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RESEARCH ARTICLE

Hexane and Chloroform Fractions of *Laetiporus sulphrueus* var. *miniatus* Inhibit Thrombin-treated Matrix Metalloproteinase-2/9 Expression in Human Oral Squamous Carcinoma YD-10B Cells

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Abstract

Laetiporus sulphrueus var. *miniatus* is widely distributed worldwide, and has commonly been used as a medicinal mushroom. In the present study, we investigated the effects of water extract and solvent fractions from the *Laetiporus miniatus* as possible antioxidant, anti-thrombin and anti-invasive agents against phorbol 12-myristate 13-acetate (PMA)- or thrombin-induced matrix metalloproteinase-2 (MMP-2) and MMP-9 activities. Samples were fractionated into n-hexane, $CHCl_3$, ethyl acetate, n-butanol, and water fractions, and individually analysed. The water fraction had the highest extraction yield at 34.90% (w/w), while the n-butanol fraction demonstrated the highest anti-oxidative activity at 81.44%. In the thrombin inhibitory activity test, the water fraction exhibited the highest activity at 94.64%. Even at the concentration of 40 μg/mL, evaluation of anti-proliferating activity in YD-10B cells did not reveal any cytotoxic effects. Although MMP-9 expression in YD-10B cells increased after the addition of PMA and thrombin, MMP-2 did not. Additionally, MMP-2/-9 levels in PMA-treated YD-10B cells (i.e., both mRNA expression and protein activation) were highly inhibited in the hexane and chloroform fractions. Compared with MMP-2 levels, MMP-9 mRNA expression and proteolytic activity were inhibited to a greater extent by the hexane and chloroform fractions in thrombin-treated YD-10B cells. Taken together, these results support that thrombin induces tumor invasion through MMP-2/9 and suggest that the *L. miniatus* may act as an effective functional food, conferring anti-oxidative, anti-thrombotic and anti-cancer activities.

Keywords: Cancer invasion, Human oral squamous carcinoma YD-10B cells, *Laetiporus sulphrueus* var. *miniatus*, Matrix metalloproteinase-2/9, Thrombin

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Introduction

Cancer cells have been both directly and indirectly associated with the dysregulation of the blood coagulation cascade [1]. Vascular injury or inflammation may disrupt blood flow, resulting in the formation of a blood clot or thrombus that finally interferes with hemostasis. The thrombus consists of two components, a plug of aggregated platelets and fibrinogen.

Thrombin plays a key role in the activation of the coagulation cascade, and represents, at present, one of the most intriguing targets for the development of new therapeutic agents for the treatment of thrombotic disorders [2]. Currently, several pharmacotherapies (e.g., antiplatelet, antithrombotic, anticoagulant and thrombolytic agents) are approved for the treatment of thrombotic disorders [3-5]. Antithrombotic agents, particularly, are a special focus of interest in treating metastasis. Several studies have reported that thrombin induces tumor cell invasion, which appears to correlate with matrix metalloproteinase-9 (MMP-9) expression [6-8]. Work in C6 glioma cells [6] and U2-OS cells [8] have demonstrated that thrombin-induced invasion and MMP-9 expression are regulated by the PI3K pathway. In addition, upregulation of MMP-9 expression has been reported in invasive human oral squamous cell carcinoma [9, 10].

Redox imbalance of antioxidants, reactive oxygen species (ROS) and reactive nitrogen species (RNS), can contribute to cancer development. At specific levels, ROS acts as a second messenger in certain cellular signaling pathways and plays a critical role in various biological functions such as cell division, aging, survival and death. Thus, at uncontrolled levels, ROS can cause a wide variety of human disorders and diseases, including cancers [11, 12]. Several studies indicate that antioxidants exhibit anti-invasive activity against cancer cells by inhibiting MMP expression [13, 14].

Mushrooms have various physiological properties, including anti-oxidative, anti-inflammatory, anti-thrombotic and anti-cancer effects, as well as promote immune responses. Consequently, its extracts have been the focus of intense research with a view towards developing new functional foods, medicines and cosmetics.

Laetiporus miniatus, an edible medicinal mushroom growing on living or dead oak tree trunks, belongs to the family Polyporaceae in the order Polyporales. Its fruiting bodies have striking golden or yellow color [15, 16]. Previous studies have reported various compounds derived from *L. miniatus*, which include polysaccharides, benzofuran glycoside, acetylenic acid, lanostanoid triterpene and β-1,3-1,4-glucanase [16-19]. Other investigations have shown that extracts and specific fractions obtained from *L. miniatus* have various positive biological and pharmacological activities, including anti-tumor [20-22], anti-inflammatory [23, 24], anti-microbial, and anti-fungal activities. Some of these properties observed were seen with the hexane fraction isolated from *L. miniatus* lipid extracts [25]; while, specifically, anti-oxidant activities were seen with its hot water soluble and hot alkali soluble polysaccharides [26]. In addition, this mushroom is known to act as a chemo-

therapeutic and an immune-modulating agent [25]. However, little is known about the effects of *L. miniatus* in the context of oral cancer migration. Accordingly, the aim of this study was to assess the potential therapeutic and anti-invasive activities of the aqueous and organic solvent extracts from *L. miniatus* on human oral squamous carcinoma cells.

Materials and Methods

Chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH), fibrinogen, thrombin, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Rockville, MD, USA). H-D-phenylalanine-L-pipecolyl-L-arginine-paranitroaniline dihydrochloride was purchased from Chromogenix (Orangeburg, NY, USA).

Preparation of fractionations from a *L. miniatus*

The fruiting bodies of *L. miniatus* were collected from Chiaksan mountain. Five hundred gram of dried whole mushrooms of *L. miniatus* was milled into a powder and extracted with 50% aqueous methanol (5 L) for 3 days at room temperature, filtered and concentrated under reduced pressure. The residue (10 g) was diluted with 300 mL H_2O and then successively partitioned with n-hexane, $CHCl_3$, EtOAc, and n-BuOH (300 mL). Each fraction was evaporated under vacuum and lyophilized with a freeze dryer (Ilshin Lab Co. Ltd., Yangju, Korea) to yield a powder. The yields of fractions were given in Fig. 1. Dimethyl sulfoxide (DMSO) was added to completely dissolve the fractionated extracts and to dilute each extract to a final concentration of 10 mg/mL.

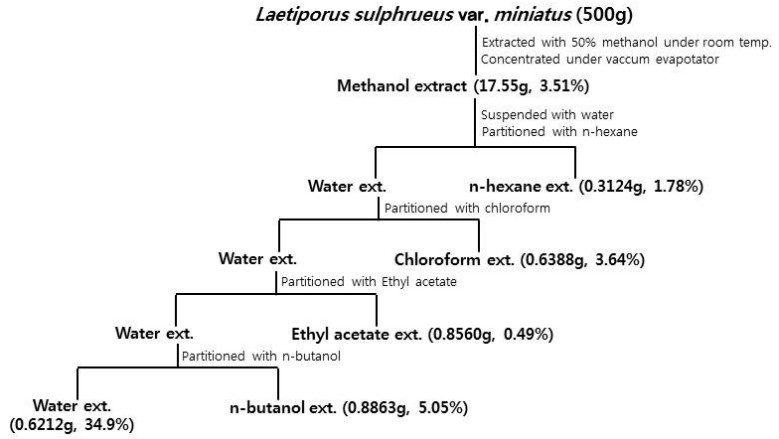

Fig. 1. The fraction yields of *Laetiporus miniatus* extract. Fraction yields were described as the percent of dry substance of fractions based on the dry methanolic extract. The percentage yield of 50% methanolic extract was 3.51% (w/w).

Cell culture

Human oral squamous carcinoma cell line YD-10B was obtained from Korea Cell Line Bank (Seoul, Korea). Cells were grown in RPMI-1640 with 10% Fetal Bovine Serum (Life technologies, Gaithersburg, MD, USA) at 37°C in humidified atmosphere with 5% CO_2 incubator.

Estimation of antioxidant activity

The free radical-scavenging activity was determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) according to the method of Blois [27] and Kim et al. [28]. A total volume of 0.4 mL of each fraction solution and 5.6 mL of 1×10^{-4} M DPPH ethanol solution were mixed. The mixtures were incubated for 10 min at room temperature. The absorbance of each well was measured at 525 nm using UV-vis spectrophotometer (UV-1201; Shimadzu, Kyoto, Japan). Each assay was performed in triplicate and DPPH activity was taken as the average of three independent tests.

DPPH activity was calculated as an inhibition percentage according to the following equation:

$$\text{Scavenging effect (\%)} = \{1 - [A_{525(\text{sample})} / A_{525(\text{Blank})}]\} \times 100$$

$A_{525(\text{Blank})}$ was the absorbance of the control (deionized water, instead of the sample).

Estimation of thrombin inhibitory activity

Thrombin inhibitory activity was measured by the method of Doljak et al.[29]. 50 μL of thrombin solution (0.5 NIH units/mL) was diluted by 40 uL of Hanks' balanced salt solution (HBSA) buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 0.1% bovine serum albumin, pH 7.5). The diluted thrombin solution was mixed with 10 uL of solvent fractions (10 mg/mL) and incubated for 15 min. After 50 μL of 3 mM, H-D-phenylalanine-L-pipecolyl-L-arginine-paranitroaniline Dihydrochloride (substrate) was added to a thrombin mixture, the mixture was incubated for 5 min at room temperature, and the absorbance of each well was measured at 405 nm using UV-vis spectrophotometer (UV-1201, Shimadzu). Thrombin inhibitory activity was calculated as a percentage according to the following equation:

$$\text{Inhibiting ability (\%)} = \{1 - [A_{405(\text{sample})} / A_{405(\text{Control})}]\} \times 100$$

$A_{405(\text{control})}$ was the absorbance of the control (thrombin solution, assay buffer, and substrate).

Cell viability assay

MTS cell proliferation assay was performed using Cell Counting Kit-8. YD-10B (2×10^4) cells were seeded in 96-well plates. After 16 hr incubation, cells were treated with solvent fractions at a various concentrations (0, 20, 40, 80 and 100 μg/mL), and incubated for 48hr. After treatment, CCK-8 solution (10 μL) was added to each well. After the plates were incubated for 3hr at 37°C, the absorbance of each well was measured at 450 nm using spectra Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). Result was expressed as a percentage of growth, with control cells treated with DMSO alone having 100% of growth.

Reverse transcription-polymerase chain reaction (RT-PCR)

TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract total RNA from YD-10B cells. PCR was carried out using ReverTra ACE PCR RT Master Mix Kit containing (Toyobo, Osaka, Japan) 1 μg of total RNA. The samples were processed through 30 cycles consisting of 1 minute at 94°C, 1 minute at 58°C for MMP-2, 58°C for MMP-9, 58°C for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and 1 minute at 72°C. A fragment of the human GAPDH gene amplified as an internal control. The oligodeoxynucleotides were synthesized as specific primers: MMP-2 forward primer, GCGACAAGAAGTATCGCTTC; MMP-2 reverse primer, TGCCAAGGTCAATGTCAGGA; MMP-9 forward primer, CCATTTCGACGATGACGAGTT; MMP-9 reverse primer, CTTGTCGCTGTCAAAGTTCGAG; GAPDH forward primer, GAAGGTGAAGGTCGGAGT; GAPDH reverse primer, GAAGATGGTGATGGGATTTC. Amplified products were electrophoresed on a 1.5% agarose gel containing ethidium bromide, visualized under UV light, and analyzed by use of a densitometer.

Gelatin Zymography

For the gelatinase activity, the supernatants of YD-10B cell cultures underwent electrophoresis on an 8% polyacrylamide gel containing 0.1% (w/v) gelatin (from porcine skin, Type A). The gel was incubated at room temperature for 1hr in the presence of 2.5% triton X-100, 50 mM Tris-Cl pH 7.5, 10 mM $CaCl_2$ and 1.0 uM $ZnCl_2$) and subsequently at 37°C overnight in a buffer containing 10 mM $CaCl_2$, 0.15 M NaCl and 50 mM Tris-Cl (pH 7.5). The gel was then stained for protein with 0.25% Coomassie blue and proteolysis was detected as a white zone within a dark zone. For quantification, gelatin zymograms were using a GelQuant.NET program.

Statistical analysis

Statistical analysis for anti-oxidant and anti-thrombin assay was performed with one-way ANOVA using Instat statistical software (GraphPad Software, Inc., La Jolla, CA, USA).

For the statistical analysis of the cell viability assay, RT-PCR and gelatinase activity were obtained using Student's *t*-test. Data were expressed as the mean ± standard derivation (SD) and considered statistically significant for $p < 0.05$. All results were presented as the average of at least three separate experiments.

Results and Discussion

Anti-oxidative effects of the water extract and solvent fractions from *L. miniatus*

ROS and RNS are reactive chemical species that trigger cell damage, which, in turn, can cause a wide variety of degenerative diseases. However, at a specific concentration, ROS also acts as a second messenger in certain cellular signaling pathways, and plays an important role in various biological functions such as cell division, aging, survival and death. Thus, when ROS reaches uncontrolled levels, it is can cause a wide variety of human disorders and diseases, including cancers [11, 12]. It is also known that this mushroom possesses antioxidant properties, with components harboring vitamin C, vitamin E and polyphenol compounds, for example [30]. Therefore, we investigated whether the water extract and solvent fractions of *L. miniatus* could function as potential preventive agents of various diseases by measuring their anti-oxidative effects by examining DPPH radical scavenging. As shown in Fig. 2, the highest activity was observed in the butanol fraction (81.44%), followed by the ethyl acetate fraction (77.70%); those of water, chloroform and hexane fractions were 39.82%, 25.52%, and 9.86%, respectively. These results suggest that the butanol and ethyl acetate fractions of *L. miniatus* might serve as potential antioxidant sources.

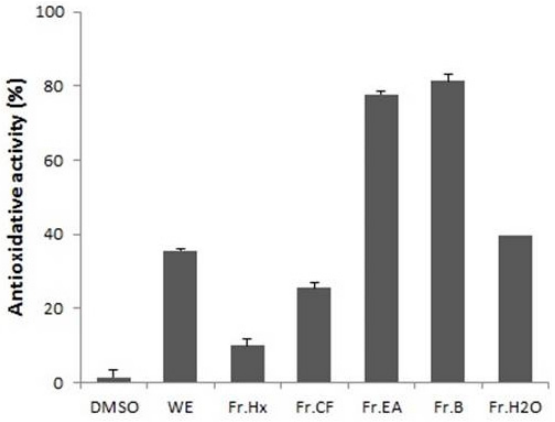

Fig. 2. Anti-oxidative activities of solvent fractions obtained from *Laetiporus miniatus* by DPPH assay (F-value 1178.59; $p < 0.001$). DMSO, dimethyl sulfoxide; WE, water extract; Fr.Hx, hexane fraction; Fr.CF, chloroform fraction; Fr.EA, ethyl acetate fraction; Fr.B, butanol fraction; Fr.H_2O, H_2O fraction; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

Thrombin inhibition effects of the water extract and solvent fractions from *L. miniatus*

Thrombin, which plays a critical role in blood coagulation, catalyzes the conversion of fibrinogen into fibrin, which, in turn, leads to the formation of a blood clot. Recently, there has been greater progress made towards the development of new therapeutic drugs, derived from medicinal foods that present with little side effects, for the treatment of thrombotic disorders [31, 32]. In this study, we sought to investigate whether the water extract and solvent fractions of *L. miniatus* could function as potential preventive agents of vascular disease by measuring its antithrombotic activity. As shown in Fig. 3, the highest inhibition (94.64%) was observed with the water extract, followed by the water fraction (at 90.10% of inhibition), with the fractions of butanol, chloroform, ethyl acetate and hexane fraction exhibiting 89.41%, 73.44%, 69.14%, and 64.97%, inhibition, respectively. Of note, these results corresponded to previous studies, where thrombin inhibition was significantly higher in the butanol fractions and organic solvent factions [33], suggesting that the fractions of *L. miniatus* might be evaluated for the treatment of thrombotic diseases and potentially used as a thrombin inhibitor.

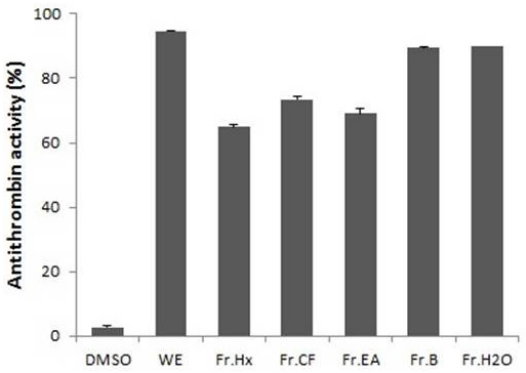

Fig. 3. Thrombin inhibitory activities of solvent fractions obtained from *Laetiporus miniatus* (F-value 689.46; $p < 0.001$). DMSO, dimethyl sulfoxide; WE, water extract; Fr.Hx, hexane fraction; Fr.CF, chloroform fraction; Fr.EA, ethyl acetate fraction; Fr.B, butanol fraction; Fr. H_2O, H_2O fraction.

Cell viability effects of the water extract and solvent fractions from the *L. miniatus*

To determine whether the cytotoxic effects of the water extract and solvent fractions from *L. miniatus* was attributable directly to cell death, the MTS cell proliferation assay was performed using the human oral squamous carcinoma cell line YD-10B (Fig. 4). Cells were treated with different concentrations (0, 20, 40, 80 and 100 μg/mL) of water extract and solvent fractions from *L. miniatus* for 48hr. At the high concentration of 100 μg/mL (as compared to control), cell viability effects of the water extract, ethyl acetate, butanol and water fractions remained constant with values of 88.01%, 95.84%, 92.18% and 83.02%,

respectively; but major changes were observed with the hexane and chloroform fractions (Fig. 3). No significant cytotoxic effects were observed at concentrations of up to 40 μg/mL from the water extract nor the solvent fractions of *L. miniatus*.

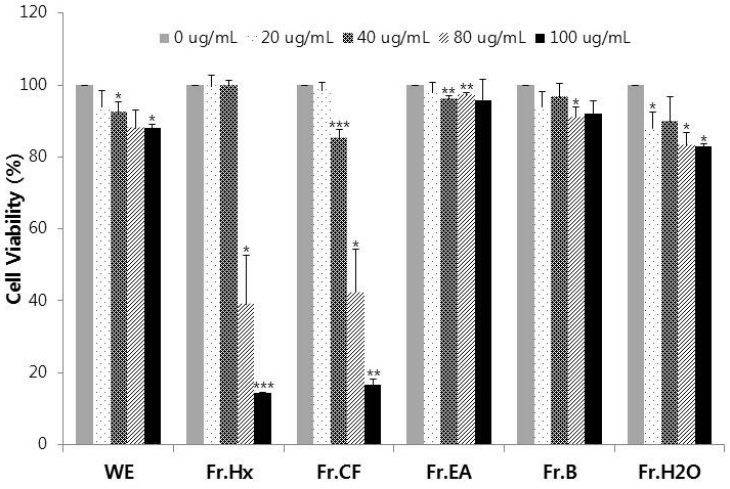

Fig. 4. In vitro cytotoxicity effects of solvent fractions obtained from *Laetiporus miniatus* in YD-10B cells. YD-10B cells were treated with solvent fractions obtained from *L. miniatus* at different concentrations for 48 hr. Data represent the mean ± SD of three independent experiments. WE, water extract; Fr.Hx, hexane fraction; Fr.CF, chloroform fraction; Fr.EA, ethyl acetate fraction; Fr.B, butanol fraction; Fr.H_2O, H_2O fraction.

Anti-thrombin effects of the water extract and solvent fractions from *L. miniatus* in YD-10B oral cancer cells

YD-10B cells demonstrate highly invasive activity through the collagen or Matrigel-coated transwell, both *in vitro* three-dimensional culture and *in vivo* tumorigenicity [34]. Many reports suggest that thrombin regulates the gelatinolytic activity of MMP-2 and MMP-9 in cancer cell invasion [7-10, 35]. However, the effect of thrombin on human oral squamous carcinoma cells is not fully understood. Thus, in this study we sought to evaluate whether the effects of the water extract and solvent fractions from *L. miniatus* are due to the regulation of thrombin-treated invasion. YD-10B cells were treated with different concentrations (0, 2.5, 3.5 and 4.5 U/mL) of thrombin for 24h in serum-free media. The YD-10B cells showed markedly low MMP-9 expression, with MMP-2 expression relatively higher than that of MMP-9. Additionally, while stimulation with thrombin (3.5 U/mL) led to a significant increase of MMP-9 expression, the expression of MMP-2 revealed no significant induction (Fig. 5).

PMA is widely used as a tumor promoter for the stimulation of cell invasion in various types of cancer cells [36-38]. In PMA-treated YD-10B cells, mRNA expression and protein activation of MMP-2/-9 were highly inhibited in the hexane and chloroform fractions (Fig. 6). When MMP-2 and MMP-9 mRNA expressions were analyzed in thrombin-treated

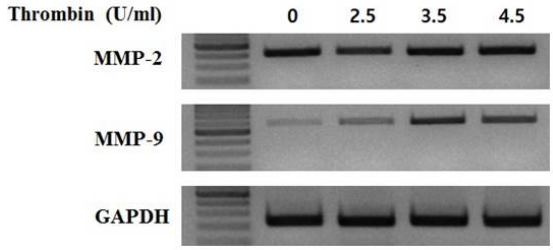

Fig. 5. Thrombin effects on mRNA expressions of MMP-2/9 in YD-10B cells. YD-10B cells were treated with thrombin at different concentrations for 24 hr. MMP, matrix metalloproteinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

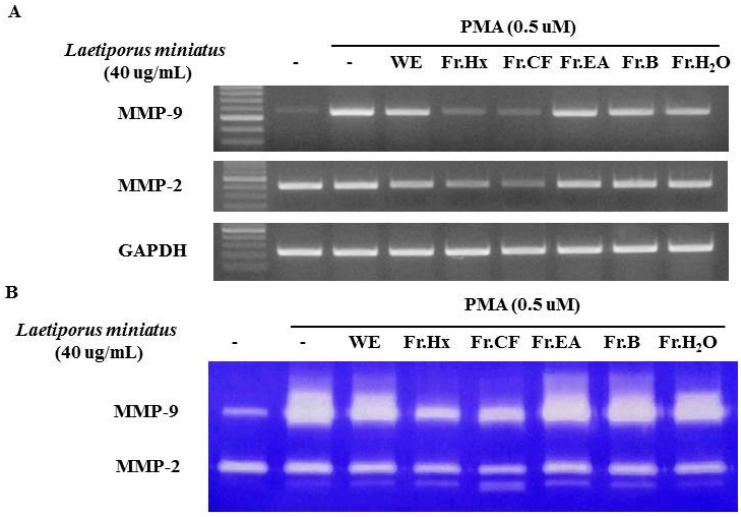

Fig. 6. Effect of solvent fractions obtained from *Laetiporus miniatus* on mRNA expressions and protein activities of MMP-2/9 in PMA-treated YD-10B cells. YD-10B cells were treated with the indicated concentration (40 μg/mL) of solvent fractions obtained from *L. miniatus* 2 hr prior to PMA (0.5 uM) stimulation. (A) 24 hr later, the levels of MMP-2/9 mRNA were determined by RT-PCR. GAPDH were used as the internal control. The relative expressions of MMP-2/9 mRNA were analyzed the band intensity using a GelQuant.NET program; (B) Activities of MMP-2/9 protein in the conditioned media were determined by gelatin zymography. The relative invasion activity of MMP-2/9 mRNA were analyzed the band intensity using a GelQuant.NET program. WE, water extract; Fr.Hx, hexane fraction; Fr.CF, chloroform fraction; Fr.EA, ethyl acetate fraction; Fr.B, butanol fraction; Fr.H_2O, H_2O fraction; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

YD-10B cells, only MMP-9 mRNA demonstrated significant induction by thrombin (3.5 U/mL). Specifically, upon stimulation with thrombin, the expression of MMP-9 mRNA increased 13-fold. More importantly, the mRNA expression of MMP-9 was markedly inhibited (by 50%) by the hexane and chloroform fractions, whereas activation of MMP-2 was only mildly inhibited (Fig. 7A). Protein activation of MMP-2 and MMP-9 in thrombin-treated YD-10B cells was confirmed by gelatin zymography (Fig. 7B). There was no significant difference in protein levels of MMP-2 and MMP-9 when induced by thrombin.

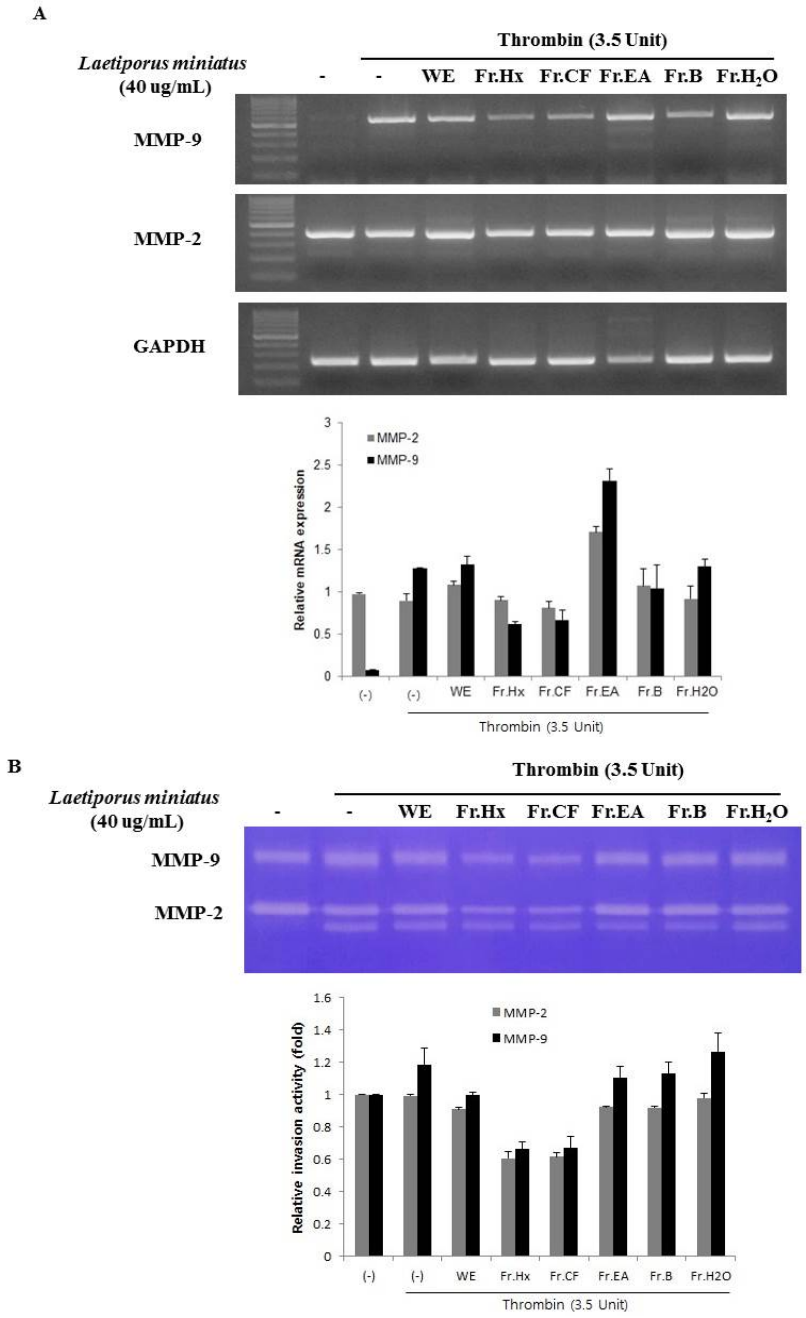

Fig. 7. Effect of solvent fractions obtained from *Laetiporus miniatus* on mRNA expressions and protein activities of MMP-2/9 in thrombin-treated YD-10B cells. YD-10B cells were treated with the indicated concentration (40 μg/mL) of solvent fractions obtained from *L. miniatus* 2 hr prior to thrombin (3.5 uM) stimulation. (A) 24 hr later, the levels of MMP-2/9 mRNA were determined by RT-PCR. GAPDH were used as the internal control. The relative expressions of MMP-2/9 mRNA were analyzed the band intensity using a GelQuant.NET program; (B) Activities of MMP-2/9 protein in the conditioned media were determined by gelatin zymography. The relative invasion activity of MMP-2/9 mRNA were analyzed the band intensity using a GelQuant.NET program. WE, water extract; Fr.Hx, hexane fraction; Fr.CF, chloroform fraction; Fr.EA, ethyl acetate fraction; Fr.B, butanol fraction; Fr.H_2O, H_2O fraction; MMP, matrix metalloproteinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Whereas, the hexane and chloroform fractions of *L. miniatus* were inhibited by 50%, MMP-2 and MMP-9 activations compared to the thrombin only treated group. This finding may lend further support to previous studies [20, 23], where acetyl eburicoic acid derived from the fruiting bodies of *L. miniatus* demonstrated potent anti-cancer effects on HL-60 human myeloid leukemia cells, resulting from apoptosis activation. Therefore, we suggest that the hexane and chloroform fractions of *L. miniatus* might serve as potential anti-invasive agents in oral cancer, although further experiments will need to be carried out in order to identify the active compounds present within the hexane and chloroform fractions of *L. miniatus*.

REFERENCES

1. Kakar P, Lip GY. Hypertension: endothelial dysfunction, the prothrombotic state and antithrombotic state and antithrombotic therapy. Expert Rev Cardiovasc Ther 2007;5: 441-50.
2. Turpie AG. Oral, direct factor Xa inhibitors in development for the prevention and treatment of thromboembolic diseases. Arterioscler Thromb Vasc Biol 2007;27:1238-47.
3. Meadows TA, Bhatt DL. Clinical aspects of platelet inhibitors and thrombus formation. Circ Res 2007;100:1261-75.
4. Hirsh J, O'Donnell M, Eikelboom JW. Beyond unfractionated heparin and warfarin: current and future advances. Circulation 2007;116:552-60.
5. Hart RG, Benavente O, McBride R, Pearce LA. Antithrombotic therapy to prevent stroke in patients with atrial fibrillation: a meta-analysis. Ann Intern Med 1999;131: 492-501.
6. Kim J, Lee JW, Kim SI, Choi YJ, Lee WK, Jeong MJ, Cha SH, Lee HJ, Chun W, Kim SS. Thrombin-induced migration and matrix metalloproteinase-9 expression are regulated by MAPK and PI3K Pathways in C6 glioma cells. Korean J Physiol Pharmacol 2011; 15:211-6.
7. Choi MS, Kim YE, Lee WJ, Choi JW, Park GH, Kim SD, Jeon SJ, Go HS, Shin SM, Kim WK, et al. Activation of protease-activated receptor 1 mediates induction of matrix metalloproteinase-9 by thrombin in rat primary astrocytes. Brain Res Bull 2008;76: 368-75.
8. Radjabi AR, Sawada K, Jagadeeswaran S, Eichbichler A, Kenny HA, Montag A, Bruno K, Lengyel E. Thrombin induces tumor invasion through the induction and association of matrix metalloproteinase-9 and beta-1 integrin on the cell surface. J Biol Chem 2008; 283:2822-34.
9. Juarez J, Clayman G, Nakajima M, Tanabe KK, Saya H, Nicolson GL, Boyd D. Role and regulation of expression of 92-kDa type-IV collagenase (MMP-9) in 2 invasive squamous cell carcinoma cell lines of the oral cavity. Int J Cancer 1993;55:10-8.
10. Chiang YY, Tsai MH, Lin TY, Chiang IP. Expression proprofile of metastasis-related genes in invasive oral cancers. Histol Histopathol 2008;23:1213-22.
11. Wink DA, Hines HB, Cheng RY, Switzer CH, Flores-Santana W, Vitek MP, Ridnour LA, Colton CA. Nitric oxide and redox mechanism in the immune response. J Leukoc

Biol 2011;89:873-91.

12. Williams MS, Kwon J. T cell receptor stimulation, reactive oxygen species, and cell signaling. Free Radic Biol Med 2004;37:1144-51.
13. Nam H, Kim MM. Eugenol with antioxidant activity inhibits MMP-9 related to metastasis in human fibrosarcoma cells. Food Chem Toxicol 2013;55:106-12.
14. Gweon EJ, Kim SJ. Resveratrol induces MMP-9 and cell migration via the p38 kinase and PI-3K pathways in HT1080 human fibrosarcoma cells. Oncol Rep 2013;29:826-34.
15. Overeem C van. *Laetiporus miniatus* (Jungh.) Icon Fung Malay1925;12:1.
16. Imazeki R, Hongo T. Colored llustrations of mushrooms of Japan. Vol. II. Osaka: Hoikusha;1989.
17. Hong MR, Kim YS, Joo AR, Lee JK, Kim YS, Oh DK. Purification and characterization of a thermostable beta-1,3-1,4-glucanase from *Laetiporus sulphureus* var. *miniatus.* J Microbiol Biotechnol 2009;19:818-22.
18. Hwang HS, Lee SH, Baek YM, Kim SW, Jeong YK, Yun JW. Production of extracellular polysaccharides by submerged mycelial culture of *Laetiporus sulphureus* var. *miniatus* and their insulinotropic properties. Appl Microbiol Biotechnol 2008;78:419-29.
19. Yoshikawa K, Bando S, Arihara S, Matsumura E, Katayama S. A benzofuran glycoside and an acetylenic acid from the fungus *Laetiporus sulphureus* var. *minatus.* Chem Pharm Bull (Tokyo) 2001;49:327-9.
20. León F, Quintana J, Rivera A, Estévez F, Bermejo J. Lanostanoid tripenes from *Laetiporus sulphureus* and apoptosis induction on HL-60 human myeloid leukemia cells. J Nat Prod 2004;67:2008-11.
21. He JB, Tao J, Miao XS, Bu W, Zhang S, Dong ZJ, Li ZH, Feng T, Liu JK. Seven new drimane-type sesquiterpenoids from cultures of fungus *Laetiporus sulphureus.* Fitoterapia 2015;102:1-6.
22. Zhang JW, Wen GL, Zhang L, Duan DM, Ren ZH. Sulphureuine B, a drimane type sesquiterpenoid isolated from *Laetiporus sulphureus* induces apoptosis in glioma cells. Bangladesh J Pharmacol 2015;10:844-53.
23. Saba E, Son Y, Jeon BR, Kim SE, Lee IK, Yun BS, Rhee MH. Acetyl eburicoic acid from *Laetiporus sulphureus* var. *minatus* suppresses inflammation in murine macrophage RAW 264.7 cells. Mycology 2015;43:131-6.
24. Oh SH, Choi SY, Lee NR, Lee JN, Kim DS, Lee SH, Park SM. Cell migration and anti-inflammatory effect of Red Ginseng extracts fermented with *Laetiporus sulphureus.* J Soc Cosmet Sci Korea 2014;40:297-305.
25. Sinanoglou VJ, Zoumpoulakis P, Heropoulos G, Proestos C, Ćirić A, Petrovic J, Glamoclija J, Sokovic M. Lipid and fatty acid profile of the edible fugus *Laetiporus sulphurous.*Antifungal and antibacterial properties. J Food Sci Technol 2015;52:3264-72.
26. Klaus A, Kozarski M, Niksic M, Jakovljevic D, Todorovic N, Stefanoska I, Van Griensven LJ. The edible mushroom *Laetiporus sulphureus* as potential source of natural antioxidants. Int J Food Sci Nutr 2013;64:599-610.
27. Blois MS. Antioxidant determinations by the use of a stale free radical. Nature 1958; 181:1199-200.
28. Kim YJ, Kim CK, Kwon YJ. Isolation of antioxidative components of *Perillae semen.*

Korean J Food Sci Technol 1997;29:38-43.

29. Doljak B, Stegnar M, Urleb U, Kreft S, Umek A, Ciglaric M, Strukelj B, Popovic T. Screening for selective thrombin inhibitor in mushrooms. Blood Coagul Fibrinolysis 2001;12:123-8.
30. Kozarski M, Klaus A, Jakovljevic D, Todorovic N, Vunduk J, Petrovic P, Niksic M, Vrvic MM, Griensven LV. Antioxidants of edible mushrooms. Molecules 2015;20: 19489-525.
31. Yun YP, Do JH, Ko SR, Ryu SY, Kim JH, Song HC, Park YD, Ahn KS, Kim SH. Effects of Korean red ginseng and its mixed prescription on the high molecular weight dextran-induced blood stasis in rats and human platelet aggregation. J Ethnopharmacol 2001;77:259-64.
32. Vitseva O, Varghese S, Chakrabarti S, Folts JD, Freedman JE. Grape seed and skin extracts inhibit platelet function and release of reactive oxygen intermediates. J Cardiovasc Pharmacol 2005;46:445-51.
33. Kim KH, Lee HJ, Lee JH, Jang YS, Kim DK, Shim BS, Cho KH, Ko SG, Ahn KS, Kim SH. Blockade of glycoprotein IIb/IIIa mediates the antithrombotic activity of butanol fraction of *Actinostemma lobatum* Maxim. J Ethnopharmacol 2008;116:431-8.
34. Kim EJ, Che ZM, Park YJ, Hwang YS, Kim KY, Jung DW, Jeon NK, Choi YW, Lee EJ, Kim J. Morphogenesis and biological significance of spindle cell transformation in a spindle cell carcinoma. Cancer Lett 2009;275:61-71.
35. Koo BH, Park MY, Jeon OH, Kim DS. Regulatory mechanism of matrix metalloproteinase-2 enzymatic activity by factor Xa and thrombin. J Biol Chem 2009;284: 23375-85.
36. Hwang YS, Park KK,Chung WY. Kalopanaxsaponin a inhibits the invasion of human oral squamous cell carcinoma by reducing metalloproteinase-9 mRNA stability and protein trafficking. Biol Pharm Bull 2012;35:289-300.
37. Weng CJ, Chau CF, Hsieh YS, Yang SF, Yen GC. Lucidenic acid inhibits PMA-induced invasion of human hepatoma cells through inactivating MAPK/ERK signal transduction pathway and reducing binding activities of NF-kappaB and AP-1. Carcinogenesis 2008; 29:147-56.
38. Park SK, Hwang YS, Park KK, Park HJ, Seo JY, Chung WY. Kalopanaxsaponin a inhibits PMA-induced invasion by reducing metalloproteinase-9 via PI3K/Akt- and PKCdelta-mediated signaling in MCF-7 human breast cancer cells. Carcinogenesis 2009;30:1225-33.