ cells/well. After 24 h, the cell culture medium was changed with the control medium or the medium containing samples. After incubation of 48 h, cells were washed and 100 μL of MTT solution (1 mg/mL) was added and incubated for 4 h. Next, in order to solubilize the formed formazan crystals, 100 μL of dimethyl sulfoxide was added and formazan amount was determined by measuring the absorbance at 540 nm using a GENios® microplate reader (Tecan Austria GmbH, Grödig, Austria). Viability of cells was quantified as a percentage compared to the control, and dose response curves were developed.

**Determination of MMP activity by gelatin zymography**

Activities of MMP-2 and -9 in HT1080 cells treated with samples were determined by gelatin zymography. HT 1080 cells in serum-free medium were seeded in 24-well plates with a density of 2×10$^5$ cells/well and pre-treated with different concentrations of sample for 1 h. MMP expression was stimulated by treatment with phorbol 12-myristate 13-acetate (PMA, 10 ng/mL) and cells were incubated for 24 h. Total protein contents were normalized by the Bradford protein determination method. Cell conditioned medium was subjected to substrate gel electrophoresis. A same amount of protein containing conditioned media was applied under non-reducing conditions on 10% polyacrylamide gels containing 1.5 mg/mL gelatin. After electrophoresis, polyacrylamide gels were washed with 50 mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100 at room temperature to remove sodium deoxycholate. Gels were then incubated for 48 h at 37°C in a developing buffer containing 10 mM CaCl$_2$, 50 mM Tris-HCl, and 150 mM NaCl to digest gelatin by MMP. Areas of gelatin hydrolyzed by MMP were visualized as clear zones against blue background by 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 from sample-treated cells using TRIzol reagent. Changes in the steady-state concentration of mRNA for MMP-2 and MMP-9 were assessed by RT-PCR. Briefly, total RNA (2 μg) 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 ATT AC-3’ for MMP-2; forward 5’-CAC TGT CCA CCC CTC AGA GC-3’ and reverse 5’-CAT TTG CTC GAC CTC GTC ATC AG-3’ and reverse 5’-TGC AGT TTG CCA GAC ATG AG-3’ for tissue inhibitor of metalloproteinase (TIMP)-1; forward 5’-TGG TCC AGG GTT TCT TAC TCC-3’ for MMP-2; forward 5’-GCC ACC CAG AAG ACT GTG GAT-3’ and reverse 5’-TGG TCC AGG GTT TCT TAC TCC-3’ for
Western blot analysis

Western blotting was performed according to standard procedures. Briefly, HT1080 cells were lysed in RIPA lysis buffer (Sigma-Aldrich Co., St. Louis, USA) at 4°C for 30 min. Cell lysates (35 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane (Amersham Biosciences UK Ltd., Buckinghamshire, UK), blocked with 5% skim milk and hybridized with primary antibodies (diluted 1:1,000) against ALP and collagen I. After incubation with horseradish-peroxidase-conjugated secondary antibody at room temperature, immunoreactive proteins were detected using a chemiluminescent ECL assay kit (Amersham Biosciences UK Ltd.) according to the manufacturer's instructions. Western blot bands were visualized using a CAS-400SM Davinch-Chemi imager™ (Davinch-K).

Statistical analysis

The data were presented as mean±SD. Differences between the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range tests. Differences were considered significant at P<0.05. The statistical software package, SAS v9.1 (SAS Institute Inc., Cary, NC, USA), was used for these analyses.

RESULTS AND DISCUSSION

MMPs are known to be a part of different important pathways including metastasis, oxidative stress, and fibrosis (11,12). Hence, potent MMP-inhibitors are always of high interest in several pharma and nutraceutical fields. To obtain natural MMP-inhibiting compounds, marine organisms hold a great deal of potential as they are present in unique and challenging environments. Various organisms, especially marine plants and metabolites have been identified as potential MMP-inhibitors and possible mechanism of actions for isolated compounds have been suggested (13,14). In order to provide valuable insights on that matter, L. tetragonum was studied in regard to its MMP-inhibition efficiency, which will help its future utilization by isolating and elucidating the bioactive substances.

The extract was tested for its cytotoxic presence in human fibrosarcoma cell line HT1080 for 48 h at 5 different concentrations (5, 10, 20, 50, and 100 μg/mL) (Fig. 1). The cytotoxicity test revealed that these concentrations were cytocompatible and the possible inhibition of MMP-2 and -9 was not due to cytotoxic influence.

To test the effect of brown algae on MMP enzymatic activity, brown algae species were tested for their efficiency to inhibit MMP-2 and -9 activity following PMA stimulation. Gelatinolytic activity of MMP-2 and -9 secreted from fibrosarcoma cell line HT1080 were evalu-

![Fig. 1. Cytotoxicity of Limonium tetragonum extract treatment for 48 h on human fibrosarcoma cell line HT1080 at the concentrations of 5, 10, 20, 50, and 100 μg/mL. Control: cells without sample treatment.](image)

![Fig. 2. Effect of Limonium tetragonum extract on the enzymatic activities of MMP-2 and -9 in HT1080 cells. Gelatinolytic activities of MMP-2 and -9 were evaluated by gelatin zymography and detected by electrophoresis on gelatin containing 10% polyacrylamide gel. Band sizes of multiple assays (n=3) were calculated and depicted as percentage against the blank and control group. Means with different letters (a-d) are significantly different (P<0.05) by Duncan’s multiple range test. Blank, cells without sample treatment and phorbol 12-myristate 13-acetate (PMA) stimulation. Control, cells stimulated with PMA without sample treatment.](image)
MMP Inhibitory Effect of *L. tetragonum*

RNA and total protein levels MMP-2 and -9 were evaluated by RT-PCR and western blotting along the levels of TIMP-1 and -2. TIMPs are known inhibitors of MMPs and are also known to elevate the activity of MMP-2 in some occasions (15). RT-PCR and Western blotting results indicated the presence of the extract was able to suppress the expression of MMP-2 and -9 in both mRNA and protein levels (Fig. 3A). The presence of TIMPs is expected to be an indicator for inhibited MMP activity as part of the cellular response for extracellular stimuli (16). Hence, PMA stimulation caused TIMP levels to de-

![Graphs showing mRNA and protein expression levels](image)
crease and MMP expression to increase (Fig. 3B). However, treatment with the extract did not affect TIMP levels following the PMA stimuli. The expected results were to suppress MMP expression while regulating TIMP expression in order to balance the extracellular matrix degradation. On the other hand, protein levels of TIMP-1 and -2 were slightly elevated. Overall, unregulated TIMP expression showed that L. tetragonum did not intervene with the TIMP-linked cellular inhibition of MMP-2 and -9 but only affected the enzymatic activity and gene expressions of MMPs. Considering that both MMP and TIMP mRNA were suppressed by treatment, it might be suggested that the extract inhibited overall MMP activity through a direct enzyme-bound inhibition rather than regulation of the expression of TIMPs at gene level. However, current results indicated that the extract was unable to regulate TIMP expression as expected in order to regulate MMP activity to prevent metastatic and unwanted fibrotic degradation. Under these conditions, utilization of crude extracts of L. tetragonum might be limited to support nutrition rather than as a potential nutraceutical as the extract only inhibited MMPs enzymatically and probably not with specific binding considering the different types of metabolites present. In order to understand the intervention on MMP-related intracellular pathways, levels of mitogen-activated protein kinases (MAPK)-related proteins, namely p38, p-extracellular signal-regulated kinase (ERK), and p-c-Jun N-terminal kinase (JNK), were also evaluated (Fig. 4). As expected, PMA stimulation resulted in elevated expressions of ERK, JNK, and p38 as well as their phosphorylation in cellular activity. Reports showed that the inhibition of the MAPK pathway is closely related to the down-regulation of MMP secretion (17, 18). The activation of the MAPK pathway induced by PMA as shown in Fig. 4 depicted high amount of protein levels and linked phosphorylation. However, treatment with L. tetragonum extract at the concentration of 20 μg/mL did not show any changes in the aforementioned increment in protein expressions suggesting that the anti-MMP effect of L. tetragonum was observed only in inhibiting enzymatic activity rather than in intracellular pathway regulation. However, among all the MAPK proteins tested, phosphorylated p38 levels were inhibited by treatment. Kim et al. (19) reported that upregulation of MMP-2 and -9 were induced by transforming growth factor (TGF)-β-linked p38 expression but not ERK and JNK signaling in human breast epithelial cells. Likewise, current data suggested that L. tetragonum treatment-induced downregulation of MMP-2 and -9 activities and mRNA expression were not linked to phosphorylation of ERK and JNK but only p38. Therefore, it could be suggested that inhibition of MMP-2 and -9 by L. tetragonum was exerted through TGF-β pathways as well as enzymatic inhibition. Nevertheless, current results indicate that L. tetragonum is a source of MMP inhibitors that might be used for future development of anti-tumor compounds. Some flavonoids, especially quercetin and derivatives from other sources were already reported to possess anti-MMP bioactivity (20, 21). Assessment of the true potential of L. tetragonum were hindered by a lack of in vitro mechanism predictions which could lead to in vivo studies. Up to now, only hepatoprotective effects of L. tetragonum extract were studied in vivo following an in vitro study. After the confirmation of hepatoprotective presence of L. tetragonum in liver cells (5), Kim et al. (22) reported that the EtOAc fraction of L. tetragonum extract protected liver cells against alcohol toxicity by a suggested mechanism through ethanol metabolism and antioxidant enzyme activities. In this context, current data regarding the anti-MMP effects of L. tetragonum extract could pave the way for in vivo studies for the development of non-invasive natural products for tumor treatment. L. tetragonum was studied for its containment of flavonoids similar to that of quercetin derivatives based on its chemical structure (6). An evaluation of L. tetragonum and its constituents will provide valuable insights for its utilization as a functional food. In the current state, L. tetragonum is suggested as a potential nutraceutical due to its anti-MMP presence.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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