Methanol extract of *Elaeagnus glabra*, a Korean medicinal plant, inhibits HT1080 tumor cell invasion

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Abstract. *Elaeagnus glabra* (Thunb.), an evergreen shrub with alternate leaves, has been used as a medicinal plant in Korea. Since many plant-derived molecules have inhibitory effects on tumor cell invasion, primarily via suppression of the activity of matrix metalloproteinases (MMPs), we investigated the effect of the methanol extract of *E. glabra* on tumor cell invasion. The invasiveness of HT1080 human fibrosarcoma cells were reduced in a dose-dependent manner following 24 h treatment of up to 200 μg/ml of the *E. glabra* extract, at which concentration no cytotoxicity occurred. Furthermore, gelatinolytic activities, and the protein and mRNA levels of MMP-2 and MMP-9 were also suppressed with increasing concentrations of the extract. Given that MMP-2 and MMP-9 are instrumental in tumor cell invasion, it is very likely that the reduction in tumor cell invasion by *E. glabra* extract is a consequence, at least in part, of suppressed expression of both MMP-2 and MMP-9. Isolation of a molecule(s) responsible for the extract inhibition of tumor cell invasion would pave the way for the development of a new generation of metastasis inhibitors.

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that remodel and degrade the extracellular matrix (ECM). More than 25 MMPs have been identified to date and MMPs are classified based on their substrate specificity and structural similarity (1-3). The ECM has a complex structure that influences the function and migration process of its resident cells by offering specific contextual information (4). MMP-mediated degradation of ECM is a hallmark in several pathologic conditions such as arthritis, inflammation, cancer, angiogenesis, cardiovascular, pulmonary, ocular, gastrointestinal and oral diseases (5,6).

Invasion and metastasis, both fundamental properties of malignant cancer cells, are the end result of a complex series of steps involving multiple tumor-host interactions (7,8). Cancer cells metastasize through a series of the following sequential steps: escape from the primary tumor, migration and invasion of surrounding tissues, entrance into the vasculature, transport through the circulatory system, extravasation and growth in a secondary organ (7,9,10). Among these steps, cancer cell migration and invasion of surrounding tissues are mediated in part by MMPs, especially MMP-2 and MMP-9 (11-13).

Many plant-derived compounds possess antitumor activity (14-16) and many Korean medicinal plants have been shown to exert an inhibitory effect on MMP-9 (17,18). *Elaeagnus glabra* (Thunb.) is an evergreen shrub or small tree with alternate leaves, belongs to the Elaeagnaceae family, inhabits East Asia (especially Korea, Japan and China) and is reported to have anti-bacterial, procoagulant, anti-asthmatic and anti-diarrheal effects (19). Other species of the *Elaeagnus* genus have been documented to possess therapeutic effects as well. For example, *E. angustifolia* has anti-nociceptive, anti-inflammatory and muscle relaxing activities (20-22) and *E. multiflora*, anti-oxidant and anti-inflammatory activities (23).

In this study, we investigated the inhibitory effect of the methanol extract of the bark derived from *E. glabra* on tumor invasion using a human fibrosarcoma cell line HT1080.

Materials and methods

Plant extract. The methanol extract of *Elaeagnus glabra* bark was purchased from the Plant Extract Bank (Daejeon, South Korea).

Cell culture. A human fibrosarcoma cell line HT1080 was purchased from the American Type Culture Collection (ATCC, USA). The cells were cultured in minimum essential medium (MEM; Gibco, USA) containing penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum

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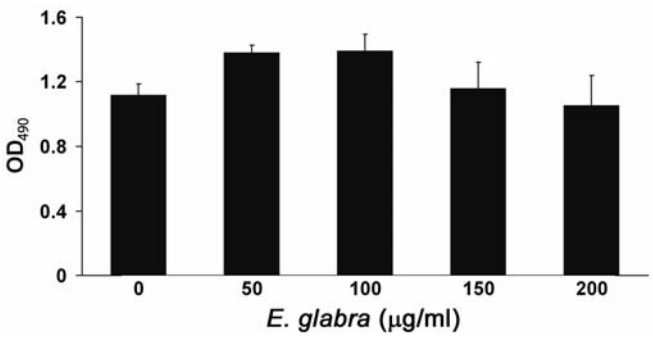

Figure 1. The methanol extract of *E. glabra* at concentrations up to 200 μg/ml does not exert cytotoxicity on HT1080 cells. HT1080 cells in serum-free MEM were left untreated or treated with the indicated concentration of the *E. glabra* extract, incubated for 24 h and subjected to MTT assay for quantifying cell growth. The bar graph shows the absorbance at 490 nm measured on an ELISA reader (n=3 independent experiments; mean ± standard deviation is shown).

(FBS; Gibco; denoted as complete MEM hereafter) at 37˚C in 5% CO_2 air. After the cells adhered, the media was replaced with serum-free MEM and the indicated concentration of the extract was added. Conditioned media were collected and the cells were harvested after a 24-h incubation at 37˚C in 5% CO_2 air.

MTT cell viability assay. HT1080 cells were seeded onto a 96-well culture plate at a density of $4x10^4$ cells/well in 200 μl of complete MEM and incubated overnight. On the second day of culture, the media was replaced with 200 μl of serum-free MEM and treated with the indicated concentration of *E. glabra* extract (0-200 μg/ml). On the third day, 100 μg of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, USA) was added to each well and incubated for 4 h. The media was then discarded and 100 μl of dimethyl sulfoxide (DMSO; Sigma) was added. Absorbance was measured at 490 nm on an ELISA reader.

Invasion assay. HT1080 cells ($5x10^4$) in 250 μl of complete MEM were seeded in the upper chamber of a 10-well chemotaxis chamber (Neuro Probe, USA) and serum-free MEM was placed in the lower chamber. A Matrigel-coated membrane was inserted between the two chambers. Following overnight incubation at 37˚C, the media in the upper chamber was replaced with serum-free MEM and treated with the indicated concentration of *E. glabra* extract. Upon an additional 24-h incubation at 37˚C in 5% CO_2 air, the membrane was fixed and stained with a Hemacolor rapid staining kit (Merck, Germany) as per manufacturer's instructions.

Gelatin zymography. The quantity of protein in the conditioned media was determined with a BSA protein assay kit (Pierce, USA). Subsequently, the conditioned media was mixed with an equal volume of 2X sample loading buffer (62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 4% sodium dodecyl sulfate (SDS) and 0.01% bromophenol blue; Bio-Rad, USA) and loaded onto a 7.5% acrylamide:bis-acrylamide (29:1; Bio-Rad) gel containing 625 μg/ml gelatin (Sigma). Upon electrophoresis at 100 V for 2 h, the gel was soaked in 1X zymogram renaturation buffer (Bio-Rad) on a rocker for 1.5 h at room temperature to remove residual SDS, rinsed in distilled water, incubated at 37˚C for 18 h in 1X zymogram development buffer (Bio-Rad), stained with 0.25% (w/v) coomassie brilliant blue R-250 (Bio-Rad) and then destained in destaining buffer (10% acetic acid and 20% methanol).

Western blotting. HT1080 cells were treated with the indicated concentration of methanol extract of *E. glabra* for 24 h and the conditioned media were harvested. The cells were then scraped into 1X cell lysis buffer (Cell Signaling, USA) and incubated for 10 min on ice. The resulting cell lysate was cleared by centrifugation at 6,700 x g at 4˚C for 5 min. The supernatant containing cytosolic proteins was collected and the protein concentration of the supernatant and the conditioned media was measured with a BSA protein assay kit. The conditioned media or the cell lysate, with the same amount of protein, was mixed with an equal volume of 2X sample loading buffer, boiled for 5 min, cooled on ice for 5 min and then analyzed by 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the separated proteins were transferred to a nitrocellulose membrane (Amersham, USA). The membrane was blocked with 5% skim milk in 1X TBST [0.01 M Tris (pH 7.6), 0.1 M NaCl and 0.1% Tween-20] for 2 h at room temperature with shaking and incubated with the indicated primary antibody, followed by HRP-conjugated secondary antibody. The immunoreactive protein bands were visualized with enhanced chemiluminescent reagents (Amersham).

Northern blotting. HT1080 cells treated with the indicated concentration of methanol extract of *E. glabra* for 24 h were washed with ice-cold 1X phosphate buffered saline (PBS) twice and the total RNA was extracted with TRIzol Reagent (Invitrogen, USA) as per manufacturer's instructions. The extracted total RNA (20 μg) was separated on a 1% agarose-formaldehyde gel in 1X 4-morpholinepropanesulfonic acid (MOPS) buffer [20 mM MOPS (pH 7.0), 1 mM EDTA (pH 8.0) and 2 mM sodium acetate]. The separated RNA was transferred onto a Hybond-N membrane (Amersham) by capillary transfer in 20X SSC [3 M NaCl and 0.3 M sodium citrate (pH 7.0)] and immobilized by UV cross-linking. The membrane was incubated in Rapid-Hyb buffer (Amersham) harboring human MMP-2 and MMP-9 cDNA probes radio-labeled with [α-^{32}P]dCTP using a random primer method, washed and exposed to X-ray film.

Statistical analysis. Intensity of bands in images was quantitated with the NIH ImageJ software (24). Results are expressed as mean ± standard deviation. Statistical significance was determined at $p<0.05$ using Student's t-test.

Results

E. glabra extract is not toxic to HT1080 cells. HT1080 is a human fibrosarcoma cell line that has been extensively used to study the migration and invasion of tumor cells. Moreover, HT1080 cells express high levels of MMP-2 and MMP-9 (25), which play an important role in tumor cell invasion.

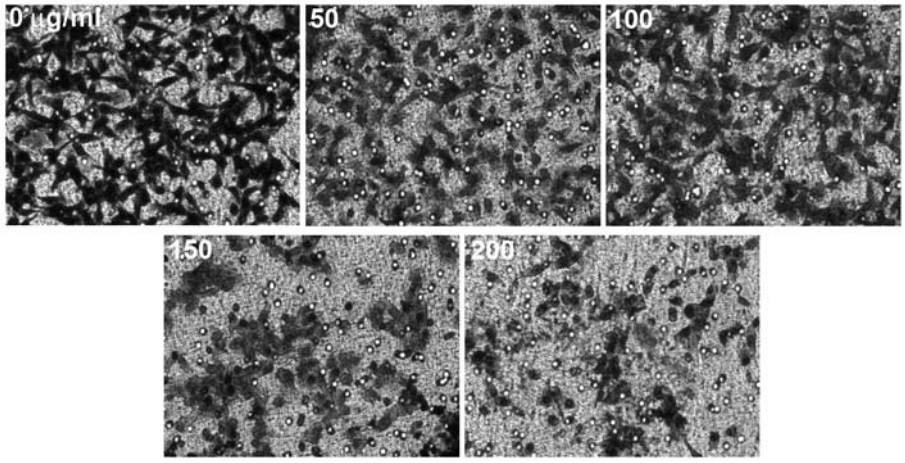

Figure 2. The methanol extract of *E. glabra* impedes invasiveness of HT1080 cells. A 10-well chemotaxis chamber was used to measure HT1080 cell invasiveness upon treatment with the indicated concentration of the *E. glabra* extract. A Matrigel-coated membrane inserted between the upper and lower chambers was stained with a Hemacolor rapid staining kit. The stained area represents cells which have migrated from the upper chamber. The number in each panel denotes the concentration of the *E. glabra* extract added. Each image is representative of 3 independent experiments.

Therefore, we chose HT1080 cells to investigate the effect of *E. glabra* on tumor cell invasion.

To test if the *E. glabra* extract is cytotoxic, HT1080 cells were cultured with *E. glabra* extract (0-200 μg/ml) for 24 h and then subjected to MTT assay. The methanol extract of *E. glabra* did not significantly inhibit the growth of HT1080 cells at concentrations ranging between 0 and 200 μg/ml (Fig. 1), suggesting that the *E. glabra* extract has no significant effect on the HT1080 cell survival. Thus, we performed all subsequent experiments with the extract concentrations ranging between 0 and 200 μg/ml.

E. glabra extract inhibits HT1080 cell invasion. Since many natural plant products exhibit an anti-invasiveness effect (26), we questioned whether the *E. glabra* extract inhibits tumor cell invasion. To this end, we carried out a Matrigel invasion assay with HT1080 cells treated with the *E. glabra* extract. The extract inhibited the invasive activities of HT1080 cells in a dose-dependent manner (Fig. 2), demonstrating that the *E. glabra* extract has an anti-invasiveness effect, at least with respect to HT1080 cells.

E. glabra extract inhibits MMP-2 and MMP-9 activities. Since MMP-2 and MMP-9 play a pivotal role in tumor cell invasiveness, we wished to assess the effect of *E. glabra* extract on MMP-2 and MMP-9 enzyme activities. For this goal, we performed gelatin zymography with conditioned media harvested from the extract treated HT1080 cells. The gelatinolytic activities of both MMP-2 and MMP-9 were reduced with increasing concentrations of the extract (Fig. 3), suggesting that a decrease in HT1080 cell invasion is a consequence, at least in part, of reduced activities of both MMP-2 and MMP-9.

E. glabra extract reduces MMP-2 and MMP-9 protein levels. MMP enzyme activity is regulated at both transcriptional and post-transcriptional levels (27). To determine whether the reduced MMP-2 and MMP-9 enzyme activities were caused by a decrease in MMP-2 and MMP-9 protein levels, HT1080

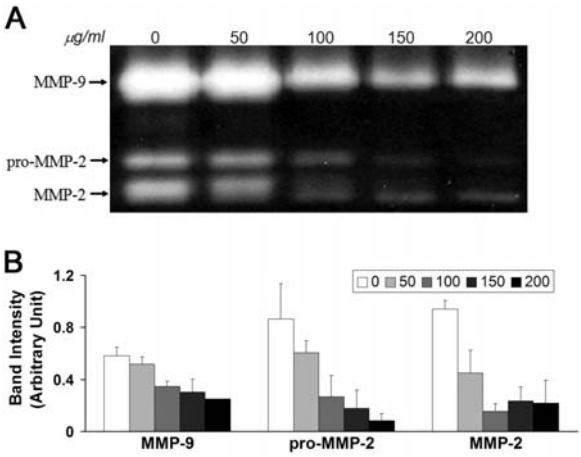

Figure 3. The methanol extract of *E. glabra* inhibits gelatinolytic activities of MMP-2 and MMP-9. (A) The conditioned media harvested from the HT1080 cells treated for 24 h with the indicated concentration of the *E. glabra* extract were analyzed by gelatin zymography. The white bands represent MMP-mediated gelatin digestion. The image is representative of three independent experiments. Quantitation of band intensity is shown in B (n=3). Numbers in the box represent the concentration of the extract in μg/ml added to the cells. Bars represent the average intensity of each band ± standard deviation.

cells treated with the *E. glabra* extract were processed for Western blotting along with their conditioned media. MMP-2 and MMP-9 protein levels were reduced in a dose-dependent manner in both conditioned media (Fig. 4B and D) and cells treated with the extract (Fig. 4A and C), indicating that decreased gelatinolytic activities of MMP-2 and MMP-9 in the extract treated cells ensue from, at least in part, the down-regulation of MMP-2 and MMP-9 proteins.

E. glabra extract suppresses MMP-2 and MMP-9 mRNA levels. To examine whether the decreased MMP-2 and MMP-9 protein levels in the HT1080 cells treated with the *E. glabra* extract result respectively from the reduction in MMP-2 and MMP-9 mRNA levels, we turned to Northern blotting to measure their mRNA levels. Expression of both

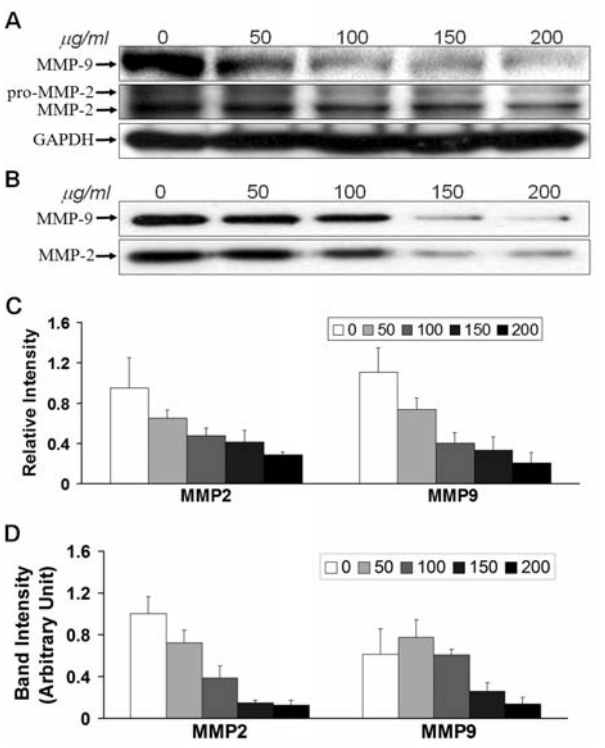

Figure 4. The methanol extract of *E. glabra* reduces expression of MMP-2 and MMP-9 proteins in HT1080 cells. The cells were treated with the indicated concentration of the *E. glabra* extract for 24 h. Subsequently, the cells (A and C) and their conditioned media (B and D) were processed for Western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Each image is representative of 3 independent experiments. Quantitation of MMP-2 and MMP-9 band intensity in A normalized to that of GAPDH is shown in C (n=3). Quantitation of band intensity in B is shown in D (n=3). Numbers in the box in C and D represent the concentration of the extract in μg/ml added to the cells. Bars indicate the average intensity of each band ± standard deviation.

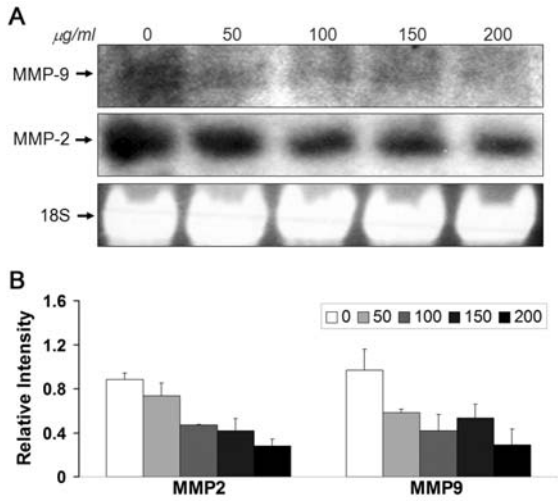

Figure 5. The methanol extract of the *E. glabra* suppresses expression of MMP-2 and MMP-9 mRNAs in HT1080 cells. (A) The cells were treated with the indicated concentration of *E. glabra* extract for 24 h and then processed for Northern blotting. Ribosomal RNA 18S was used as a loading control. Images are representative of 3 independent experiments. Quantitation of band intensity in A normalized to that of ribosomal RNA 18S is shown in B (n=3). Numbers in the box represent the concentration of the extract in μg/ml added to the cells. Bars denote the normalized average intensity of each band ± standard deviation.

MMP-2 and MMP-9 mRNA decreased in the extract treated cells with increasing concentrations of the extract (Fig. 5), signifying that the decrease in MMP-2 and MMP-9 protein levels in the extract treated cells reflects, at least in part, the reduction in MMP-2 and MMP-9 mRNA levels, respectively.

Discussion

Herein, we show that the methanol extract of *E. glabra* suppresses cell invasion, gelatinolytic activities, and protein and mRNA expressions of both MMP-2 and MMP-9 in HT1080 cells. In light of the instrumental role for MMP-2 and MMP-9 in tumor cell invasion, it is very likely that *E. glabra* extract inhibition of MMP-2 and MMP-9 expression leads to a decrease in gelatinolytic activity of MMP-2 and MMP-9, which in turn dampens tumor cell invasion. However, we cannot rule out the following possibilities: i) the *E. glabra* extract inhibits tumor cell invasion independently of reduction in MMP-2 and MMP-9 activity or expression; ii) the *E. glabra* extract directly inhibits MMP-2 and MMP-9 proteins independently of a reduction in MMP-2 and MMP-9 expression, leading to suppression of tumor cell invasion; iii) the *E. glabra* extract promotes activity of MMP inhibitors, e.g., tissue inhibitors of metalloproteinase (TIMP), thereby repressing MMP activity. Further work is required to clarify this issue.

Even though we demonstrate here that the *E. glabra* extract inhibits HT1080 tumor cell invasion, which molecule(s) in the extract is responsible for the inhibitory effect remains unknown. There have been several studies on plant-derived molecules inhibiting MMP or tumor cell invasion. For instance, polyphenol, epigallocatechin gallate and epicatechin gallate, all isolated from green tea, individually inhibit MMP-2 and MMP-9 (28,29); obovatal isolated from *Magnolia obovata*, MMP-2 and tumor cell invasion (30); curcumin, isolated from *Curcuma longa*, MMP-2, MMP-9 and tumor metastasis (31); and quercetin, a flavonol present in many vegetables and fruits including onions and apples, MMP-2 and MMP-9 (32). Therefore, it would be of high interest to explore whether it is one of these molecules, a novel molecule(s), or both that accounts for the inhibitory effect of the *E. glabra* extract on tumor cell invasion.

Although MMP is a promising target for anticancer therapy, efforts to develop MMP inhibitor with few side effects have been unsuccessful thus far. In this regard, screening natural products, especially plant products, for anti-MMP activity followed by determining which molecule in the product is responsible for the activity would shed light on the development of a new generation of MMP inhibitors.

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