Research Paper

Mongolian *Chelidonium majus* Suppresses Metastatic Potential of Hepatocellular Carcinoma Cells through TIMP Up-regulation and MMP Down-regulation

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In this study, the antimetastatic effect of *Chelidonium majus* ethanol extract on hepatocellular carcinoma *in vitro* was investigated. The viability of SK-Hep1 and normal liver cells was determined using a cell counting kit-8 assay. Wound healing assays were performed to investigate SK-Hep1 cell migration and various metastatic characteristics including adhesion, aggregation and invasion were also measured using these cells. Furthermore, the proteolytic activity of extracellular matrix metalloproteinase-9 was measured using gelatin zymography. Expression levels of matrix metalloproteinase-2, matrix metalloproteinase-9, membrane type 1-matrix metalloproteinase and tissue inhibitor of metalloproteinase-1 were measured using reverse transcription-polymerase chain reaction and Western blotting. *Chelidonium majus* ethanol extract significantly inhibited the proliferation of SK-Hep1 hepatocellular carcinoma cells in a dose-dependent manner. Moreover, metastatic characteristics including adhesion, migration, aggregation and invasion were significantly suppressed by *Chelidonium majus* ethanol extract treatment. Further, this preparation downregulated the expression of matrix metalloproteinase-2, matrix metalloproteinase-9 and membrane type 1-matrix metalloproteinase, but upregulated tissue inhibitor of metalloproteinase-1 and tissue inhibitor of metalloproteinase-2, in a dose-dependent manner. Additionally, the proteolytic activity of matrix metalloproteinase-9 was greatly diminished with 400 µg/ml of *Chelidonium majus* ethanol extract. Taken together, it was suggested that *Chelidonium majus* ethanol extract might exert an antimetastatic effect on hepatocellular carcinoma cells by inhibiting proliferation, adhesion, migration, aggregation and invasion through the downregulation of matrix metalloproteinases and upregulation of tissue inhibitor of metalloproteinases. Thus, this extract could represent a promising therapeutic agent for this disease.

Key words: *Chelidonium majus*, SK-Hep1 cells, metastasis, matrix metalloproteinase

Cancer is the second leading cause of global mortality, causing 8.7 million deaths in 2015[1]. In particular, Mongolia has the highest incidence of liver cancer, which is almost eight times higher than the global average[2]. Unsurprisingly, extensive research is being conducted on potential resources to develop optimal drugs for the treatment of malignant tumors. Despite the advances in research and therapeutics, the incidence of cancer has not decreased in recent years and instead, appears to increase annually[1,3]. The spread of cancer cells from the primary site to other body parts, known as metastasis, is mainly responsible for the incurable nature of malignant tumors and 90 % of cancer-associated deaths[4]. Hepatocellular carcinoma (HCC), a primary liver malignancy, is characterized by its ability to spread locally within the liver, invade blood vessels, and subsequently metastasize to distant organs[5-7]. The aggressiveness of HCC has been a dilemma for decades,

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and the current treatment methods are limited to liver transplantation and surgical resection[5]. Recently, researchers have focused on identifying alternative sources of cancer drugs such as natural products from medicinal plants[8]. Botanical resources are available abundantly and associated with minimal side effects, making them excellent candidates for future anticancer drug discovery.

Chelidonium majus L., an herbal plant belonging to the family Papaveraceae, is an important ingredient in traditional Chinese medicine and Western phytotherapy[9-11]. It is well known that this herbal plant have the phytochemical composition such as alkaloids and phenolic compounds (kaempferol, quercetin, caffeic acid, ferulic acid). Mongolian traditional herbalists have used *C. majus* to relieve pain, cough, fever, and swelling from microbial infection and to treat stomach and liver diseases[11]. Numerous studies have reported the hepatoprotective[12], antimicrobial[13], immunomodulatory[9] and antiinflammatory[14] effects of crude extracts or purified compounds from *C. majus in vitro* and *in vivo*. In addition, this plant has been previously reported to exert potent anticancer effects[15-17].

Cancer drug discovery has primarily been focused on the inhibition of carcinogenesis; however, to date, no antimetastatic drugs are clinically available, and pharmacists are developing an interest in designing drugs with both anticancer and antimetastatic effects[18,19]. Studies have shown Mongolian *C. majus* to be an excellent candidate for the development of a novel anticancer drug, but its antimetastatic potential has not been investigated. Therefore, in the present study, the effect of *C. majus* ethanol extract (CME) on the growth, adhesion, migration, aggregation and invasion of the SK-Hep1 human HCC cell line *in vitro* was investigated.

MATERIALS AND METHODS

Preparation of CME:

C. majus was collected from Handgait, Ulaanbaatar, Mongolia. The air-dried plants were ground and 50 g of the powdered material was macerated in 500 ml of 80 % ethanol for 48 h. Then, the suspension was filtered, the ethanol was evaporated using a rotary vacuum evaporator and the extract was freeze-dried. A 10-mg/ml stock solution of CME was prepared by dissolving freeze-dried powder in Dulbecco's modified Eagle's medium (DMEM, Gibco, Rockville, MD, USA).

Cell culture:

Human SK-Hep1 HCC cells were obtained from the Korean Cell Line Bank and Chang liver cells were obtained from Konkuk University in Korea. Both cell lines were cultured in DMEM containing 10 % fetal bovine serum (FBS; Gibco), and 1 % penicillin-streptomycin (Gibco) in a humidified 5 % CO_2 atmosphere at 37°.

Cell viability analysis:

Cells were seeded at 1×10^5 cells/well in a 96-well plate containing DMEM with 10 % FBS and incubated for 24 h. Cells were incubated with different concentrations of CME (0, 50, 100, 200, and 400 μg/ml) for 24 h. Viability was measured using a cell counting kit (CCK)-8 assay (Dojindo, Tokyo, Japan) according to the manufacturer's instructions. Optical density was measured at 450 nm using an Infinite F50 microplate reader (Tecan, Mannedorf, Switzerland). Viability of cells treated with CME was expressed as a percentage of untreated cell viability.

Cell adhesion assay:

In brief, a 6-well plate was coated with 0.1 % gelatin overnight at 37° and air-dried for 30 min. SK-Hep1 cells (10^5 cells/ml) were suspended in DMEM containing CME (0, 100, 200, and 400 μg/ml), plated into the wells and incubated at 37° with 5 % CO_2 for 6 h. Then, the medium was removed and cells were washed twice with phosphate-buffered saline (Gibco). The attached cells were counted under a microscope (Olympus CK40, Tokyo, Japan).

Cell aggregation assay:

SK-Hep1 cells (10^5 cells/ml) were suspended in DMEM containing CME (0, 100, 200, and 400 μg/ml). Then, the cell suspension (20 μl) was incubated on the undersurface of a 60-mm culture dish lid (Sigma-Aldrich, St. Louis, MO, USA). Cell aggregates were monitored under a microscope after 0, 2, 4, 6, 12, and 24 h. Single and multiple cell aggregates were counted as a single particle.

Cell migration assay:

The mobility of cancer cells was studied using the wound healing assay. SK-Hep1 cells (10^5 cells/ml) were plated in 6-well plates; when cells were 100 %

confluent, a wound was created using a plastic pipette tip in the middle of the wells. Then, cells were treated with increasing concentrations of CME (0, 100, 200, 400 µg/ml). Wound closure was subsequently observed under a microscope and images were acquired at 0, 24, and 48 h.

Cell invasion assay:

Transwell chambers with 8-µm pore-size filters (Sigma-Aldrich) in a 6-well plate were used to examine the invasion of SK-Hep1 cells. Matrigel (BD Biosciences, Bedford, MA, USA) was diluted in cold DMEM (1:20) and added to the filters, which were air-dried under a laminar hood overnight. SK-Hep1 cells (10^5 cells/ml) were suspended in DMEM containing CME (0, 100, 200, and 400 µg/ml) and added to the upper compartment of invasion chambers. DMEM containing 10 % FBS was added to the lower compartment as a chemoattractant. After 24 h, cells on the lower surface were fixed with 70 % ethanol and stained with 0.2 % crystal violet for 10 min. Invading cells were observed using a microscope. Then, crystal violet was extracted with 10 % acetic acid for 5 min and the absorbance (nm) was measured at 595 using an ELISA plate reader (Tecan). The invasion of treated cells was expressed as the percentage of control cells.

Gelatin zymography:

Secreted matrix metalloproteinase (MMP)-9 activity was studied using gel zymography as described in the Gelatin Zymography protocol by Abcam, UK. SK-Hep1 cells (10^5 cells/ml) were exposed to CME (0, 100, 200, and 400 µg/ml) for 48 h and activity in treated cells was expressed as a percentage of that in control cells.

Reverse transcription-polymerase chain reaction (RT-PCR):

Total RNA was isolated from SK-Hep1 cells (10^5 cells/ml) exposed to the indicated concentrations of CME (0, 100, 200, and 400 µg/ml) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, extracted RNA (1 µg) was reversed transcribed into cDNA using M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania) and metastasis-related gene expression was quantified using the Maxime PCR PreMix (i-MAX II, iNtRON Biotechnology, Seongnam, Korea). PCR conditions consisted of an initial denaturation step at 95° for 5 min, followed by 30 amplification cycles consisting of denaturation for 40 s at 95°, annealing for 40 s (temperature 56-62°), and extension for 1 min at 72°. Primer sequences are shown in Table 1 and their relative expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels, as a control.

Western blot analysis:

SK-Hep1 cells (10^5 cells/ml) were allowed to adhere overnight in 6-well plates and cultured with 0, 100, 200, and 400 µg/ml CME. After 24 h, protein was extracted from cells using a protein extraction solution (iNtRON Biotechnology) and the protein concentration was standardized using a modified Bradford assay. Then, 25 µg of each protein sample was separated using 10 % SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5 % skim milk and probed with the following primary antibodies (1:1000 dilution), antiGAPDH, antiMMP-2, antiMMP-9, antimembrane type 1 (MT1)-MMP, and antitissue inhibitor of metalloproteinase-1 (TIMP1, Cell Signaling Technology, Danvers, MA, USA). After incubation with horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution), the membranes were treated with enhanced chemiluminescence Westsave Gold reagent (AbFrontier, Seoul, Korea) for 10 min and exposed to radiographic film (Agfa HealthCare, Greenville, SC, USA). Relative expression was normalized to that of GAPDH, which served as the control.

Statistical analysis:

All experiments were performed in triplicate and repeated at least three times. Quantitative data were expressed as means±standard deviations. Differences between means were calculated using an analysis of variance (ANOVA) followed by Duncan's multiple

TABLE 1: LIST OF PRIMERS TO DETECT METASTASIS-RELATED GENES

Name	Forward primer (5'→3')	Reverse primer (5'→3')
GAPDH	CGAGATCCCTCCAAAATCAA	AGGTCCACCACTGACACGTT
MMP-2	GGCCCTGTCACTCCTGAGAT	GGCATCCAGGTTATCGGGGA
MMP-9	CGGAGCACGGAGACGGGTAT	TGAAGGGGAAGACGCACAGC
MT1-MMP	TGGGTAGCGATGTCTTC	AGTAAGCAGTCTGGGT
TIMP1	GATCCAGCGCCCAGAGAGACACC	TTCCACTCCGGCATT

range test. P<0.05 was considered statistically significant and these were calculated using SAS/STAT® software.

RESULTS AND DISCUSSION

Chang liver cell viability was not altered by CME upto 400 µg/ml (fig. 1A); however, CME significantly inhibited SK-Hep1 HCC cell proliferation in a dose-dependent manner (fig. 1B). Moreover, this preparation exerted a stronger antiproliferative effect against SK-Hep1 cells (half-maximal inhibitory concentration (IC_{50}) = 369.35 µg/ml) than against Chang normal liver cells (IC_{50} = 4407.69 µg/ml) after a 24-h treatment. Therefore, the following experiments were performed using 100, 200, and 400 µg/ml CME.

As shown in fig. 2A, cell adhesion to gelatin-coated surfaces was significantly decreased by 52.01±5.91 % and 82.04±2.09 % following a 6-h treatment with 200 and 400 µg/ml CME, respectively, compared to that in untreated cells. Additionally, 100 µg/ml of CME inhibited the colony formation of SK-Hep1 cells by 89.61±4.33 % after a 24-h treatment, based on a cell aggregation assay (fig. 2B).

Cell motility was assessed to determine whether CME could inhibit HCC migration using a scratch wound healing assay. As shown in fig. 3, the wound size was reduced by 28.09±3.64 % in cells treated with 200 µg/ml CME compared to that in untreated cells after 24 h; moreover, untreated wounds healed completely

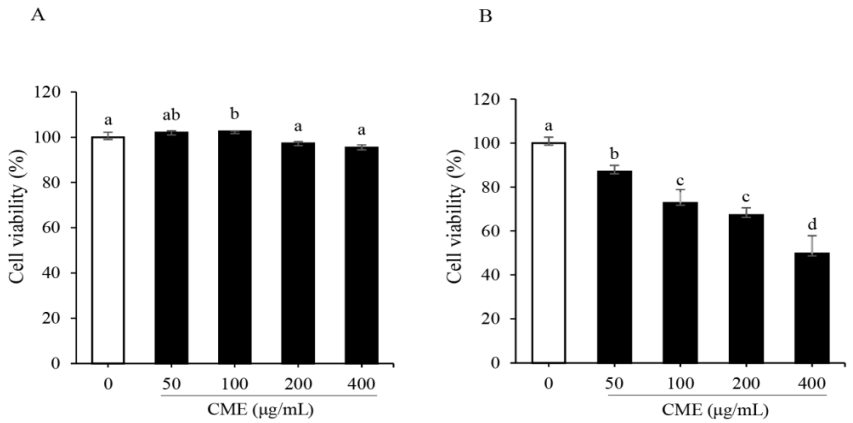

Fig. 1: Effects of *Chelidonium majus* extract (CME) on the viability of Chang liver cells and SK-HEP hepatocellular carcinoma cells
(A) Chang liver cells and (B) SK-Hep1 cells were treated with 50-400 µg/ml CME for 24 h and cell viability was assessed using a cell counting kit (CCK)-8 assay. Data are presented as the mean±standard deviation (SD; n=3). Untreated Chang liver cells and HCC cells were considered 100 % viable. Bars with different superscript letters represent significant differences at p<0.05 based on the Duncan's multiple range test

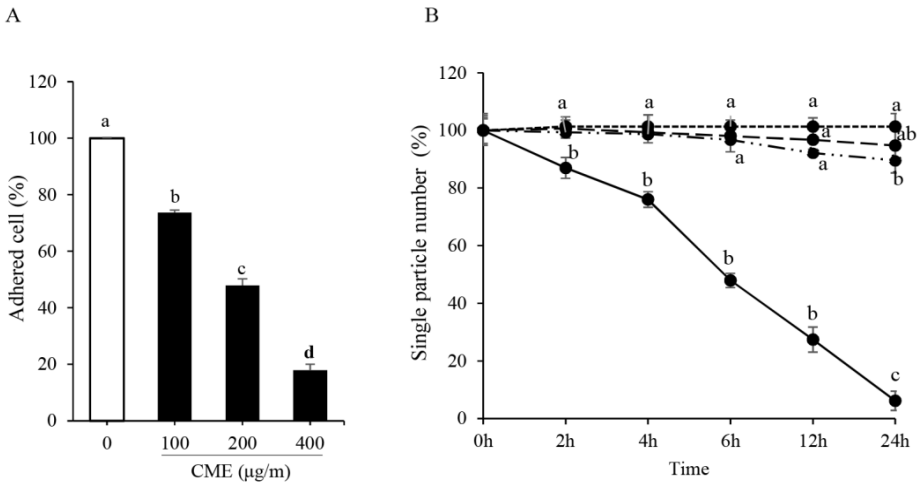

Fig. 2: Effects of *Chelidonium majus* extract (CME) on adhesion and aggregation of SK-Hep1 cells
(A) Adhesion was measured using gelatin-coated surfaces. Adhered cell counts were indicated relative to that of untreated controls (%). (B) Cell aggregates were observed under a microscope (Olympus CK40) and counted at different time intervals. The number of aggregated cells was indicated relative to that in the untreated control at each time point (%). Results are presented as the mean±SD (n=3). Bars with different superscript letters represent significant differences at p < 0.05 based on the Duncan's multiple range test. (—●—) 0 µg/ml; (- ● -) 100 µg/ml; (--●--) 200 µg/ml; (----●----) 400 µg/ml

after 48 h. Next, a Matrigel-coated chamber assay was performed to confirm the inhibitory effect of CME on HCC cell migration. CME significantly suppressed HCC cell migration in a dose-dependent manner (fig. 4). Further, invasion was reduced by 29.23±0.89, 55.13±2.25, and 74.43±2.10 % after CME treatment with 100, 200, and 400 µg/ml, respectively (fig. 4).

As shown in fig. 5, CME treatment suppressed the expression of MMP-2 and MMP-9 in a dose-dependent manner. MMP-2 mRNA expression was significantly decreased to 32, 67, and 73 % of levels in untreated cells, in response to 100, 200, and 400 µg/ml CME, respectively. Similarly, MMP-9 mRNA expression was significantly and gradually reduced with increasing concentrations of CME (100-400 µg/ml). Moreover, the mRNA expression of MT1-MMP was significantly reduced in a dose-dependent manner to 21.73±5.05, 40.83±6.79, and 45.57±4.78 % of control levels after treatment with 100, 200, and 400 µg/ml CME, respectively. In contrast, the mRNA expression of TIMPs

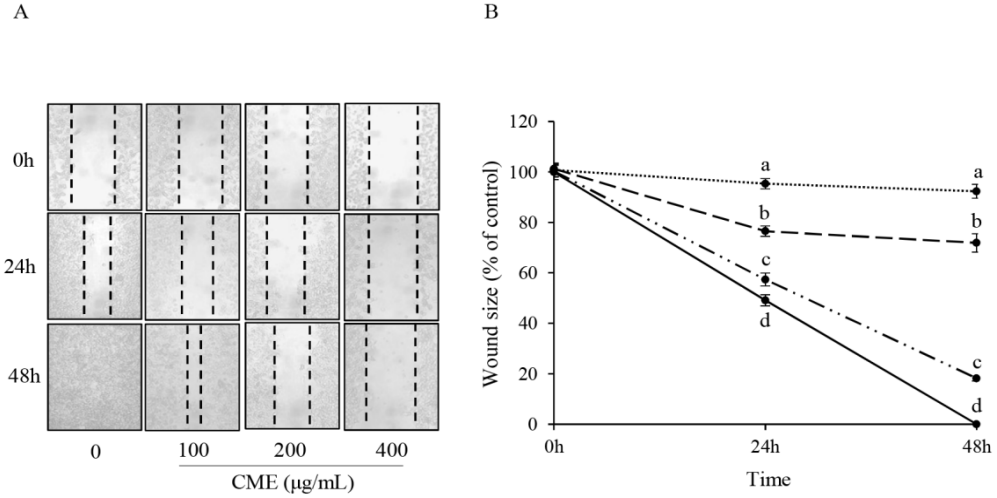

Fig. 3: Effects of *Chelidonium majus* extract (CME) on the migration in SK-Hep 1 cells
(A) Motility of SK-Hep1 cells, as assessed by wound closure assays. (B) Wound sizes were observed with a microscope (Olympus CK40) and measured at the indicated time points. Amount of migration was indicated relative to that in untreated controls (%). Data are presented as the mean±SD (n=3). Bars with different superscript letters represent significant differences at p < 0.05 based on the Duncan's multiple range test. (—●—) 0 µg/ml; (- ● -) 100 µg/ml; (--●--) 200 µg/ml; (----●----) 400 µg/ml

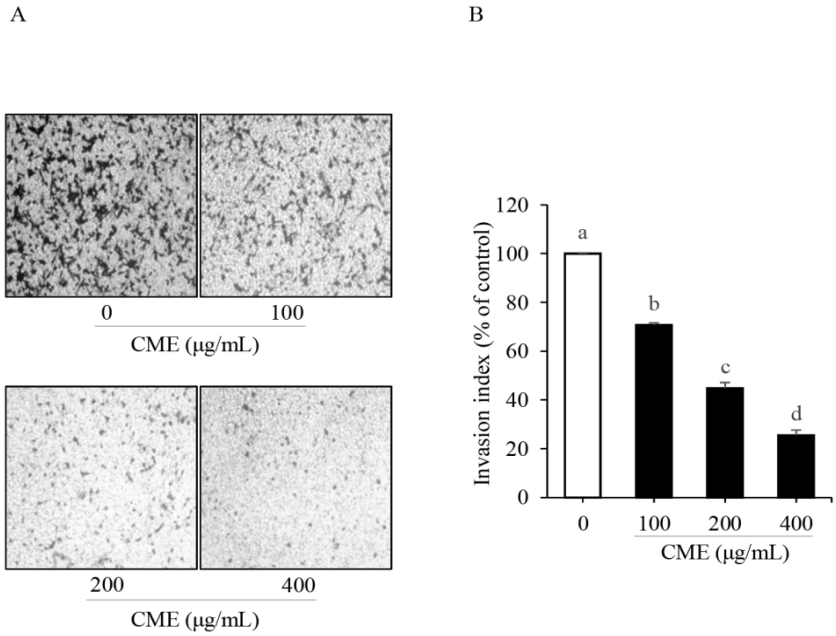

Fig. 4: Effects of *Chelidonium majus* extract (CME) on invasion of SK-Hep 1 cells
(A) Cells that invaded transwell chambers were stained with 0.2% crystal violet and observed under ×40 magnification (Olympus CK40). (B) Relative invasive activity, as determined by extracting the stain and quantifying absorbance at 595 nm. Data are presented as the mean±SD (n=3). Untreated SK-Hep1 cells were considered 100 % viable. Bars with different superscript letters represent significant differences at p<0.05 based on the Duncan's multiple range test

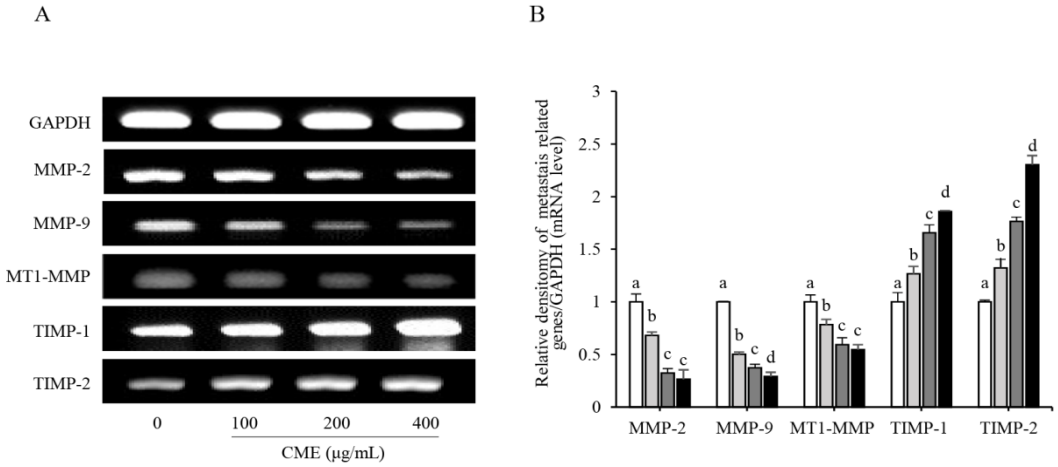

Fig. 5: Effects of *Chelidonium majus* extract (CME) on mRNA expression in SK-Hep1 cells
(A) SK-Hep1 cells were incubated with or without CME (100–400 µg/ml) for 24 h. The expression levels of MMP-2, MMP-9, MT1-MMP, TIMP-1, and *TIMP-2* were measured using reverse transcription-polymerase chain reaction (RT-PCR). mRNA expression was quantified using the Maxime PCR PreMix. (B) Bands were quantified using ImageJ software and expressed as the ratio to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. Data are presented as the mean±SD (n=3). The viability of untreated cells was set to 1.0. Bars with different superscript letters represent significant differences at p<0.05 based on the Duncan's multiple range test. (□) 0 µg/ml; (▫) 100 µg/ml; (▪) 200 µg/ml; (■) 400 µg/ml

(TIMP-1 and TIMP-2) was significantly increased by CME treatment, compared to that in untreated cells, in a dose-dependent manner. Furthermore, protein levels of MMPs, MTI-MMP, and TIMPs followed the same trend in treated HCC cells (fig. 6). Specifically, CME treatment significantly increased the expression of TIMP-1 and TIMP-2 compared to levels in untreated cells. However, TIMP-1 expression in cells treated with 200 µg/ml CME was not significantly changed compared to that in cells treated with 400 µg/ml CME. Additionally, TIMP-2 expression in cells treated with 100 µg/ml CME was not significantly different from that in cells treated with 200 µg/ml CME.

MMP activation was measured by gelatin zymography to elucidate the inhibitory effect of CME on the production of gelatinases in HCC cells (fig. 7). The patterns of MMP-9 secretion, based on zymography assays, was similar to those of mRNA and protein expression. MMP-9 activity was significantly suppressed after CME treatment when compared to that in untreated cells. As shown in fig. 7, MMP-9 activity was decreased to 40.22±5.44, 48.56±3.36, and 81.38±4.09 % of control levels in response to 100, 200, and 400 µg/ml of CME, respectively. However, MMP-9 activity was not significantly different between 100 and 200 µg/ml CME treatment groups.

HCC, the most common hepatic malignancy, is an aggressive tumor associated with frequent intrahepatic and extrahepatic invasion[20]. Consequently, its prognosis remains poor in the late stage[21]. Extrahepatic metastasis is a multi-step process consisting of migration from the primary liver site, invasion of the blood vessels or lymph nodes, the formation of aggregates to facilitate travel, and adherence to distant secondary sites such as the lung, bones, and adrenal gland[22-25]. Cancer cells exhibit altered expression of extracellular matrix (ECM)-degradative proteins, which mainly include MMPs[25] that are highly expressed in HCC cells and are associated with the growth, expansion, and invasion of malignant tumors[26]. MMPs comprise a family of proteolytic enzymes that create space for cell migration by rearranging the structure of the ECM and manipulating intracellular signaling and junctions[27]. Among family members, MMP-2 and MMP-9 are gelatinases that play a key role in angiogenesis by upregulating vascular endothelial growth factors and breaking down basement membrane components to reduce cell-to-cell interactions and facilitate cell motility[28]. In this study, an examination of MMP-2 and MMP-9 expression revealed that CME down-regulated their transcription in a concentration-dependent manner. Huang *et al*. reported that β-mangostin, a dietary xanthone, exerts antimetastatic activity through the inhibition of MMP-2 and MMP-9 mRNA and protein expression in human HCC cells[29]. These results are consistent with our findings showing that CME could reduce colony formation, adhesion, invasion, and migration via the suppression of MMP-2 and MMP-9 expression.

Moreover, CME treatment suppressed MT1-MMP transcription, whereas it upregulated the expression of TIMP-1 and TIMP-2. MMP-2 and MMP-9 are

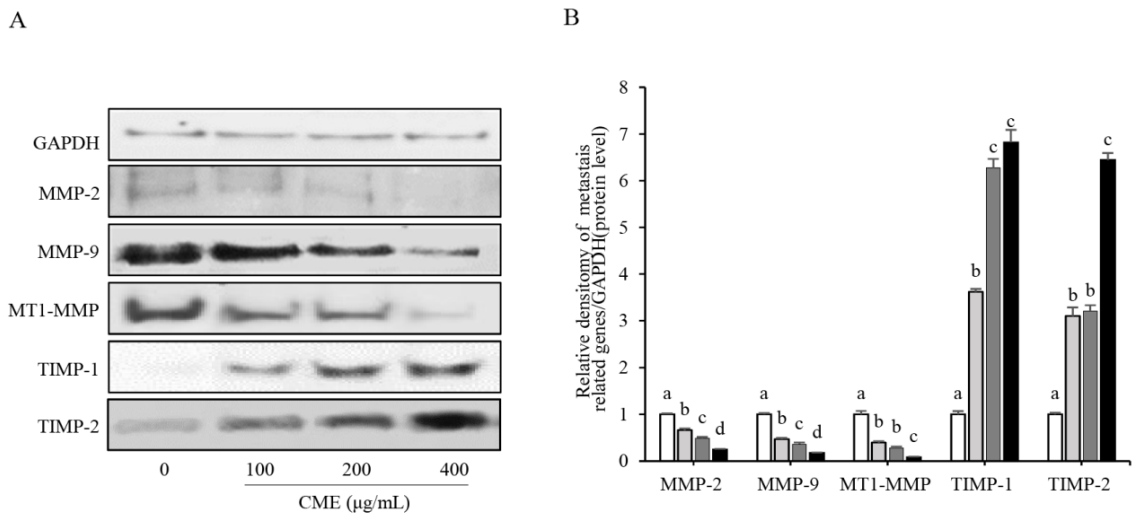

Fig. 6: Effects of *Chelidonium majus* extract (CME) on metastasis-related protein expression in SK-Hep1 cells
(A) SK-Hep1 cells were incubated with or without CME (100–400 μg/mL) for 24 h. Protein expression levels of MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2 were determined by western blotting. (B) Bands were quantified using ImageJ software and expressed as the ratio to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. The viability of untreated cells was set to 1.0. Data are expressed as the mean±SD (n=3); values with different superscript letters represent significant differences at $p < 0.05$ based on the Duncan's multiple range test. (□) 0 μg/ml; (▫) 100 μg/ml; (▪) 200 μg/ml; (■) 400 μg/ml

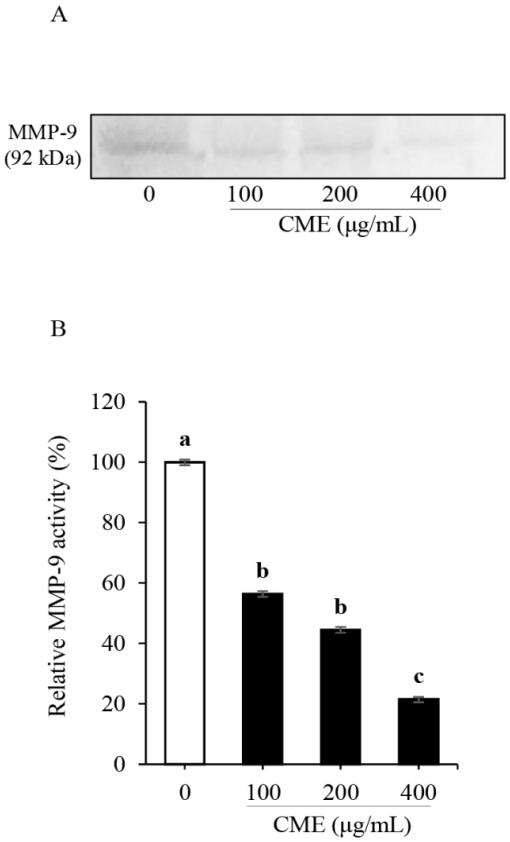

Fig. 7: Effect of *Chelidonium majus* ethanol (CME) extract on proteolytic activity in SK-HEP1 cells after 24 h
(A) The activity of extracellular matrix metalloproteinase (MMP)-9 was measured using gelatin zymography and (B) relative densitometry was calculated using ImageJ software. Data are presented as the mean±SD (n=3). The viability of untreated cells was set to 100%. Values with different superscript letters represent significant differences at $p<0.05$ based on the Duncan's multiple range test

generally secreted as inactive proenzymes and are either activated by other MMPs or suppressed by their inhibitors[30]. MT1-MMP, a well-known activator of MMP-2, forms a complex with the latent form of MMP-2 (proMMP-2) and TIMP-2 to activate MMP-2[31]. This trimolecular complex cleaves proMMP-2 to generate the MMP-2/MT1-MMP complex, which subsequently stimulates proMMP-9, as reported previously[32]. Thus, MT1-MMP directly activates MMP-2 but indirectly stimulates MMP-9, which consequently promotes tumour metastasis[33]. In contrast, TIMP-1 is a negative regulator of MMP-9, and the balance between MMP-9 and TIMP-1 interactions has been reported to modulate inhibitory effects on liver cancer cell invasion[34]. This study demonstrated that CME suppresses HCC cell metastatic potential by up-regulating TIMP-1 and TIMP-2 and down-regulating MMP-2 and MMP-9.

MMP-2 and MMP-9 (gelatinase-A and gelatinase-B, respectively) degrade type IV collagen and gelatin, major structural components of the ECM and basement membrane. Particularly, the latent form of MMP-9 (92 kDa) is highly expressed in the human SK-Hep1 HCC cell line compared to MMP-2 levels[32]. Furthermore, to verify the relationship between MMP expression and the metastasis-inhibitory potential of CME, the proteolytic activity of secreted MMPs was measured by gelatin zymography. CME treatment inhibited the gelatin-degrading activity of MMP-9 in a concentration-dependent manner by 40-81 %.

Despite the steady development of chemotherapeutics with high efficacy and safety, the prognosis of HCC remains poor due to the high rates of recurrence and metastasis. Currently, there is no effective chemotherapeutic regimen that can prevent metastasis. Therefore, the evaluation of traditional medicinal plants and their application to drug discovery has been the center of attention over the past few years[35]. This study was performed to evaluate the antimetastatic effect of *C. majus* used as a Mongolian traditional medicine. These results suggest that CME suppresses all critical processes related to metastasis such as cell migration, invasion, aggregation, and adhesion by down-regulating the predominant metastasis-related gelatinases and their activators while upregulating their natural inhibitors. Thus, CME, as an MMP inhibitor, might represent a promising antimetastatic agent. Although it was confirmed that the antimetastatic effect of *C. majus in vitro*, animal studies were not performed in this study. Hence, further studies are needed to address antimetastatic properties of *C. majus* in a relevant animal model.

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