J Food Sci Nutr
Vol 11, p 191~197 (2006)

J Food Science and Nutrition

Inhibitory Effect of Methanol Extract of *Magnolia officinalis* on Matrix Metalloproteinase-2

Dong-Yup Lee[1], Cheorl-Ho Kim[2] and Dong-Soo Kim[1†]

[1]*Department of Food Science and Technology, Kyungsung Universirty, Busan 608-736, Korea*
[2]*Department of Life Science, Sungkyunkwan University, Gyeonggi 440-746, Korea*

Abstract

Matrix metalloproteinase-2 (MMP-2) is a key enzyme involved in tumor invasiveness. The plant of *Magnolia officinalis* Rehd. et Wils. is often included as an ingredient in various herbal remedies recommended for cancer theraphies in Korea. Various extracts prepared from stems of *M. officinalis* were tested for cytotoxic activity on human hepatocellular carcinoma cell line, SK-Hep cells using the XTT assay method. Then, the inhibitory effect was examined on MMP-2 activity using gelatin zymography. Methanol (MeOH) extract of *M. officinalis* caused the strongest inhibition of the MMP-2 activity, as measured by gelatin zymography method for enzyme activity. IC_{50} values of fractions on MMP-2 activity were in a range of 4.9~11.3 μg/mL. Among each fraction, butanol and ethylacetate (EtOAc) fractions showed the strong inhibitory activities (IC_{50}=10.7 and 4.9 μg/mL, respectively). When the *M. officinalis*'s constituents such as magnolol, honokiol, (-)-epigallocatechin gallate (EGCG) and ovovatol were examined for inhibitory effects on MMP-2 activity, EGCG showed strong inhibitory activity. However, MeOH extract of *M. officinalis* was dose-dependently inhibited to MMP-2 activity. The MeOH extract, hexane and EtOAc fractions (IC_{50} of >200 μg/mL) exhibited weak cytotoxicity activity, while butanol (IC_{50}=80 μg/mL) and chloroform fractions (IC_{50}=90 μg/mL) exhibited relatively strong cytotoxic activity. From these results, *M. officinalis* could be suitable for cancer treatment and chemopreventive drugs.

Key words: *Magnolia officinalis* Rehd. et Wils, human hepatocellular carcinoma, matrix metalloproteinase-2, MeOH extract, epigallocatechin gallate, cancer chemopreventive drugs

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that degrade specific components of the extracellular matrix. At present, they are known to include at least 20 enzymes and are classified into five groups according to their domain structure and substrate specificity: collagenases, gelatinases, stromelysins, membrane-type MMPs and others (1). Among MMPs, gelatinases (MMP-2 and MMP-9), which are able to degrade type IV collagen (a major component of the basement membrane), have been studied extensively and have been shown to be involved in tumor invasiveness, metastasis, and angiogenesis (2,3). The 72-kDa gelatinase A (MMP-2) is the most widely distributed of all the MMPs. MMP-2 has been found in a variety of malignant tumors, including breast, lung, gastric, and esophageal carcinomas (4-7). Based on these observations, MMPs have become attractive targets for development of anticancer drugs, and much attention has been directed to the design and synthesis of the effective MMP inhibitors over the last decade (8). In fact, some of promising MMP inhibitors, such as marimastat, AG3340, and CGS-27023A, are currently used in clinical trials (9-11).

Recently, we have found that *Euonymus alatus* and *Magnolia officinalis* Rehd. et Wils. have anti-cancer activities (12). Especially, *M. officinalis* has been used as a traditional medicine for curing gastric ulcer and stomach cancer and the components of which have been investigated, and magnolol, honokiol and ovovatol are well known (13-18).

Anticancer properties have been associated with the components of various natural products including green tea polyphenols, resveratrol, limonene and organosulfur compounds from garlic (19,20). Therefore, we investigated inhibitory activity of MMP-2 by the methanol extract containing several fractions from *M. officinalis*. The aim of this study was to investigate the effects of *M. officinalis* on MMP-2 activity.

†Corresponding author. E-mail: kdskim@ks.ac.kr
Phone: +82-51-620-4714, Fax: +82-51-622-4986

MATERIALS AND METHODS

Processing of the plant material and isolation of compounds

The stems of *M. officinalis* Rehd. et Wils. were collected in Kyungju city, the Republic of Korea, and the sample and voucher specimen (number 4-93-761) are kept in the herbarium of the College of Oriental Medicine, Dongguk University. The dried plant samples were extracted 3 times with methanol at 70°C for 5 hr. The extracts were filtered through a 0.45 μm filter and lyophilized. The w/w yield of the methanol extracts was about 2.25%. The methanol extract (100 g) was suspended in water (500 mL) and successively reextracted by 500 mL each (3 times) of hexane (yield: 11.7 g), chloroform (yield: 27.8 g) and ethyl acetate (yield: 25.5 g) and butanol (yield: 7.79 g). All fractions including the final remaining water fraction (yield: 30.8 g) were concentrated under reduced pressure using a rotary evaporator and then freeze-dried. For the bioassay test, samples were dissolved in dimethyl sulfoxide (DMSO) and further diluted in incubation buffer. Components such as magnolol, honokiol, (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG) were extracted from magnolia bark according to the method of Fujita et al. (21). The yield of each compound was below 1 mg/100 g raw plant materials. The drugs were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 40 mM.

Cell culture

SK-Hep1, human hepatocellular carcinoma cell line, was obtained from the Korean cell line bank. These cell lines were grown in Dulbecco modified Eagle medium (DMEM) containing 100 U of penicillin per mL, 100 μg of streptomycin per mL, and 10% fetal bovine serum at 37°C in 5% CO_2-air. Cells were grown to sub-confluence and were rinsed with phosphate-buffered saline (PBS) and then incubated in serum-free medium for 24 hr. The serum-free medium contained gelatinase such as MMP-9. The amount of gelatinase in the conditioned media were estimated and quantified by cell numbers.

XTT proliferation assay

The effect of the extracts from *M. officinalis* Rehd. et Wils. on proliferation of SK-Hep1 was investigated using a commercially available proliferation kit (XTT II, Boehringer Mannheim, Mannheim, Germany). Briefly, the cells were plated in 96-well culture plates at a density of 10,000 cells per well in DMEM culture medium, adding the XTT reagent and incubation for 2~24 hr. During incubation an orange color was formed, the intensity of which could be measured and quantitated with a spectrophotometer, in this instance with an ELISA reader.

The extracts were added to various final concentrations (and control: 0 μg/mL) in triplicates. After 72 hr of culture, 50 mL of XTT reaction solution (sodium 3'-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate; mixed in proportion 50:1) was added to the wells. The optical density was read at 490 nm wavelength in an ELISA plate reader after 4 hr incubation of the plates with XTT in an incubator (37°C and 5% CO_2+95% air). All determinations were confirmed using replication in at least three identical experiments. The data shown are for only one experiment, but representative for all replications.

Cell proliferation and [^{3}H] thymidine incorporation

Cells were plated at a density of 1×10^6 cells/well in 6 well plates. After 24 hours, the cells were treated with MeOH extract dissolved in DMEM at final concentrations of 1 to 100 mg/mL for 2 hours, and then cell were labeled with [^{3}H] thymidine at 1 mCi/mL during 20 hours. And then thymidine incorportion was measured as described by Nebigil and Malik (22). All determinations were confirmed using replication in at least three identical experiments. The data shown are for only one experiment, but representative for all replications.

MMP-2 inhibition assay

Each fraction and extracts were used for MMP-2 inhibition assay. MMP-2 activity was monitored according to a previously published fluorescence assay with slight modifications (23). A mixture of 30 μL of enzyme solution (0.5 unit/mL) and 2 μL of each fraction dissolved in DMSO were incubated with 366 μL of buffer (50 mM Tris·Cl, 10 mM $CaCl_2$, 0.2 M NaCl, 0.02% (w/v) NaN_3, 0.05% (w/v) Brij·35, pH 7.5) at 37°C for 60 min. A solution of 2 μL of MOCAc-Pro-Leu-Gly-Leu-A2pr (Dnp)-Ala-Arg-NH_2 dissolved in DMSO was added to the solution, and the reaction was performed at 42°C for 3 hr. The reaction was quenched with 500 μL of acetic acid, followed by measurement of fluorescence intensity (excitation 328 nm, emission 393 nm) of the solution. Percentage of inhibition was calculated from the difference of fluorescence, subtracted the blank value, between the presence and absence of inhibitor (fractions). Each inhibitor concentration was run 2~4 times. The IC_{50} was calculated from a least-squares fit of the percent inhibition and inhibitor concentration using a 4-parametric function.

Gelatin zymography assay

Zymography was performed as described previously (24). Culture supernatants were resuspended in a sample

buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.00625% (w/v) bromophenol blue and loaded without boiling in 7.5% acrylamide/bisacrylamide (29.2:0.8) separating gel containing 0.1% (w/v) gelatin. Electrophoresis was carried out at a constant voltage of 100 V. After electrophoresis, the gels were soaked in 0.25% Triton X-100 (2×30 min) at room temperature and rinsed in NanoPure water. For inhibitory effect of all fractions on gelatinolytic activity of MMP-2, each fraction was freshly solubilized in the Tris-HCl buffer used for incubation of gel; the gel slab was cut into slices corresponding to the lanes and then, put in different tanks containing the stated concentrations of the fractions. The gel slab containing gelatin was incubated at 37°C for 20 hr in the incubation buffer containing 50 mM Tris-HCl (pH 7.6), 20 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij-58 with or without 50 µg/mL of each fractions from *M. officinalis*. The gel was then stained for 15~30 min in 0.1% (w/v) Coomassie blue R-250 in 30% methanol and 10% acetic acid, and destained in the same solution without the Coomassie blue dye. Proteolysis was detected as a white zone in a dark field.

Densitometric and statistical analysis

The intensity of the bands obtained from zymogram studies was estimated with Gel-Print System (Core Bio Corp., Seoul, Korea). The values are calculated by percent of control and expressed as means±SE.

RESULTS AND DISCUSSION

Extraction and fractionation of the plant material

The stems of *M. officinalis* Rehd. et Wils. were extracted with methanol (MeOH), as illustrated in Fig. 1. The yield of the MeOH extracts was about 2.25%. The MeOH extract (100 g) was reextracted by hexane (yield: 11.7 g), chloroform ($CHCl_3$, yield: 27.8 g) and ethyl acetate (EtOAc, yield: 25.5 g) and butanol (BuOH, yield: 7.79 g). All fractions including the final remaining water fraction (yield: 30.8 g) dissolved in DMSO were used.

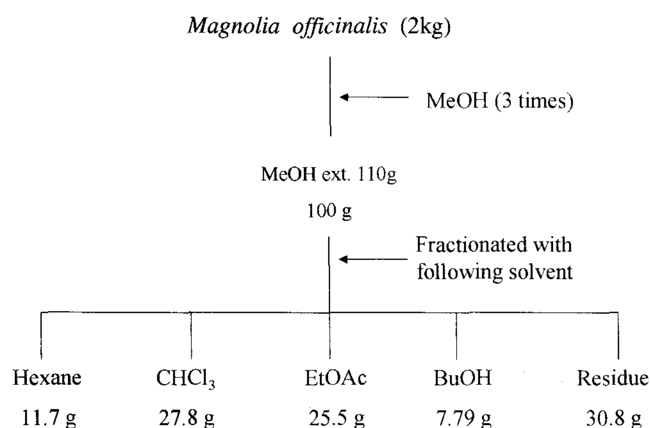

Fig. 1. Schematic extraction procedure.

Cytotoxicities of each fraction and the methanol extract against SK-Hep1 cells

The cytotoxic effects of the MeOH extract, BuOH fraction, $CHCl_3$ fraction, EtOAc and hexane fraction at various concentrations were evaluated in a standardized *in vitro* cytotoxicity assay against the following human hepatocellular carcinoma cell line, SK-Hep1 cells. Compounds concentrations of 0, 1, 10, 50, 100, 500, and 1,000 µg/mL were incubated for 24 hr in cultures in 96-well microplates (volume 100 mL/well). After 24 hr the number of cells surviving was determined quantitatively using a colorimetric XTT assay in a microtiter plate reader. Although all fractions and the methanol extract had weak cytotoxic activity, typical concentration dependent cytotoxic effect of these was detected against SK-Hep1 cells. Dose-dependent cytotoxic effect of each fraction and the methanol extract against SK-Hep1 cells is shown in Fig. 2. They had weak cytotoxic activity in typical concentration. The hexane, EtOAc fractions and the MeOH extract exhibited weak cytotoxic activity (IC_{50} of >200 µg/mL). However, the BuOH (IC_{50}=80 µg/mL) and $CHCl_3$ fractions (IC_{50}=90 µg/mL) exhibited relatively higher cytotoxic activity (Table 1A).

Inhibition of each fraction and the methanol extract on MMP-2 activity by zymography

We investigated the effects on the gelatinolytic activity of MMP-2 of haxane, EtOAc, $CHCl_3$ and BuOH fractions from the methanol extract at 50 µg/mL each, to examine

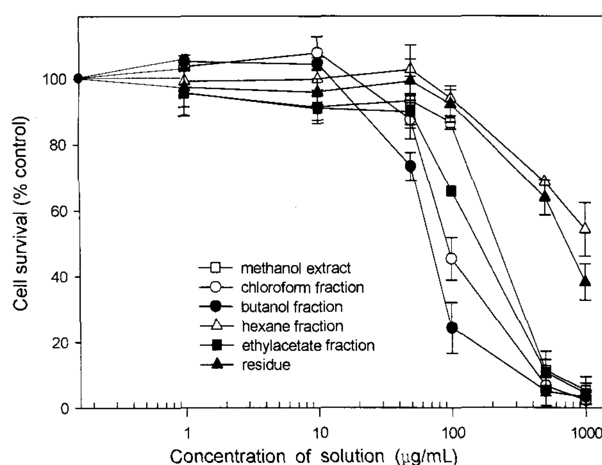

Fig. 2. Effects of methanol extract and further fractions of *M. officinalis* on SK-Hep1 cell growth.
Cell viability in the presence or absence of the methanol extract (□) or butanol (●), chloroform (○), ethyl acetate (■), hexane (△) and residue (▲) fractions are measured as described in the Material and Methods section. Data points are mean values and SE obtained from three replicates.

Table 1. Cytotoxic and MMP-2 inhibitory activities of the various fractions

A) Cytotoxicities

Fraction	IC_{50} (μg/mL)
MeOH extract	>100
Hexane	>100
EtOAc	>100
$CHCl_3$	90
BuOH	80
Residue	>100

B) IC_{50} values for MMP-2 inhibition

Fraction	IC_{50} (μg/mL)
MeOH extract	11.3
EtOAc	4.9
BuOH	10.7

whether the medicinal extracts of *M. officinalis* have inhibitory activity on MMP-2 enzyme activity or not. The water soluble residue and MeOH-extracts were subjected to MMP-2 zymographic determination using SDS-PAGE.

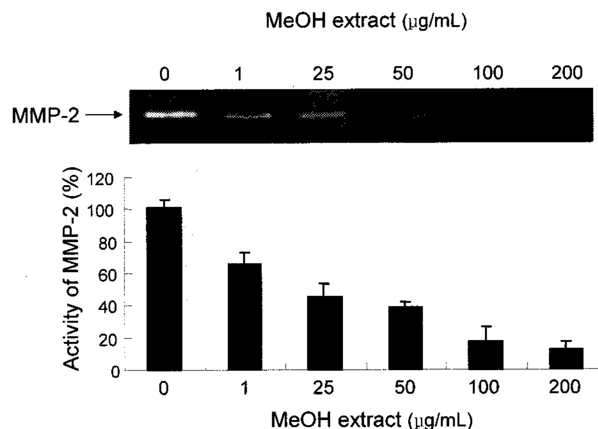

Fig. 3. Effect of MeOH extract on the activity of MMP-2 by gelatin zymography. Zymography was performed on conditioned media of SK-Hepl cells in the presence of increasing concentrations of MeOH fraction. The experiments were repeated 3 times and resulted in a similar outcome.

As shown in Fig. 3, MeOH extract showed a dose-dependent inhibition on MMP-2 activity, while the residue

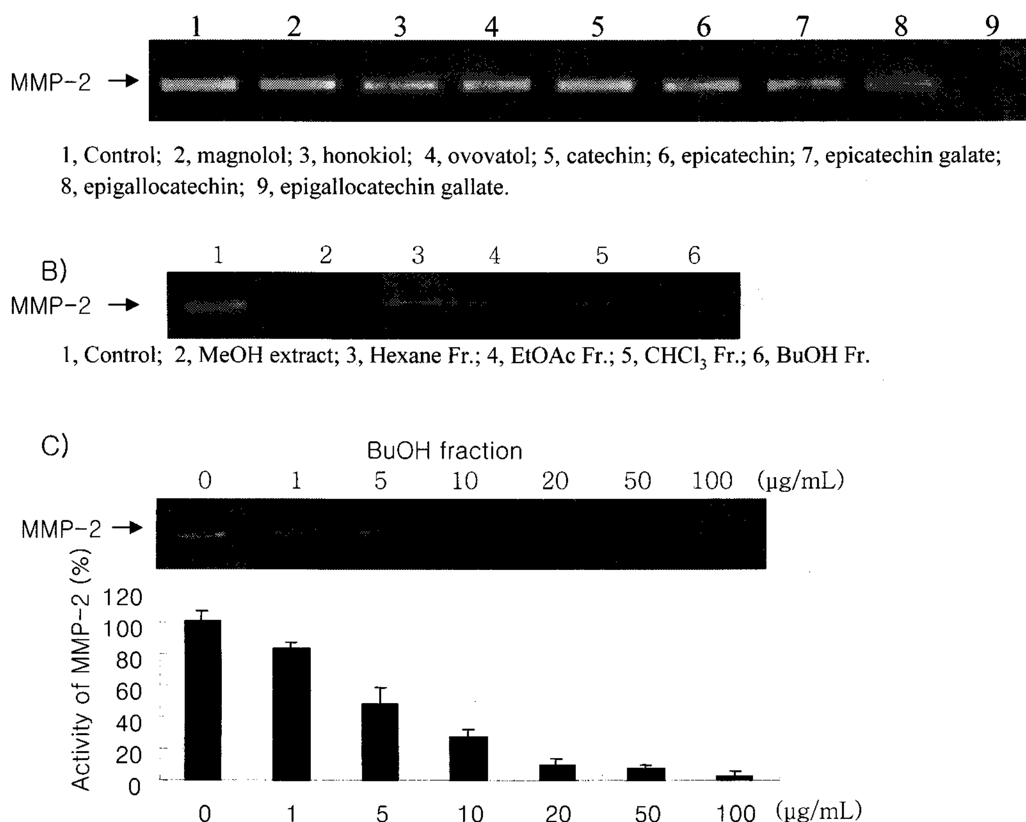

Fig. 4. Zymography of MMP-2 in the presence of various extracts, fractions and compounds. A) Gelatin zymography of conditioned media of SK-Hepl cells incubated with or without 50 μg/mL of magnolol (lane 2), honokiol (lane 3), ovovatol (lane 4), catechin (lane 5), epicatechin (lane 6), epicatechin gallate (lane 7), epigallocatechin (lane 8), epigallocatechin gallate (lane 9) and residue fraction of *M. officinalis* in the presence of 0.1% DMSO for the control (lane 1) for 18 hr. B) Gelatin zymography of conditioned media of SK-Hepl cells incubated with or without 50 μg/mL of methanol extract (lane 2), hexane (lane 3), ethyl acetate (lane 4), chloroform (lane 5), butanol fraction (lane 6) and residue fraction of *M. officinalis* in the presence of 0.1% DMSO for the control (lane 1) for 18 hr. C) Effect of BuOH fraction on the activity of MMP-2 of conditioned media SK-Hepl cells in the presence of increasing concentrations of BuOH fraction. The experiments were repeated 3 times and resulted in a similar outcome.

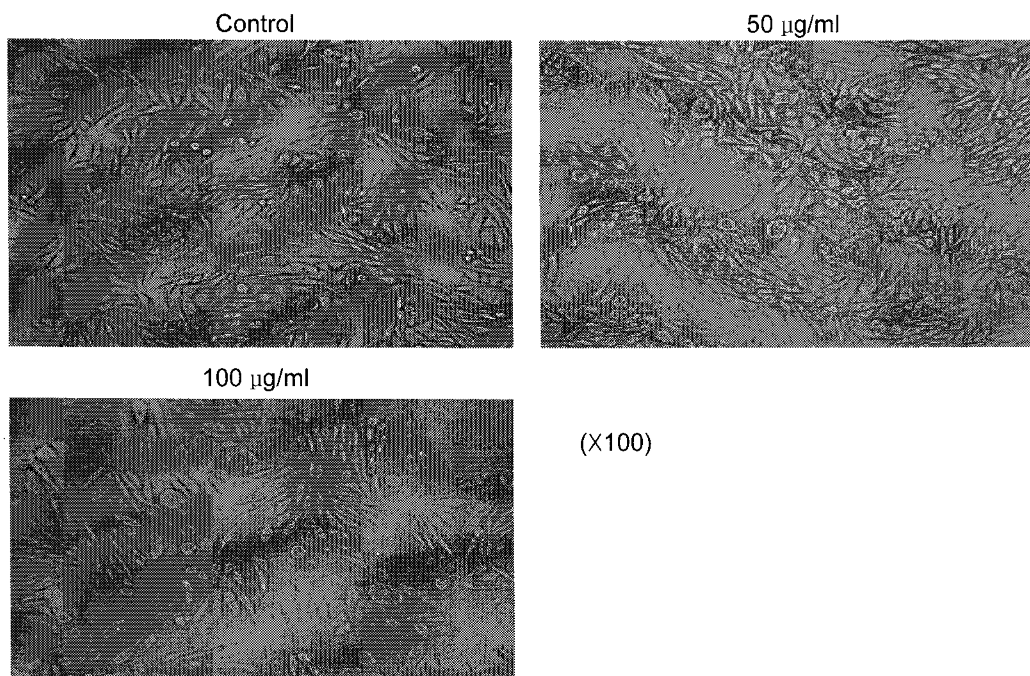

Fig. 5. Effects of BuOH fraction on DNA synthesis in SK-Hep-1.

did not. Therefore, the MeOH extract was further fractionated by serial fractionation method using organic solvent as described in Materials and Methods. The inhibitory effects were examined on the gelatinolytic activity of MMP-2 (Fig. 4A, B) for the concentration of 50 μg/mL of each fraction, including hexane, EtOAc, $CHCl_3$ and BuOH, and magnolol, honokiol, ovovatol, C, EC, ECG and EGCG of the methanol extract prepared from *M. officinalis* according to the method of Fujita et al. (21). The controls using either DMSO or methanol showed no effect on proteolytic activity of MMP-2 (data not shown). Proteolytic activity was indicated by the presence of clear bands on a dark background and the intensity of the band was shown proportional to activity of MMP-2. The control line that conditioned medium was incubated in Tris-$CaCl_2$-buffer containing 0.1 M DMSO only showed presence of MMP-2. C, EC, hexane, EtOAc and H_2O lines also showed low inhibitory activity.

ECG, EGC and $CHCl_3$ lines showed weak inhibition of proteolytic activity of MMP-2 (IC_{50}>50 μg/mL). However, MeOH, BuOH and EtOAc lines showed the strongest inhibitory activity (IC_{50}=11.3, 10.7 and 4.9 μg/mL, respectively) (Table 1B). Furthermore, BuOH fraction inhibited MMP-2 in a dose-dependent manner (Fig. 4C). These inhibitory effects were higher than that of EGCG, a MMP-2 inhibitor from plant sources (12), which showed high inhibitory activity (IC_{50}=12 μg/mL). Treatment of SK-Hep1 cells with up to 100 μg/mL of BuOH fraction inhibited their growth (Fig. 5) and

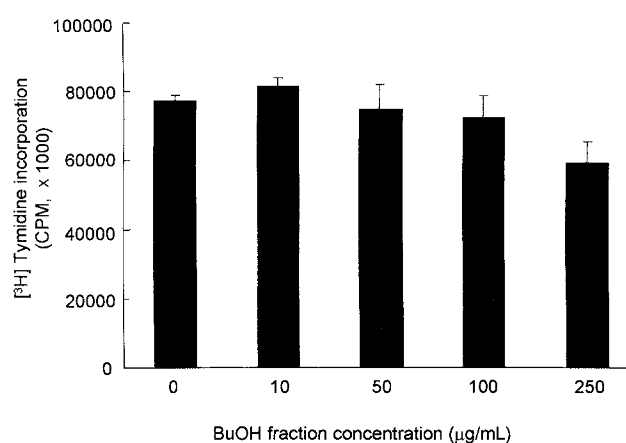

Fig. 6. Microscopical observations in cultured SK-Hep1 cells. Cells were treated BuOH fraction at 100 μg/mL and 250 μg/mL or without of BuOH fraction for 24 hr.

changed indeed morphology of cells (Fig. 6).

MMPs, especially MMP-2, play a key role in tumor invasion. Since tumor invasiveness is a critical process influencing the grade of malignancy, estimation of MMP activity *in vivo* may be clinically important in both cancer diagnosis and therapy with anticancer drugs that target MMPs. MMP-2 has been implicated as playing an important role in cancer invasion and metastasis (25-29) and proMMP-2 is activated at the cell surface by membrane-type-1 MMP (MT-1 MMP) (30,31). As a MMP-2 specific inhibitor, a fluorine-18 labeled MMP inhibitor, $[^{18}F]$SAV03 has been developed (32,33).

Methanol extract of *M. officinalis* has been fractio-

nated using solvents of increasing polarity to analyze the nature of active principles responsible for the cytotoxic and MMP-2 inhibitory activity. The cytotoxic activities of all fractions and the methanol extract were shown in Fig. 1. The order of potency appears to be as follows: BuOH fraction>crude extract=MeOH extract>$CHCl_3$ fraction>EtOAc fraction>hexane fraction. One important band was detected in the zymographic assays (Fig. 3 and 4). As shown in Fig. 3, high polarity fraction, $CHCl_3$ and BuOH fraction, had also inhibitory effect of proteolytic activity on MMP-2. Although treatment of SK-Hep1 cells with up to 50 μg/mL of BuOH fraction neither inhibited their growth nor changed indeed morphology (Fig. 2), BuOH fraction inhibited MMP-2 in a dose dependent manner (IC_{50}=10.7 μg/mL). Understanding the molecular mechanisms by which the major compound of the BuOH fraction from *M. officinalis* interact with and inhibit MMP-2 activities, thus restraining matrix degradation and cell invasion, is therefore important in exploiting its properties for cancer prevention and treatment. These results indicate that MeOH extract is an antitumor agent with low cytotoxicity acting on MMP-2 and may serve as a leader compound with development of antitumor drugs.

ACKNOWLEDGMENTS

This study was supported by Kyungsung University Research Grants in 2004.

REFERENCES

1. Cawston TE. 1996. Matrix metalloproteinase inhibitors and the prevention of connective tissue breakdown. *Pharmacol Ther* 70: 163-182.
2. Hiraoka NE, Allen IJ, Apel MR, Weiss SJ. 1998. Matrix metalloproteinases regulateneovascularization by acting as pericellular fibrinolysins. *Cell* 95: 365-377.
3. Liotta LA, Tryggvaason K, Garbisa S, Hart I, Foltz CM, Shafie S. 1980. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* 284: 67-68.
4. Shima IY, Sasaguri J, Kusukawa H, Yamana H, Fujita T, Kakegawa, Morimatsu M. 1992. Production of matrix metalloproteinase-2 and metalloproteinase-3 related to malignant behavior of esophageal carcinoma. A clinicopathologic study. *Cancer* 70: 2747-2753.
5. Tokuraku MH, Sato S, Murakami Y, Okada Y, Seiki M. 1995. Activation of the precursor of gelatinase A/72 kDa type IV collagenase/MMP-2 in lung carcinomas correlates with the expression of membrane-type matrix metalloproteinase (MT-MMP) and with lymph node metastasis. *Int J Cancer* 64: 355-359.
6. Talyensaari-Mattila AP, Paakko M, Hoyhtya G, Turpeenniemi-Hujanen T. 1998. Matrix metalloproteinase-2 immunoreactive protein: a marker of aggressiveness in breast carcinoma. *Cancer* 83: 1153-1162.
7. Sier CF, Kubben KJ, Ganesh S, Heerding MM, Griffioen G, Hanemaaijer R, van Krieken JH, Lamers CB, Verspaget HW. 1996. Tissue levels of matrix metalloproteinases MMP-2 and MMP-9 are related to the overall survival of patients with gastric carcinoma. *Br J Cancer* 74: 413-417.
8. Whittaker MC, Floyd D, Brown P, Gearing AJH. 1999. Design and therapeutic application of matrix metalloproteinase inhibitors. *Chem Rev* 99: 2735-2776
9. Bramhall SR, Rosemurgy A, Brown PD, Bowry C, Buckels JA. 2001. Marimastat Pancreatic Cancer Study Group, Marimastat as first-line therapy for patients with unresectable pancreatic cancer: a randomized trial. *J Clin Oncol* 19: 3447-3455.
10. Levitt NC, Eskens F, Propper DJ, Harris AL, Denis L, Ganesan TS, Mather RA, McKinley L, Planting A, Talbot DC, Van der Burg M, Wilner S, Verweij J. 1998. A phase one pharmacokinetic study of CGS 27023A, a matrix metalloproteinase inhibitor. *Proc Am Soc Clin Oncol* 17: 213-214.
11. Price A, Shi Q, Morris D, Wilcox ME, Brasher PMA, Rewcastle NB, Shalinsky D, Zou H, Applet K, Johnston RN, Yong VW, Edwards D, Forsyth P. 1999. Marked inhibition of tumor growth in a malignant glioma tumor model by a novel synthetic matrix metalloproteinase inhibitor AG3340. *Clin Cancer Res* 5: 845-854.
12. Cha BY, Park CJ, Lee SG, Lee YC, Kim DW, Kim JD, Seo WG, Kim CH. 2003. Inhibitory effect of methanol extract of *Euonymous alatus* on matrix metalloproteinase-9. *J Ethnopharm* 85: 163-167.
13. Chang HM, But PH. 1986. *Pharmacology and applications of Chinese materia medica.* World Scientific Publishers, Singapore. Vol I, p 878-880.
14. Nagase H, Ikeda K, Sakai Y. 2001. Inhibitory effect of magnolol and honokiol from *Magnolia obovata* on human fibrosarcoma HT-1080 invasiveness *in vitro*. *Planta Medica* 67: 705-708.
15. Ikeda K, Nagase H. 2002. Magnolol has the ability to induce apoptosis in tumor cells. *Biol Pharm Bull* 25: 1546-1549.
16. Yang SE, Hsieh MT, Tsai TH, Hsu SL. 2002. Down-modulation of Bcl-XL, release of cytochrome c and sequential activation of caspases during honokiol-induced apoptosis in human squamous lung cancer CH27 cells. *Biochem Pharmacol* 63: 1641-1651.
17. Teng CM, Chen CC, Ko FN, Lee LG, Huang TF, Chen YP, Hsu HY. 1988. Two antiplatelet agents from *Magnolia officinalis*. *Thromb Res* 50: 757-765.
18. Hirano T, Gotoh M, Oka K. 1994. Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells. *Life Sci* 55: 1061-1069.
19. Kaegi E. 1998. Unconventional therapies for cancer: 2. Green tea. The task force on alternative therapies of the Canadian breast cancer research initiative. *Canad Med Assoc J* 158: 1033-1035.
20. Demeule M, Brossard M, Page M, Gingras D, Beliveau R. 2000. Matrix metalloproteinase inhibition by green tea catechins. *Biochimica et Biophysica Acta* 1478: 51-60.
21. Fujita MH, Itokawa, Sashida Y. 1973. Studies on the components of *Magnolia obovata Thunb.* II. On the components of the methanol extract of the bark. *Yakugaku Zasshi* 93: 422-428.
22. Nebigil C, Malik KU. 1990. Prostaglandin synthesis elicited by adrenegic stimuli in rabbit aorta is mediated via *alpha*-1 and *alpha*-2 adrenergic receptors. *J Pharmacol*

Exp Ther 254: 633-640.

23. Knight CG, Willenbrock F, Murphy G. 1992. A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS Lett* 296: 263-266.
24. Choi NS, Kim BY, Lee JY, Yoon KS, Han KY, Kim SH. 2002. Relationship between acrylamide concentration and enzymatic activity in an improved single fibrin zymogram gel system. *J Biochem Mol Biol* 35: 236-238.
25. Aimes RT, Quigley JP. 1995. Matrix metalloproteinase-2 is an interstitial collagenase. *J Biol Chem* 270: 5872-5876.
26. Woessner JF. 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 5: 2145-2154.
27. Chung TW, Kim JR, Suh JI, Lee YC, Chang YC, Chung TH, Kim CH. 2004. Correlation between plasma levels of matrix metalloproteinase (MMP)-9/MMP-2 ratio and alpha-fetoproteins in chronic hepatitis carrying hepatitis B virus. *J Gastroenterol Hepatol* 19: 565-571.
28. Chung TW, Lee YC, Kim CH. 2004. Hepatitis B viral HBx induces matrix metalloproteinase-9 gene expression through activation of ERKs and PI-3K/AKT pathways: Involvement of invasive potential. *FASEB J* 18: 1123-1125.
29. Sato H, Takino T, Okada, Y, Cao J, Shinagawa A, Yamamoto E, Seiki M. 1994. A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature* 370: 61-65.
30. Chung TW, Lee YC, Ko JH, Kim CH. 2003. Hepatitis B virus X protein (HBx) is a negative regulator of PTEN in liver cells: Involvement of AKT activation. *Cancer Res* 63: 3453-3458.
31. Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI. 1995. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J Biol Chem* 270: 5331-5338.
32. Furumoto S, Iwata R, Ido T. 2002. Design and synthesis of fluorine-18 labeled matrix metalloproteinase inhibitors for cancer imaging. *J Labelled Compd Radiopharm* 45: 975-986.
33. Furumoto S, Takashima K, Kubota K, Ido T, Iwata R, Fukuda H. 2003. Tumor detection using $^{(18)}$F-labeled matrix metalloproteinase-2 inhibitor. *Nucl Med Biol* 30: 119-125.

(Received August 21, 2006; Accepted September 12, 2006)