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Anti-metastatic Effects of *Celastrus orbiculatus* Extract in B16F10 Melanoma Cells

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Abstract – *Celastrus orbiculatus* has been widely used as a traditional medicine for the treatment of many diseases including rheumatoid arthritis and odontalgia. In the present study, anti-metastatic activity of a methanolic extract from *C. orbiculatus* (MCO) was studied. A gelatin zymographic assay revealed that MCO has potent inhibitory effects on MMP-2 and MMP-9 activities in B16F10 melanoma cells. Moreover, MCO attenuated MMP expression via down-regulation of NF-κB translocation to the nucleus. Melanoma cell migration and invasion were also down-regulated by MCO. In addition, MCO significantly suppressed lung metastasis in an *in vivo* model. These results strongly suggest that MCO may possibly be used as a valuable anti-metastatic agent for cancer treatment.
Keywords – *Celastrus orbiculatus*, metastasis, invasion, MMP

Introduction

Cancer metastasis, a hallmark of malignancy, represents the spread and growth of cancer cells from the primary neoplasm to distal sites (Weiss, 1990). It is the principal cause of mortality among cancer patients and to date, there are no available therapeutic options. Thus, it is critical to develop effective anti-metastatic agents. Metastasis of cancer cells is generally described as a cascade of several events including primary tumor dissociation, migration, invasion, adhesion and proliferation at a target site (Arvelo and Cotte, 2006). Throughout the metastatic process, the rate-limiting step is the breakdown of connective tissue barriers such as the extracellular matrix (ECM) and basement memebrane (BM; Yoon *et al*., 2003), and therefore, the degradation of ECM and BM are crucial events in the cascade of metastasis.

Matrix metalloproteinases (MMPs) are a multigene family of zinc-dependent endopeptidases and they play a crucial role in the proteolysis of the ECM and BM which is essential for invasion, metastasis and angiogenesis of cancers (McCawley and Matrisian, 2000). Although other MMPs are involved in metastatic process, the two gelatinases, MMP-2 and MMP-9, are abundantly expressed in various cancer cells and recognized as key enzymes for tumor invasion and metastasis (Liabakk *et al*., 1996; Johnsen *et al*., 1998). Therefore, inhibitors of MMP-2 or MMP-9 are attractive therapeutic targets to inhibit tumor invasion and metastasis.

Celastrus orbiculatus Thunb. (Celastraceae) is widely distributed in Korea and China. *C. orbiculatus* has been used as a traditional medicine in Korea for the treatment of various diseases including rheumatoid arthritis, insomnia and contusions. It has been reported that *C. orbiculatus* has cytotoxic activities (Zhang *et al*., 2005; Zhang *et al*., 2006) and sesquiterpenoids in this plant showed anti-inflammatory activity (Guo *et al*., 2006). Moreover, anti-inflammatory activity of the flavonones from this plant has also been reported (Hwang *et al*., 2001; Min *et al*., 1999).

Studies on the anti-metastatic activity of the methanol extract of *C. orbiculatus* (MCO) are limited. Thus, the present studies were undertaken to investigate the possibility of using the methanolic extract of *C. orbiculatus* (MCO) as an anti-metastatic agent. In this study, we describe its anti-metastatic effects by blocking MMPs activities, leading to inhibition of cancer cell migration and invasion.

Experimental

Preparation of the stem and leaves of *C. orbiculatus* – The plant materials were purchased from Hainyakupsa (Chonbuk, South Korea) in October 2009. A voucher specimen (WH069) was deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University. An extract was obtained twice from the dried

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sample (1000 g) with 15,000 mL of MeOH under sonification for 2 h. The resultant methanolic extract was concentrated into 52.3 g (Yield: 5.23%) using a rotary evaporator. The sample was lyophilized and then stored at –20 °C until use.

Animals – Male C57BL/6 mice (5 weeks old) weighing 16 - 20 g were supplied by Damul Science (Dajeon, Korea). All animals were housed at 22 ± 1 °C with a 12 h light/dark cycle and fed a standard pellet diet with tap water *ad libitum*.

Cell and cell culture – B16F10 murine melanoma cells were obtained from the Korean cell line bank (KCLB, Korea) and cultured in DMEM containing 10% heat-inactivated FBS supplemented with penicillin (100 U/ml), streptomycin (100 Ag/ml), and sodium bicarbonate (2.2 g/l) at 37 °C in a 5% CO_2 and humidified air atmosphere. Cultures used in subsequent experiments were at less than 60 passages.

Gelatin digestion assay – An agarose solution (1%) was prepared in collagenase buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 0.15 M NaCl, pH 7.8) with 0.15% porcine gelatin and allowed to solidify in wells of a 6-well plate (3 ml/well) for 1 h at room temperature. Different concentrations of MCO (1 µl total volume) were incubated with 10 µl of bacterial collagenase-1 (0.1 mg/ml) in 89 µl of collagenase buffer for 1 h. The reaction products (10 µl) were loaded onto paper disks placed on gelatin-agarose gels and incubated for 18 h at 37 °C. The degree of gelatin digestion in agarose gel was visualized by Coomassie Blue staining after removal of the paper disks. Following destaining, the area of light translucent zone over the blue background was determined to estimate gelatinase activity.

Determination of cancer cell proliferation – To evaluate the cytotoxic activities of MCO, a MTT colorimetric assay was performed. Cells were seeded in 24-well plates at a density of 2.5×10^5 cells per well and treated with various concentration of MCO for 24 h. Then the cells were washed with PBS and incubated with 50 µl MTT (5 mg/ml) for 3 h. The number of viable cells retaining MTT was measured spectrophotometrically after solubilization at 570 nm.

Gelatin zymography – Activities of MMP-2 and MMP-9 were determined by gelatin zymography as described previously (Hrabec *et al*., 2002). B16f10 cells were incubated in the presence or absence of MCO for 24 h in FBS-free medium. The conditioned medium was concentrated using centrifugal filter devices (Millipore, MA, USA). Then the concentrated supernatant was activated with a trypsin solution (75 µg/ml trypsin in 0.1 M Tris-HCl, 10 mM $CaCl_2$ buffer, pH 8.0) and resuspended in a 2× sample buffer (125 mM Tris–HCl, 3% SDS, 40% glycerol, 0.02% bromophenol blue, pH 6.8) without boiling and electrophoresed under non-reducing conditions on 10% polyacrylamide gels containing 0.2% gelatin. After electrophoresis, the gels were washed twice with wash buffer (50 mM Tris-HCl, 2.5% Triton X-100, pH 7.5) and incubated for 18 h at 37 °C in a developing buffer containing 10 mM $CaCl_2$, 50 mM Tris-HCl, and 150 mM NaCl. The gels were stained with 0.25% Coomassie Blue R-250 in 30% methanol and 10% acetic acid, and de-stained in the same solution without the Coomassie Blue dye. Gelatinolytic bands were observed as clear zones against the blue background.

Preparation of nuclear extracts – Nuclear extracts were prepared as previously described (Baek *et al*., 2002). Briefly, the cells were allowed to swell by adding lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride). Pellets containing crude nuclei were resuspended in extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) and incubated for 30 min on ice. The samples were centrifuged at 12,000 rpm for 10 min to obtain the supernatant containing nuclear extracts. Extracts were stored at –70 °C until use.

Western blot analysis – Whole cell lysates were made by boiling B16F10 cells in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by electrophoresis in a 10% SDS-polyacrylamide gel and transferred to nitrocellulose paper. The membrane was then blocked with 5% skim milk for 2 h at room temperature and then incubated with anti-MMP-2, and anti-MMP-9 antibodies (SantaCruz, CA, USA) for 3 h. After washing three times with PBS containing 0.05% tween 20, the blot was incubated with secondary antibody (anti-rabbit) for 2 h and the antibody specific proteins were visualized by an enhanced chemiluminesence detection system according to the recommended procedure (Millipore Corporation. MA, USA).

Matrigel invasion assay – B16F10 cells (1×10^5) were added into the upper compartment of BioCoat Matrigel invasion chambers (BD biosciences, USA) and cultured in serum-free DMEM in the presence of MCO. The 8 µm filter pores were precoated with Matrigel basement membrane matrix. The lower chambers were filled with DMEM containing 10% FBS as a chemoattractant. After 22 h of incubation the noninvading cells were removed from the upper surface of the membrane. Then the lower

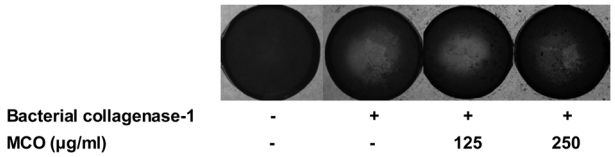

Fig. 1. Inhibitory effect of MCO on bacterial collagenase-1 as assessed by a gelatin digestion assay. A reaction mixture, containing bacterial collagenase-1, was incubated with various concentrations of MCO. Then, 5 μl of the reaction products were loaded onto paper disks placed on an agarose gel with 0.15% gelatin. The remaining bacterial collagenase-1 activity was assessed by the gelatin-digested clear zone visualized by Coomassie Blue staining.

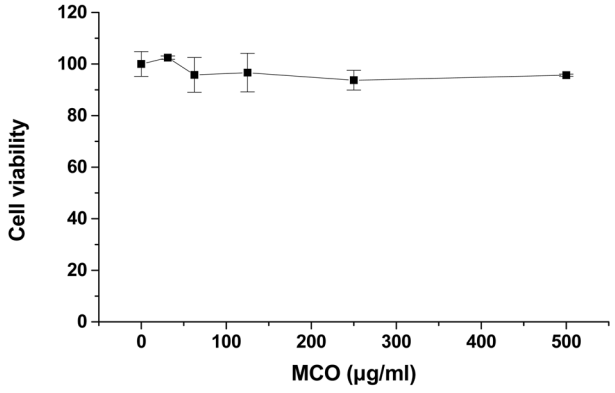

Fig. 2. Effects of MCO on the viability of B16F10 cells. Cell viability was evaluated by a MTT colorimetric assay as described in the methods. These results are expressed as means ± S.D. of three independent experiments in duplicate.

surface of the membrane was fixed and stained with methanol and 0.5% crystal violet. The membrane was photographed and the invading cells were directly counted from 6 random fields of each filter on a microscope.

Wound healing assay – For cell motility determination, wound healing assays were performed as described previously (Gao *et al.*, 2005) with minor modifications. A single linear wound was created with a sterile micropipette tip in confluent cultures of B16F10 murine melanoma cells, then washed gently with PBS to remove cellular debris. The cells were exposed to various concentrations of MCO (125, 250 μg/ml) or 0.1% DMSO as the solvent control. The wound closure was monitored and photographed at 0 and 24 h using an inverted microscope and camera (Nikon, Japan).

Evaluation of lung metastasis *in vivo* – C57BL/6 male mice (6-week-old), were injected subcutaneously with 5×10^5 B16F10 melanoma cells (0.2 ml/mouse) in FBS-free DMEM. The next day, mice were randomly divided into two groups (n = 4/group) and administered either MCO (500 mg/day/kg) or distilled water. After 14 days, animals

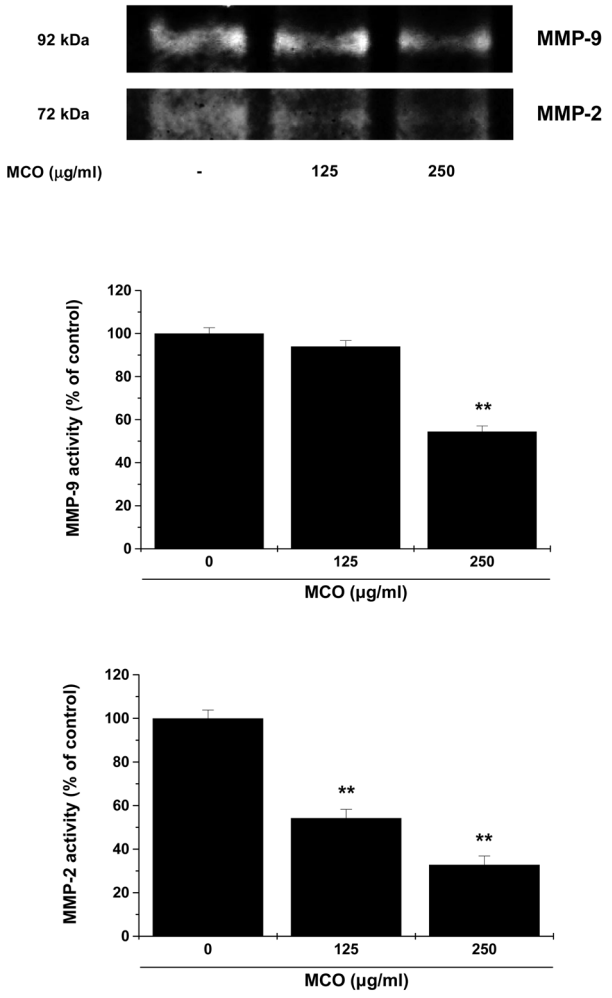

Fig. 3. Effects of MCO on MMP-2 and MMP-9 activities in B16F10 cells. B16F10 cells were treated with two concentrations of MCO for 24 h and then subjected to gelatin zymography. Determined activities of MMP-2 and MMP-9 were subsequently quantified by densitometric analysis and expressed as a percent of control. $^{**}p < 0.001$ compared with non-treated control group.

were sacrificed, and the metastatic nodules on the surface of lungs were counted using microscopy.

Densitometric and statistical analysis – The values are expressed as the mean ± S.D. or mean ± S.E.M depending on the experiment. Data between groups were analyzed by Student's unpaired 2-tailed *t*-test and *p*-values less than 0.01 were considered significant. Intensity of the bands obtained from western blotting and zymogram studies were estimated with ImageQuantTL (GE Healthcare, Sweden) and the values were expressed as mean ± S.E.M.

Results and Discussion

Metastasis, a major problem for cancer patients, is the

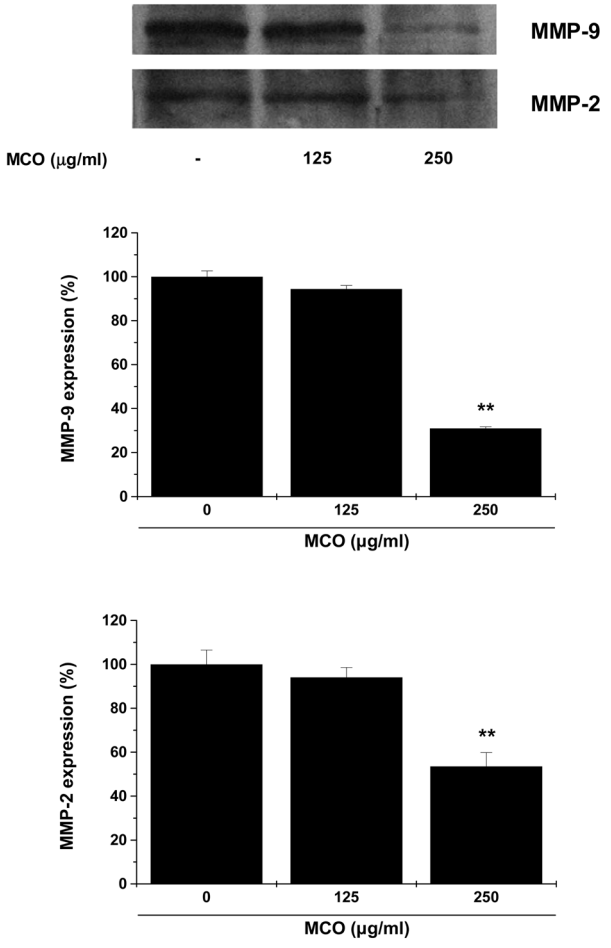

Fig. 4. Effects of MCO on the expression of MMPs in B16F10 melanoma cells. Protein extracts were prepared and samples were analyzed for MMP-9 expression by western blotting as described in the methods. Activities of MMP-9 were subsequently quantified by densitometric analysis and expressed as a percentage of the non-treated control. $^{**}p < 0.001$ compared with non-treated control group.

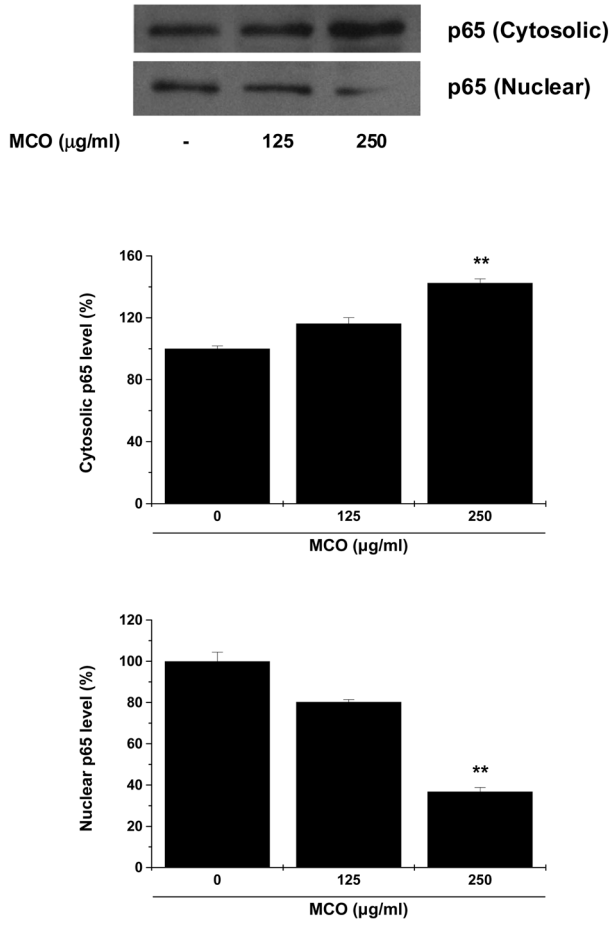

Fig. 5. Effects of MCO on NF-κB translocation in B16F10 cells. Cells were incubated with MCO for 18 h and the nuclear extracts were prepared; samples were analyzed by western blotting as described in the method and quantified by densitometry. $^{**}p < 0.001$ compared with non-treated control group.

spread of cancer cells from the primary neoplasm to secondary sites. It occur only when the cancer cells complete complex multi-step processes. Hence, any disturbance of these steps with a therapeutic target could be an attractive treatment for the inhibition of cancer metastasis. Since tumor cells must cross type IV collagen-rich basement membrane of vessel walls (Tryggvason *et al*., 1987) before entering blood vessels, degradation of the extracellular matrix is a one of the critical stages for the successful metastasis. It is well known that the proteolysis of the basement membrane is predominantly achieved by several matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9 which play a crucial role in type IV collagen degradation. Therefore, enhanced levels of MMP-2 and MMP-9 in many malignant tumor cells have been shown to be associated with the progression and invasion of tumors (Kohn and Liotta, 1995; Scorilas *et al*., 2001). In the present study, to determine whether MCO as an anti-metastatic agent, a bacterial collagenase-1 gelatin digestion assay was performed and MCO exhibited significant inhibitory effects on bacterial collagenase-1 activity (Fig. 1). Based on these results, further anti-metastatic studies with MCO were undertaken. Zymogram studies demonstrated that MCO inhibited enzymatic activities of both MMP-2 and MMP-9 in a dose-dependent manner (Fig. 3). Furthermore, MCO also down-regulated protein levels of MMP-2 and MMP-9 (Fig. 4). These results suggest that the decreased enzymatic activities of proteases by MCO were due to the suppression of protein expression. In the cell proliferation assay, MCO did not show any cytotoxicity at the treatment concentrations used in this study when incubated

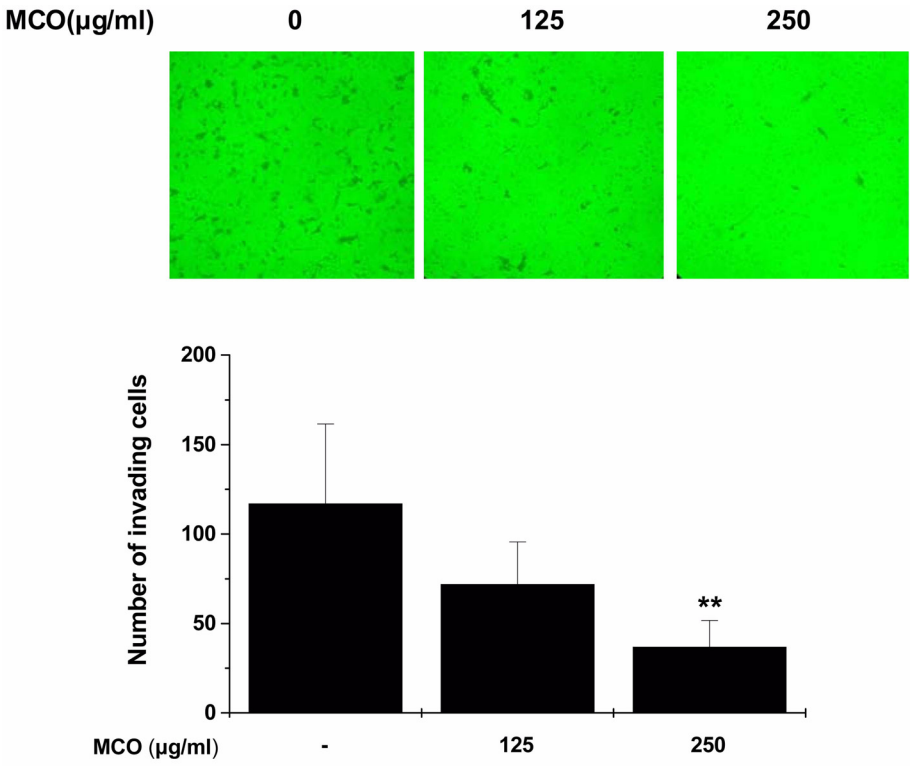

Fig. 6. Effects of MCO on cell invasion in B16F10 cells. B16F10 cells were treated with two concentrations MCO for 22 h using Matrigel-coated Transwells. Data shown are representative of three independent experiments. $^{**}p < 0.001$ compared to control group.

for 24 h with B16F10 cells (Fig. 2). Therefore, the anti-metastatic effects of MCO were not due to its cytotoxicity.

Previous reports have demonstrated that the activation of NF-κB in tumor cells may contribute to the expression of genes needed for invasion and metastasis genes, including MMP-2 and MMP-9 (Jiang *et al*., 2008). Moreover, accumulated NF-κB protein levels were observed in malignant cancers including colorectal cancer, breast cancer and malignant melanoma (Lind *et al*., 2001; Nakshatri *et al*., 1997; Yang and Richmond, 2001). Therefore, we examined whether MCO altered the translocation of NF-κB into the nucleus in B16F10 melanoma cells. MCO showed dose-dependent attenuation of NF-κB levels in the nucleus indicating down-regulation of metastatic gene expression (Fig. 5). The present result corresponded well with a report revealing that celastrol, isolated from *C. orbiculatus*, inhibited NF-κB activation (Lee *et al*., 2006).

Cancer cells must first migrate from the primary tumor as part of the invasive process in order to spread distant sites (Oppenheimer, 2006). Invasive cells are defined by several characteristics including altered adherence to the primary tumor, enhanced motility and increased proteolytic degradation of ECM components. Thus, inhibition of cell invasion and migration also could be a useful anti-metastatic strategy. We evaluated cell invasion using a Matrigel invasion assay. MCO attenuated invasion of B16F10 cells significantly in a dose-dependent manner (Fig. 6). Moreover, in a wound healing assay, the cell migration in B16F10 cells was also inhibited in the presence of MCO (Fig. 7). Recently, it has become evident that the gelatinases participate not only in degradation of the ECM matrix, but also in the stimulation the cell invasion and migration. As mentioned above, MMP-2 and MMP-9 were down-regulated by MCO, and therefore, these inhibitory actions may be a reasonable explanation for the limited invasiveness and motility of tumor cells by MCO.

In addition to anti-metastasis activity *in vitro*, the *in vivo* anti-metastatic effect of MCO was also investigated. MCO suppressed the formation of metastatic tumor nodules in the lung of C57BL/6 mice which were injected with B16F10 melanoma cells through the tail vein (Fig. 8). These results suggest that MCO exerted its anti-metastatic effect not only *in vitro* but also *in vivo*.

Recently many reports showed that celastrol, the main component of *C. orbiculatus*, has potent anti-metastatic properties. Yadav *et al*., (2010) noted that celastrol

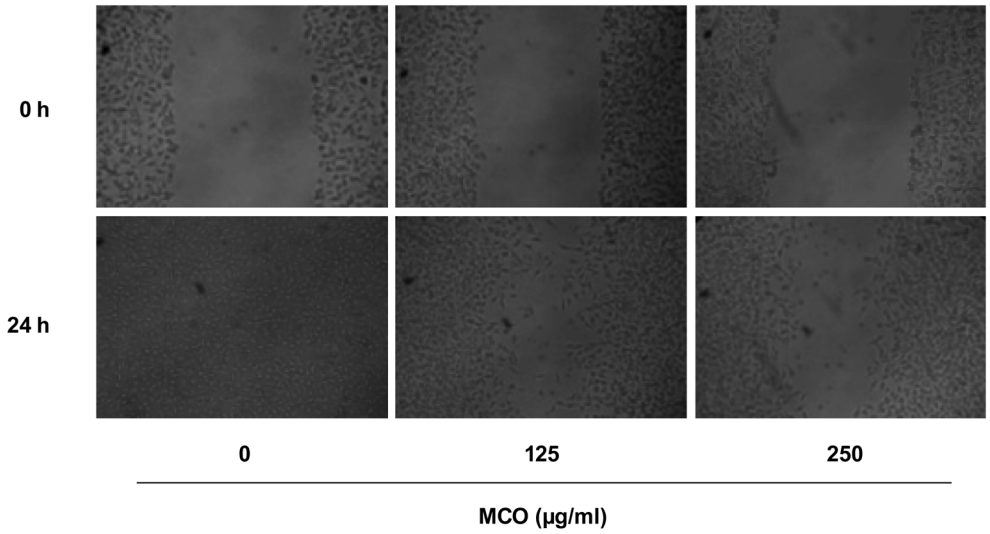

Fig. 7. Inhibitory effect of MCO on cell migration in B16F10 murine melanoma cells. B16F10 cells were plated in 12-well plates at a density of 2.5×10^5 cells/well in DMEM, supplemented with 10% FBS. Confluent monolayers were scratched using a pipet tip and then incubated in serum-free medium with or without various concentrations of MCO. Before and 24 h after scraping with the tip, cells were photographed under an inverted microscope.

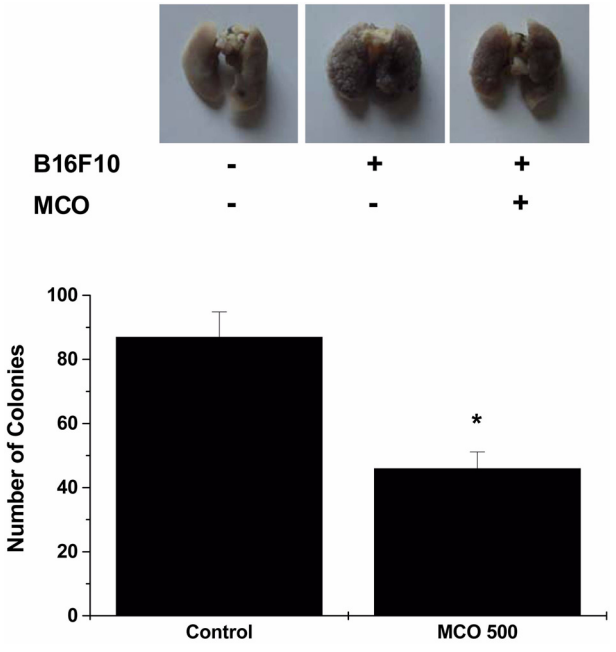

Fig. 8. Effects of MCO on lung metastasis. Lungs were photographed and observed for any metastasis on the 14th day after injection with B16F10 melanoma cells. The values represent the mean ± S.E.M. and comparisons were performed using a Student's *t*-test (*$p < 0.01$ compared to control group).

attenuates the expression of the CXCR4 chemokine receptor. In addition, Zhu *et al.* (2010) also revealed its inhibitory action on ECM adhesion. Thus, it can be concluded that celastrol at least in part, is responsible for the anti-metastatic activities of *C. orbiculatus*.

In conclusion, it was demonstrated that MCO had strong inhibitory effects on MMP-2 and MMP-9 enzyme activity and protein expression. Reduced cell invasion and migration were also apparent with MCO. Moreover, MCO reduced lung metastasis in an *in vivo* model. Based on these properties, MCO may hold great promise for cancer patients as in effective anti-metastatic agent.

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