Anti-metastatic Properties of the Leaves of *Eriobotrya japonica*

Dong Seok Cha, Tae Yong Shin, Jae Soon Eun, Dae Keun Kim, and Hoon Jeon  
College of Pharmacy, Woosuk University, Chonbuk 565-701, Korea

(Received June 22, 2010/Revised October 7, 2010/Accepted December 3, 2010)

The leaves of *Eriobotrya japonica* Lindl. have been widely used as a traditional medicine for the treatment of many diseases including gastroenteric disorders, diabetes mellitus, chronic bronchitis and asthma. In the present study, the anti-metastatic action of the EtOAc fraction of the leaves of *E. japonica* (LEJ) was investigated. LEJ showed potent inhibitory effects on MMP-2 and MMP-9 activities and expressions via down-regulation of NF-κB translocation to the nucleus in B16F10 cells. In addition, the cell migration and invasion were down-regulated by LEJ. LEJ also significantly suppressed lung metastasis in vivo. Moreover, we isolated the compounds ursolic acid and 2α-hydroxyursolic acid from LEJ and both compounds also significantly suppressed MMP-2 and MMP-9 activities, indicating that they are the active components of LEJ. The present results demonstrate that LEJ may be used as valuable anti-metastatic agent for the treatment of cancer metastasis.

**Key words:** *Eriobotrya japonica*, Matrix metalloproteinase, Cancer metastasis

---

**INTRODUCTION**

Cancer metastasis, a hallmark of malignancy, is defined as the spread and growth of cancer cells from the primary neoplasm to distant sites (Weiss, 1990). It is the principal cause of mortality among cancer patients and to date, no therapeutic option is available. Thus, it is critical to develop effective anti-metastatic agents.

Metastasis of cancer cells is generally described as a cascade of 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 membrane (BM) (Yoon et al., 2003). Therefore, the degradation of ECM and BM is a crucial event in the process of metastasis.

Matrix metalloproteinases (MMPs) are a multigene family of zinc-dependent endopeptidases that play a crucial role in the proteolysis of ECM and BM, which are essential physiological barriers that help prevent the invasion, metastasis and angiogenesis of tumors (McCawley and Matrisian, 2000). Although other MMPs are also involved in the metastatic process, the two gelatinases, MMP-2 and MMP-9, are recognized as key enzymes in tumor invasion and metastasis (Liabakk et al., 1996). They are abundantly expressed in various cancer cells and have been shown to play a crucial role in tumor invasion and metastasis (Johnsen et al., 1998). Therefore, inhibitors of MMP-2 or MMP-9 are attractive therapeutic targets against tumor invasion and metastasis.

*Eriobotrya japonica* Lindl., also known as 'loquat', belongs to the Rosaceae family. It originated in southeastern China and later became naturalized in Korea, Japan, India and many other countries. The leaves of *E. japonica* have been widely used as a traditional medicine with beneficial effects in numerous diseases, including asthma, gastroenteric disorders, diabetes mellitus and chronic bronchitis (Ito et al., 2000).

Various triterpenes, sesquiterpenes, flavonoids, tannins and megastigmane glycosides have been analyzed from the leaves of *E. japonica*, and some of them have been found to possess antitumor, antiviral, hypoglycemic and anti-inflammatory properties (Shimizu et al., 1986; De Tommasi et al., 1991; Ito et al., 2000;
Taniguchi et al., 2002; Kim and Shin, 2009). Recently, Kim et al. (2009) have shown that the methanol extract of *E. japonica* suppresses the adhesion, migration and invasion of a human breast cancer cell line. However, studies on the anti-metastatic activity of the active constituents of the leaves of *E. japonica* are extremely limited. Thus, the present studies were undertaken to investigate the inhibitory effect of the EtOAc fraction of the leaves of *E. japonica* (LEJ) and compounds isolated from LEJ on MMP-2 and MMP-9 activity and expression in B16F10 cells. We also describe its inhibitory effects on migration, invasion and lung metastasis in vivo.

**MATERIALS AND METHODS**

**General experimental procedures**

1H- and 13C-NMR spectra were determined on a JEOL JMN-EX 400 spectrometer. TLC was carried out on Merck precoated silica gel F254 plates and the spots were detected under UV and by spraying with 10% H2SO4 in ethanol followed by heating at 100–120°C for 3 min. The deionised water used was obtained from a Millipore ultra-pure water system (Millipore). All other chemicals and solvents used for extraction were analytical grade and used without further purification.

**Plant materials**

The plant materials were purchased from Hainyakupsa in October 2009. A voucher specimen (WOPE057) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University.

**Extraction and isolation**

The dried sample (2000 g) was extracted twice with 12,000 mL of MeOH by sonication for 2 h. The resultant methanolic extract was concentrated into 60.7 g (Yield: 3.035%) using a rotary evaporator. The sample was then subjected to successive solvent partitioning to give two subfractions (E21-E22). Subfraction E22 (50 mg) was purified by recrystallization from methyl alcohol to give compound 2 (9 mg). Compound 1 (Ursolic acid) was obtained by recrystallization of fraction E4 (20 mg) from methyl alcohol.

**Compound 1 (Ursolic acid)**

Colorless needles, m.p. 255-258°C; 1H-NMR (400 MHz, DMSO-d6 and pyridine-d5): δ ppm: 5.30 (1H, m, H-2), 3.22 (1H, dd, J = 10.0, 6.0 Hz, H-3), 2.60 (1H, d, J = 11.0 Hz, H-18), 1.13 (3H, s, H-23), 1.07 (3H, s, H-27), 0.94 (3H, s, H-26), 0.92 (3H, s, H-24), 0.90 (3H, d, J = 7.0 Hz, H-29), 0.89 (3H, d, J = 6.0 Hz, H-30), 0.85 (3H, s, H-5); 13C-NMR (100 MHz, DMSO-d6 and pyridine-d5): δ ppm: 179.65 (C-28), 139.00 (C-13), 125.45 (C-12), 77.83 (C-3), 55.64 (C-5), 53.25 (C-18), 47.86 (C-17), 47.74 (C-9), 42.40 (C-14), 39.87 (C-8), 39.64 (C-19), 39.28 (C-4), 39.16 (C-20), 38.99 (C-1), 37.23 (C-10), 37.19 (C-22), 33.47 (C-7), 30.94 (C-21), 28.91 (C-23), 28.40 (C-15), 27.75 (C-2), 24.65 (C-16), 23.90 (C-29), 23.56 (C-27), 21.61 (C-30), 18.70 (C-6), 17.61 (C-26), 17.59 (C-11), 16.70 (C-24), 15.79 (C-25).

**Compound 2 (2α-Hydroxyursolic acid)**

Colorless needles, m.p. 255-258°C; 1H-NMR (400 MHz, pyridine-d5): δ ppm: 5.45 (1H, t, J = 3.5 Hz, H-12), 4.10 (1H, dt, J = 3.9, 9.7 Hz, H-2β), 3.39 (1H, d, J = 9.7 Hz, H-3α), 2.61 (1H, d, J = 10.8 Hz, H-18), 1.26 (3H, s, H-23), 1.194 (3H, s, H-27), 1.06 (3H, s, H-24), 1.03 (3H, s, H-24), 0.99 (3H, d, J = 6.2 Hz, H-30), 0.96 (3H, s, H-25), 0.93 (3H, d, J = 6.6 Hz, H-29); 13C-NMR (100 MHz, pyridine-d5): δ ppm: 180.71 (C-28, COOH), 139.27 (C-13), 125.52 (C-12), 83.78 (C-3), 68.55 (C-2), 55.89 (C-5), 53.50 (C-18), 48.07 (C-17), 47.97 (C-1), 42.52 (C-14), 40.01 (C-8), 39.81 (C-4), 39.46 (C-20), 39.38 (C-19), 38.41 (C-10), 37.42 (C-22), 33.48 (C-7), 31.05 (C-21), 29.35 (C-23), 28.62 (C-15), 24.88 (C-16), 23.88 (C-27), 23.71 (C-11), 21.38 (C-29), 18.81 (C-6), 17.68 (C-24), 17.48 (2C, C-26, C-30), 16.95 (C-25).

**Animals**

Male C57BL/6 mice (5 weeks old) weighing 16-20 g were supplied by Damul Science. 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 culture**

B16F10 murine melanoma cells and HT-1080 human fibrosarcoma cells were obtained from the Korean cell line bank (KCLB) and cultured in DMEM containing 10% heat-inactivated FBS supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and sodium bicarbonate (2.2 g/L) at 37°C in a 5% CO2 and humidified air atmosphere. Cultures used in subsequent experiments were at < 50 passages.
Gelatin digestion assay

Gelatin digestion assay was 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 murine melanoma cells and human fibrosarcomas, which were then washed gently with PBS to remove cellular debris. The cells were exposed to various concentrations of the EtOAc fraction (125, 250, 500 µg/mL) and 0.2% DMSO as the solvent control. The wound closure was monitored and photographed at 0, 12, 24 and 36 h using an inverted microscope and camera (Nikon).

Matrigel invasion assay

B16F10 cells (1 × 10²) were added into the upper compartment of BioCoat Matrigel invasion chambers (BD biosciences) and cultured in serum-free DMEM in the presence of various concentrations of the EtOAc fraction. The 8 µm filter pores were precoated with Matrigel basement membrane matrix. The lower chambers were filled with DMEM containing 10% FBS as an chemoattractant. After 22 h of incubation, the noninvading cells were removed from the upper surface of the membrane. Then the lower surface of the membrane was fixed and stained with methanol and 0.5% crystal violet respectively. The membranes were photographed, and the invading cells were counted in 6 random fields of each filter.

Preparation of nuclear extract

Nuclear extracts were prepared as described previously (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 phenylmethylsulfonyl fluoride). 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% SDS, 20% glycerol and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-polyacrylamide gel electrophoresis and bands were observed as clear zones against the blue background.

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 murine melanoma cells and human fibrosarcomas, which were then washed gently with PBS to remove cellular debris. The cells were exposed to various concentrations of the EtOAc fraction (125, 250, 500 µg/mL) and 0.2% DMSO as the solvent control. The wound closure was monitored and photographed at 0, 12, 24 and 36 h using an inverted microscope and camera (Nikon).
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 MMP-9 for 3 h. After washing with PBS containing 0.05% Tween-20 three times, the blot was incubated with secondary antibody (anti-rabbit) for 2 h and the antibody specific proteins were visualized by an enhanced chemiluminescence detection system according to the recommended procedure (Millipore Corporation).

Evaluation of lung metastasis in vivo

Six week old C57BL/6 male mice were injected with $5 \times 10^5$ B16F10 melanoma cells (0.2 mL/mouse) in FBS-free DMEM subcutaneously. Mice were randomly divided into two groups (n = 4 for each group) the next day. Mice were administrated EtOAc fraction (500 mg/day/kg of body weight, respectively) or distilled water. After 14 days, animals were sacrificed, and the metastatic nodules on the surface of lungs were viewed and counted via microscopy.

Densitometric and statistical analysis

Values are expressed as the mean ± S.D. or mean ± S.E.M. depending on the experiment. Data comparing groups were analyzed by Student’s unpaired 2-tailed t-test and p-values less than 0.01 were considered significant. The intensity of the bands obtained from Western blotting and zymogram studies were estimated with ImageQuantTL (GE Healthcare) and the values are expressed as mean ± S.E.

RESULTS

Structures of isolated compound 1 and 2

Compound 1 was characterized as ursolic acid and its identity was determined by a comparison study with the data from in the literature (Jung et al., 1999). The identity of compound 2 was determined by the comparison of NMR spectral data to those reported in the literature (Numata et al., 1989; Kitajima and Tanaka, 1993). Based on the above evidence, compound 2 was determined to be 2α-hydroxyursolic acid, which was reported from this plant (Fig. 1).

Effects of LEJ on bacterial collagenase-1 activity

For the purpose of brief and easy screening of MMP inhibitors from various samples, we carried out a gelatin digestion assay using bacterial collagenase-1 as reported by Kim et al. (2006). As can be seen Fig. 2, the clear zone caused by bacterial collagenase activity was decreased following addition of LEJ (125, 250, 500 µg/mL) by approximately 9.0, 61.3 and 97.3%, respectively, compared to control group. Thus it appears highly likely that the LEJ may be an effective MMP
Anti-metastatic Properties of the Leaves of Eriobotrya japonica

inhibitor and based on these results, further studies were undertaken.

**Effect of LEJ and compounds on MMPs activity**

The effect of LEJ and its compounds on the activity of secreted MMP-2 and MMP-9 was examined using the zymography method in B16F10 cells. Trypsin activated culture supernatant of untreated control cells showed digested clear areas at 72 and 92 kDa which indicate MMP-2 and MMP-9, respectively (data not shown). However, addition of 10 mM EDTA to the zymogram development solution did not exhibit any clear bands. This result confirms that the gelatinolytic bands were created due to metalloproteinase activity (data not shown). As shown in Fig. 3, a notable reduction in band intensity of both MMP-2 and MMP-9 by the treatment of LEJ was observed in a concentration dependent manner. These results show that LEJ inhibits the enzymatic activity of MMP-2 and MMP-9 secreted from B16F10 cells. To determine the active components of the EtOAc fraction, we further performed gelatin zymography with the compounds isolated from the EtOAc fraction. As shown in Fig. 4, both compound 1 (ursolic acid) and compound 2 (2α-hydroxyursolic acid) showed significantly reduced MMP-2 and MMP-9 enzymatic activity, indicating that these compounds are responsible for the MMP inhibitory effect of the EtOAc fraction.

**Effect of LEJ on MMPs expression**

In order to determine whether LEJ could attenuate protein levels of MMP-2 and MMP-9 in B16F10 cells, we performed Western blotting. As shown in Fig. 5, untreated B16F10 melanoma cells exhibited potent expression of MMP-2 and MMP-9. Compared to the control, MMP-2 and MMP-9 expression was potently blocked in the presence of LEJ. The down-regulated protein levels were most noticeable at the maximum treatment concentration in both MMP-2 and MMP-9, demonstrating that LEJ could play a crucial role in the inhibition of MMP-2 and MMP-9 expression.

![Fig. 3. Effects of LEJ on MMP-2 and MMP-9 activity in B16F10 cells. B16F10 cells were treated with various concentration of the EtOAc fraction for 24 h and then subjected to gelatin zymography. The activity of MMP-2 and MMP-9 were subsequently quantified by densitometric analysis with the control considered to be 100% as shown just below the gel data. \(*p < 0.01, **p < 0.001\) compared with non-treated control group (means ± S.E.M., n = 3).](image)

![Fig. 4. Effects of isolated compounds on the activity of MMP-2 and MMP-9 in B16F10 cells. B16F10 cells were treated with 1.25 µg/mL of compound 1 or 2 for 24 h and then subjected to gelatin zymography. The activity of MMP-2 and MMP-9 was subsequently quantified by densitometric analysis with the control considered to be 100% as shown just below the gel data. \(*p < 0.001\) compared with non-treated control group (means ± S.E.M., n = 3).](image)
Effect of LEJ on NF-κB translocation

Because the transcriptional factor NF-κB has an important role in tumor progression and invasion we also investigated the effect of LEJ on the activation of NF-κB. To evaluate the effect of LEJ on the activation of NF-κB, Western blotting was performed using total, cytosolic, and nuclear extracts of B16F10 melanoma cells. As shown in Fig. 6, in resting conditions, the level of the NF-κB p65 subunit was constitutively enriched in nuclei of B16F10 melanoma cells. Incubation with LEJ for 18 h induced significant decrease in the levels of NF-κB in the nucleus. On the contrary, NF-κB expression in cytosol was increased by LEJ treatment in a dose dependent manner. However, LEJ did not affect the total NF-κB expression. These findings strongly...
suggest that LEJ inhibited the transcriptional activity of NF-κB in B16F10 melanoma cells.

**Effects of LEJ and isolated compounds on cell viability**

We also performed a cytotoxicity test on cancer cells using the MTT assay. B16F10 cells were pre-treated with various concentrations of LEJ or isolated compounds for 24 h. As shown in Fig. 7, the addition of LEJ had no cytotoxic effects on the cells, whereas the compounds which were isolated from LEJ altered the cell viability significantly in a dose-dependent manner. Compound 1 and 2 showed an approximately 85.71% and 27.55% decrease, respectively, in cell viability at the maximal concentration (Fig. 8). In HT-1080 cells, similar to that in B16F10 cells, the cell viability curve showed that LEJ did not affect the growth of the cells (Fig. 7). In contrast, Compound 1 and 2 showed dose-dependent cytotoxicity. As shown in Fig. 8, compound 1 had powerful cytotoxic properties compared to compound 2. These results indicate that the inhibitory properties of LEJ against tumor metastasis are not due to its cytotoxicity. In addition, the compounds isolated from LEJ had potent cytotoxic potential in B16F10 cells and HT-1080 cells.

**Effect of LEJ on cell invasion**

It is well-known that B16F10 cells have strong invasion properties in Matrigel. In this study, we investigate the inhibitory effect of LEJ on cell invasion using a BD BioCoat Matrigel chamber. Treatment with LEJ for 22 h demonstrated excellent suppression of melanoma cell invasion in a dose-dependent man-

![Fig. 7. Effects of LEJ on the viability of B16F10 and HT-1080 cells. Cell viability was evaluated by MTT colorimetric assay as described in the methods section. The results are expressed as mean ± S.D. of three independent experiments that were duplicated in each run.](image)

![Fig. 8. Effects of the isolated compounds on the viability in B16F10 and HT-1080 cells. Cell viability of B16F10 (A) and HT-1080 (B) was evaluated by the MTT colorimetric assay as described in the methods section. The results are expressed as mean ± S.D. of three independent experiments that were duplicated in each run. **p < 0.001 compared to control group.](image)

![Fig. 9. Effects of the EtOAc fraction on cell invasion in B16F10 cells. B16F10 cells were treated with various concentration of LEJ for 22 h using a Matrigel-coated transwell. Data shown are representatives of three independent experiments. **p < 0.001 compared to control group.](image)
In the 250 µg/mL and 500 µg/mL LEJ treatment groups there was a 62.20% and 69.88% inhibition of cell invasion compared to that of untreated groups. These results indicate that LEJ could prevent the spread of melanoma cells.

Effect of LEJ on cell migration
Cell migration (motility) is a critical process in invasion that allows primary tumors to metastasize. To investigate the inhibitory effect of LEJ on B16F10 murine melanoma and HT-1080 human fibrosarcoma cells migration, a wound-healing assay was performed. Treatment of B16F10 cells and HT-1080 cells with increasing concentrations of LEJ led to a concentration-dependent decrease in wound healing cell migration (Fig. 10).

Anti-metastatic effect of LEJ in vivo
Since the above results demonstrated that LEJ has potent inhibitory actions against metastasis in vitro, we wanted to confirm the anti-metastatic effects of LEJ in vivo. We induced lung metastasis through an intravenous injection of B16F10 melanoma cells using C57BL/6 mice. The mice treated with LEJ (500 mg/kg/day of body weight) had a 44.3% decrease in the number of metastatic lung colonies compared to that of the untreated control group, which was a significant difference ($p < 0.01$) (Fig. 11). In the acute toxicity test, no significant toxicity was observed at this dose level (data not shown). Thus, it can be concluded that LEJ has potent anti-metastatic activity in both in vitro and in vivo.
Anti-metastatic Properties of the Leaves of *Eriobotrya japonica*

**DISCUSSION**

Metastasis, a major problem for cancer patients, is the spread of cancer cells from the primary neoplasm to secondary sites. It occurs only when the cancer cells complete a complex multi-step process perfectly. Hence, any disturbance of these steps could be an attractive therapeutic target for preventing cancer metastasis.

Since tumor cells must cross a type IV collagen-rich basement membrane of vessel walls (Tryggvason et al., 1987) before entering blood vessels, degradation of the extracellular matrix is one of the critical stages in 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, a major component of the basement membrane. 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, in order to find a MMP inhibitor, the bacterial collagenase-1 gelatin digestion assay was performed. Among four fractions of the leaves of *Eriobotrya japonica*, only the EtOAc fraction (LEJ) exhibited a significant inhibitory effect on bacterial collagenase-1 activity. Based on these results, further studies were undertaken. Zymogram results from this study demonstrated that LEJ inhibited the enzymatic activity of both MMP-2 and MMP-9 in a dose-dependent manner. Furthermore, LEJ also down-regulated protein levels of MMP-2 and MMP-9. These results suggest that the decreased enzymatic activity of the proteases is due to the suppression of protein expression. In order to verify that the inhibitory effects of LEJ on MMPs are related to the compounds that were isolated from LEJ, we repeated the gelatin zymography with the isolated compounds. Interestingly, both compound 1 (ursolic acid) and compound 2 (2α-hydroxyursolic acid) reduced the lytic zone compared to the control in a dose-dependent manner. Thus, in this study, we have found a novel active compound from the leaves of *Eriobotrya japonica* that functions as an anti-metastatic agent.

In the cell proliferation assay, LEJ did not show any cytotoxicity at the treatment concentrations used in this study over a 24 h incubation with B16F10 or HT-1080 cells, and therefore the anti-metastatic effects of LEJ are not due to cytotoxicity. However, the compounds exhibited significant antiproliferative action in B16F10 and HT-1080 cells. The present data showed that compound 1 had powerful cytotoxic properties compared to compound 2 which had moderate cytotoxicity. However, the treatment concentration of each compounds in the zymogram analysis did not show any cytotoxicity, indicating that the compounds have inhibitory activity against MMPs at concentrations that do not induce cytotoxicity.

Previous reports have demonstrated that the activation of NF-κB in tumor cells may contribute to the expression of related invasion and metastasis genes including MMP-2 and MMP-9 (Jiang et al., 2008). Moreover, overexpressed nuclear translocation of NF-κB proteins have been observed in malignant cancers, including colorectal cancer, breast cancer and malignant melanoma (Nakshatri et al., 1997; Lind et al., 2001; Yang and Richmond, 2001). Therefore, we examined whether LEJ altered the translocation of NF-κB into the nucleus in B16F10 melanoma cells. LEJ showed dose-dependent attenuation of the NF-κB level in nucleus, indicating down-regulation of metastatic gene expression. The present results correspond well with several reports that revealed that ursolic acid reduced the levels of NF-κB (Shishodia et al., 2003).

**Fig. 11.** Effects of the EtOAc fraction on lung metastasis. The lungs were imaged and observed for any metastasis on the 14th day after injection of B16F10 melanoma cells with or without LEJ (500 mg/kg/day, *p.o*). The values represented the means ± S.E.M and the comparison were performed using Student’s *t*-test. *p < 0.01 compared to control group.
Cancer cells must first migrate from the primary tumor site at the beginning 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 could also be a useful anti-metastatic strategy. Here we evaluated the effect of LEJ on cell invasion using the Matrigel invasion assay. The results showed that LEJ significantly attenuated invasion of B16F10 cells in a dose-dependent manner. In the wound healing assay, the cell migration in both B16F10 and HT-1080 cells was inhibited in the presence of LEJ. Recently, it has become evident that the gelatinases participate not only in the degradation of the ECM matrix, but also in the stimulation of cell invasion and migration. As mentioned above, MMP-2 and MMP-9 were down-regulated by LEJ, and therefore, this inhibitory activity may provide a reasonable explanation for the limited invasiveness and motility of tumor cells treated by the EtOAc fraction.

In addition to examining anti-metastasis activity in vitro, investigation into the in vivo anti-metastatic effect of LEJ was also conducted. In the present study, we found that LEJ could suppress the formation of metastatic tumor nodules in the lung of C57BL/6 mice that were injected with B16F10 melanoma cells through the tail vein. These results suggest that LEJ exerts an anti-metastatic influence not only at an in vitro level but also on metastasis in vivo.

There is mounting evidence suggesting a relationship between the spread of tumors and inflammation. It is well-known that tumors contain a large number of infiltrating macrophages that are attracted by cytokines and chemokines, which may also promote tumor growth through an autocrine mechanism. These macrophages secrete many bioactive molecules including IL-8, VEGF, MMP-9, uPA, growth factor and PDGF, which results in an elevation of the metastatic ability of cancer cells (Brooks et al., 2010). In addition, many reports suggest that COX-2-mediated prostaglandin synthesis in the immune cells also contribute to metastatic progression (Dannenberg and Subbaramaiah, 2003; Minn et al., 2005; Wyckoff et al., 2007). Thus, the use of anti-inflammatory agents might be an effective approach for the anti-metastatic therapy. Huang et al. (2009) and Lee et al. (2008) noted that the triterpenes of LEJ had inhibitory effects on pro-inflammatory mediators. Since we isolated triterpenes from LEJ, it is possible that the anti-inflammatory activity of LEJ may be, at least in part, associated with the current anti-metastatic properties. Moreover, Szatrowski and Nathan (1991) noted that the ROS also could be related to various aspects of tumor metastasis. Because several different MMPs can be positively regulated by the intracellular redox state (Yoon et al., 2002), it is believed that ROS are involved in the process of cancer metastasis. Several reports suggest that LEJ has potent antioxidant activities (Huang et al., 2006; Eraso and Albesa, 2007), and thus, we thought that there might be a correlation between the antioxidant actions and anti-metastatic activities of LEJ.

These findings strongly indicate that LEJ and its compounds have potent anti-metastatic potential and are in agreement with literature data demonstrated by Kim et al. (2009). That report showed that the 80% MeOH extract of loquat leaf showed an inhibitory effect on the adhesion, migration and invasion of human breast cancer cells partially through the inhibition of MMP activity. In the present study, we further confirmed the anti-metastatic action of LEJ in other cancer cell lines such as B16F10 and HT-1080. Furthermore, the active triterpene compounds ursolic acid and 2α-hydroxyursolic acid were isolated from LEJ. Nevertheless, further research is required to clarify the anti-metastatic mechanism of LEJ and those compounds.

In summary, we demonstrated that LEJ has potent inhibitory effects on MMP-2 and MMP-9 enzymatic activity and protein expressions. In addition, reduced cell invasion and migration were observed in the presence of LEJ. LEJ also reduces lung metastasis induced by injection of B16F10 melanoma cells in mice. Moreover, compounds isolated from LEJ that were identified as ursolic acid and 2α-hydroxyursolic acid caused a significant decrease in MMP activity, indicating that they could be the active components of LEJ anti-metastatic activity.

ACKNOWLEDGEMENTS

This work was supported by the Grant of the Korean Ministry of Education, Science and Technology (The Regional Core Research Program/Healthcare Technology Development) and a research grant from Woosuk University.

REFERENCES


Raphael, T. J. and Kuttan, G., Effect of naturally occurring triterpenoids ursoic acid and glycyrrhizic acid on the cell-


