

A fraction of methylene chloride from *Geum japonicum* Thunberg inhibits tumor metastatic and angiogenic potential

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Abstract. The plant *Geum japonicum* Thunberg (GjT) has been used as a diuretic in traditional medicine. Herein, we report that the GjT extract blocks both the spread of human umbilical vein endothelial cells (HUVECs) on matrigel and the migration of B16 cells. We used various assays to test for cell attachment, spreading, wound healing and angiogenesis. A reverse transcription-polymerase chain reaction (RT-PCR) and a mitogen-activated protein kinase (MAPK) assay were also carried out for the mechanistic study of GjT. Our results showed that a fraction of methylene chloride fraction from GjT inhibited B16 cells during cell attachment and migration and suppressed tube formation in a dose-dependent manner. An RT-PCR analysis showed that the methylene chloride extract decreased the mRNA expression of CD44 and TIMP-2. A Western blot analysis of the phosphorylation of MAPK kinases (ERK, JNK and p38) showed that the GjT fraction increased the expression of phospho-JNK, suggesting that GjT has the potential to alleviate metastatic and angiogenic activity, via a phospho-JNK signaling pathway.

Introduction

Cancer is a dynamic process that exhibits the complicated diagnostic symptoms of various types of tissues (1). Cancer progression/metastasis, a hallmark of the disease, is a perplexing sequence of steps, whereby cells intrude neighboring tissue. It is well-known that cells penetrate into blood vessels, circulate and colonize at distant sites, thus becoming metastatic lesions (2).

Angiogenesis is the formation of new blood vessels from a pre-existing vasculature which occurs under physiological conditions (3). The process develops in a multi-step process that is comprised of the peri-vascular detachment of existing vessels, matrix degradation, the migration of endothelial cells and the formation of a functional vascular plexus (4). In a solid tumor, angiogenesis is well-characterized as a critical step for growth, invasion and metastasis. Tumor growth is caused by an imbalance of angiogenic factors in the tumor microenvironment (5). It has been reported that tumor cells promote vessel formation by angiogenic molecules, such as vascular epithelial (VEGF), basic fibroblast (bFGF) and hepatocyte growth factor (HGF), matrix metalloproteinase (MMP) and other tumor-induced molecules (6). As a result, tumor cells were developed by matrix remodeling, migration, stabilization and macrocapillary formation (3). New anti-angiogenic compounds have led to a new target in the treatment of cancer.

In clinical trials, various molecular markers such as matrix metalloproteinases (MMPs), CD44, intracellular cell adhesion molecules (ICAM), vascular cell adhesion molecules (VCAM) or urine plasminogen activator receptors (uPAR), play a key role in alleviating tumor cell proliferation during the progression of tumor cells (7). For this reason, a further understanding of the molecular mechanisms of chemotherapeutic agent(s), compound(s), fraction(s) and extract(s) is needed.

Traditional medicines have been effective in treating various degenerative diseases and they have been widely used in the world, especially in Asian countries. We have already documented that several medicinal plants (*Wisteria floribunda*, *Gastrodia elata* Blume and *Salvia miltiorrhiza* Bunge), have been shown to decrease the spread of cancer and metastasis (8-10). In the course of screening for anti-

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Abbreviations: GjT, *Geum japonicum* Thunberg; HUVECs, human umbilical vein endothelial cells; MAPKs, mitogen-activated protein kinases; CD, cluster of differentiation; TIMP, tissue inhibitor of metalloproteinase; RT-PCR, reverse transcription-polymerase chain reaction; MMP, matrix metalloproteinase

Key words: *Geum japonicum* Thunberg, angiogenesis, cell migration, mitogen-activated protein kinase

tumor agents, we determined that G_jT has a potent anti-tumor potential of alleviating tumor cell growth. The whole plant of *Geum japonicum* Thunberg (G_jT) has long been used as a diuretic and as an astringent in folk medicine in Korea, China and Japan. Xu *et al* reported that triterpene acids from G_jT exhibited activity against HIV (11,12). It has also been reported that eugenin, tannins and several other fractions are closely involved in regulating anti-herpes symptoms, hypertension and muscle ischemia (13-15).

In this study, our present results show that G_jT has the potential to inhibit metastatic and angiogenic activity. We demonstrated that an active component of G_jT exhibits anti-angiogenesis and anti-migration activity, thus suggesting that the fraction will shed light on the development of G_jT-derived traditional medicine. Therefore, our goal is to focus on the elucidation of molecular mechanisms regarding the anti-tumor effects of G_jT, by examining the inhibition of cell proliferation during the attachment, spread, migration and angiogenesis processes in B16 and HUVEC cells.

Materials and methods

Fractions of *Geum japonicum* Thunberg. *Geum japonicum* Thunberg (G_jT) was collected from the Laboratory of Molecular Physiology and Biochemistry, National Institute of Agricultural Biotechnology (NIAB) and dried in an oven at 50°C. After grinding, the powder was extracted three times with 80% methanol and the extracts were filtered with filter paper (Whatman No. 2). The extracts were concentrated in a rotary vacuum evaporator at 45°C before 80% of the methanolic fraction was freeze-dried. A fraction of the methanol was fractionated three times with an equal volume of hexane and water (1:1) and then the hexane fraction was concentrated under pressure. The fractions of methylene chloride and H₂O (1:1), ethyl acetate and H₂O (1:1) were conducted in the same way. The yield of each extract is shown in Table I.

Attachment and spreading assays. Attachment and spreading assays were carried out as previously described (9). Briefly, the B16 cells spread to the bottom of each well of the 6-well plates (Greiner, Frickenhausen, Germany) at a concentration of the G_jT extract (1x10⁴ cells/well) for 2 h (attach assay) or 24 h (spreading assay) in a 5% CO₂ incubator at 37°C. The plates were stained by a hematoxylin and eosin (H&E) stain and photographed, and the attachment ratio was measured and compared to the control.

In vitro wound healing assay. The mouse melanoma cell line B16F1 (B16, Catalog no. CRL-6323) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). B16 cells were cultured in RPMI-1640 supplemented with a 10% FBS in a 5% CO₂ incubator at 37°C. The B16 cells were attached to the bottom of each well of the 6-well plates (Greiner) and the B16 cells were plated at 1x10⁷ cells/well and allowed to attach for 3-5 h at 37°C in a 5% CO₂ atmosphere. The wound lines were made with a blade, drawn on the plate and the medium was changed. The plates were then photographed and incubated as above with a medium containing various concentration levels of the G_jT extract. The plates were

Table I. Yield of methylene chloride fraction.

Fraction	Weight, g (Yield, %)
<i>Geum japonicum</i> Thunberg power	1500.0 (100.0)
Methanol fraction	385.0 (25.7)
n-Hexane fraction	2.7 (0.7)
Methylene chloride fraction	2.3 (0.6)
Ethyl acetate fraction	18.0 (4.7)
Aqueous fraction	178.0 (48.6)

photographed at 20 h and the wound width was calculated by a microruler (<http://www.eeob.iastate.edu/faculty/DrewsC/htdocs/microruler-links.htm>) (16).

In vitro tube formation assay. An *in vitro* tube formation assay was carried out as previously described (17). Human umbilical vein endothelial cells (HUVECs), which were obtained from the ATCC (passage 2-10), were maintained in EGM-2 medium for HUVECs (Cambrex, no. CC-4176, Walkersville, MD, USA) containing 2.5% of a fetal bovine serum (FBS, Cambrex, Walkersville, MD, USA), hEGF, hydrocortisone, GA-100 (gentamycin, amphotericin-B), VEGF, hFGF-B (w/heparin), R³-IGF-1, ascorbic acid and heparin.

For an *in vitro* tube formation assay, 24-well plates were coated with matrigel (BD Bioscience, no. 354234, Franklin Lakes, NJ, USA) at a concentration of 100 µl/well, of a medium according to the manufacturer's instructions. After incubation for 2-3 h, the HUVECs were plated on coated dishes at a concentration of 2x10⁴ cells/well and incubated at 37°C in a 5% CO₂ incubator for 24-48 h. Tube formation was counted by an inverted microscope (Nikon, Tokyo, Japan). The cell images were taken by a Nikon camera (Model No. TS100, Tokyo, Japan).

RT-PCR analysis of the gene expression. Total RNA was extracted by Tri reagent (MRC, catalog no. TR 118), according to the manufacturer's manual. Total RNA (1-10 µg) from the tumor cells was converted to first-strand cDNA that was primed with random primer (or Oligo dT) in a reaction volume of 20 µl, using an RNA PCR kit (iNtRON Biotechnology, Sungnam, Korea), and 4 µl of the PCR product were used as a template. The oligo nucleotides were used in PCR amplification with specific primers for mice CD44 (forward, 5'-TCG ATT TGA ATG TAA CCT GCC-3'; reverse, 5'-TGG TGT GTT CTA TAC TCG CCC-3'), TIMP-2 (forward, 5'-GCA TCA CCC AGA AGA AGA GC -3'; reverse, 5'-GGG TCC TCG ATG TCA AGA AA-3') and GAPDH (forward, 5'- ATG TTC CAG TAT GAC TCC AC -3'; reverse, 5'- GCC AAA GTT GTC ATG GAT GA-3') as an internal control. The PCR was carried out for the products of CD44, TIMP-2 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), with denaturing at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec (9). The linear quantitation of the RT-PCR products was determined by a Gel-doc System (Bio-Rad, Universal-II, San Jose, CA, USA).

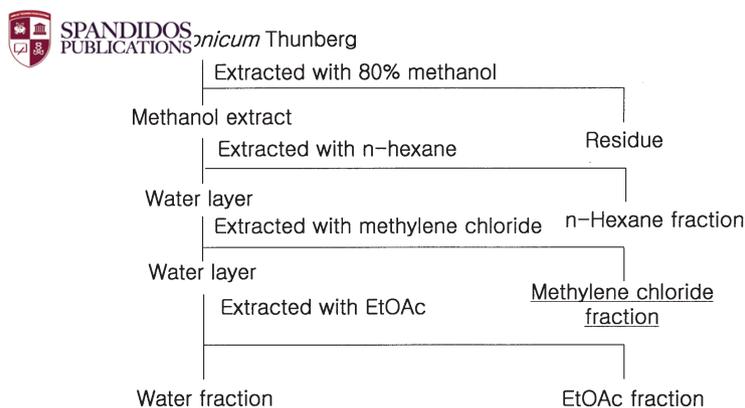


Figure 1. Fractionation procedure of the methylene chloride fraction of *Geum japonicum* Thunberg.

Western blot analysis. B16 cells were solubilized in a buffer containing 10 mM of Tris-HCl, 1% of Triton X-100, 50 mM of sodium chloride, 30 mM of sodium pyrophosphate, 50 mM of sodium fluoride, 100 μ M of sodium vanadate, 5 mM of EDTA and 1 mM of phenylmethylsulfonyl fluoride, adjusted to a pH of 7.6. The lysates were spun down by centrifugation at 15,000 x g for 20 min and supernatants were added to a 2X SDS loading buffer (125 mM of Tris-HCl, 2% glycerol, 4% SDS (w/v), 0.05% mercaptoethanol and 0.05% bromophenol blue, adjusted to a pH of 6.8). Electrophoresis was carried out using a one-dimensional SDS-polyacrylamide gel (12%) and proteins were transferred to nitrocellulose (NC) membranes. The membranes were then blocked for 2 h in a PBST (0.05% of Tween-20) containing 5% of non-fat skim milk (Seoul Milk, Seoul, Korea) and then incubated overnight in a PBST containing primary antibodies (ERK, Biosource no. 44-654; p-ERK Try185/Tyr187, Biosource no. 44-680; JNK, Cell Signaling no. 9252; p-JNK Try183/Tyr185, Cell Signaling no. 9251; p-38, Cell Signaling no. 9212; p-p38 Try180/Tyr182, Cell Signaling no. 9211), which were diluted to 1:500-2000 in 5% of a bovine serum albumin. Secondary antibodies were used in horseradish peroxidase conjugated mouse or rabbit IgG. The blots were developed by an ECL system (Pierce, no. 34094, Rockford, IL, USA) in order to identify the detection of each protein band (9,18).

Statistical analyses. Data were expressed as means \pm standard deviation and analyzed using a paired student's t-test program to compare the two groups (Newman-Keuls post test program). A P-value of <0.05 was considered statistically significant.

Results and discussion

To investigate the inhibitory mechanism of the metastatic and angiogenic activity of the *Geum japonicum* Thunberg (GjT) extract, we carried out the partial fractionation of GjT. The whole plant (1,500 g) was added with 80% methanol and extracted by a rotary mixer. The residue was discarded by centrifugation and the methanolic fraction (385 g) was re-extracted with n-hexane. The water layer was partitioned with methylene chloride and a methylene chlorode fraction was obtained (2.3 g, 0.6% yield). The flowchart of the procedure

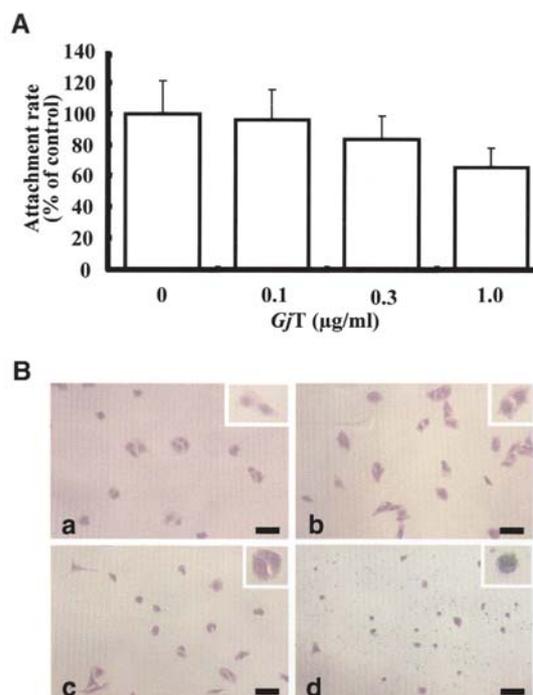


Figure 2. Changes in the cell morphology and spread of the fraction of methylene chloride of *Geum japonicum* Thunberg in B16 cells using an attachment assay. (A) The inhibition ratio of cell attachment was determined by the GjT extract after 2 h in a dose-dependent manner. (B) A comparison of cell morphology. B16 cell suspensions were treated with or without different concentrations (a, 0; b, 0.1; c, 0.3 and d, 1 μ g/ml) of the GjT extract after incubation for 24 h by an inverted microscope; scale bar, 100 μ m.

is shown in Fig. 1. We used this methylene chlorode fraction throughout the experiments.

First, we examined whether the GjT extract affected cell adhesion and spread by using an *in vitro* cell migration assay in B16 melanoma cells. The inhibition of tumor cell adhesion to the extracellular matrices and basement membrane proved to be an important step in tumor metastasis. As shown in Fig. 2A, GjT treatment (1.0 mg/ml) inhibited the attachment rate up to 30% compared to that of the control. This phenomenon can be seen by a contrast microscope (Fig. 2B). In the control, the morphology of the cells did not sustain any damage. By adding the extract at a concentration of 1.0 mg/ml, the growth of cells was significantly inhibited (Fig. 2d), while at milder concentrations, the cells showed slight damage (Fig. 2b and c). These results collectively suggest that the GjT extract, after 2 h of treatment, exhibited an inhibitory effect on tumor cell adhesion on the culture plate, in a concentration-dependent manner (Fig. 2A and B).

We confirmed also whether GjT inhibited the ability of the B16 cells to spread by a wound healing assay. As shown in Fig. 3A, a wound was made (Fig. 3A a) and then cultured onto the plates for 24 h in order to examine whether it was inhibited. GjT significantly inhibited cell spreading in a dose-dependent manner (Fig. 3A c, d and e). The relative inhibition percentage was plotted in Fig. 3B. By adding 1.0 mg/ml of GjT, the healing activity was significantly reduced by 82% (Fig. 3B). These results indicated that the GjT extract inhibited cell attachment and its spread in a concentration-dependent manner *in vitro* (Fig. 3). There are

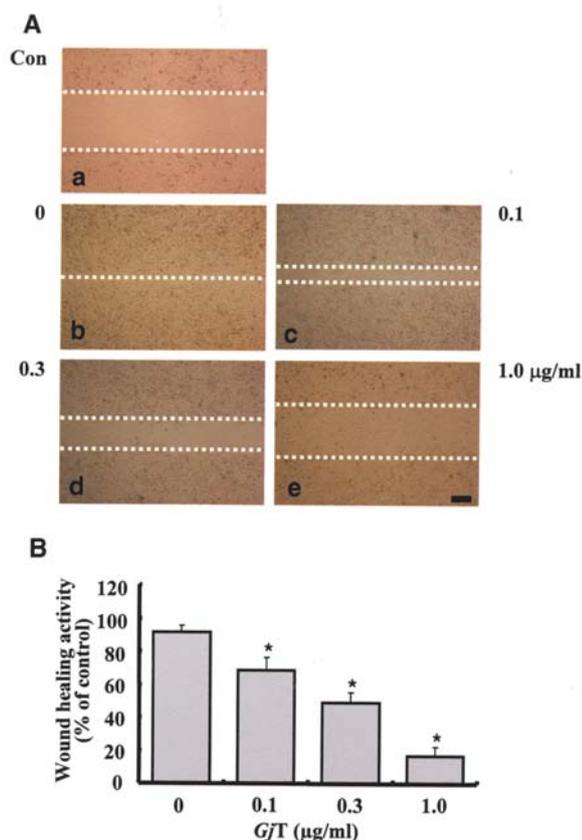


Figure 3. The *Geum japonicum* Thunberg extract inhibits cell migration *in vitro*. (A) Monolayers were scratched with a blade to make a wound and the cells were incubated with different concentrations of the GjT extract. The plate was photographed after 20 h. The GjT extract inhibited wound healing activity in a dose-dependent manner in B16 cells. (B) The quantification of the wound healing assay. Cell migration was quantified by counting the wound width 20 h after the plate was treated with or without the GjT extract. Values are means \pm SD of independent experiments conducted in triplicate; * $p < 0.05$ is considered statistically significant; scale bar, 100 μ m.

many anti-cancer therapies for which several medicinal plant extracts suppress cell migration and/or motility (19,20). The migration activity of the B16 cells decreased wound healing activity in a dose-dependent manner by the GjT extract. The most remarkable results showed that the GjT extract inhibited cell spreading and migration *in vitro*.

For this reason, we examined whether the HUVEC differentiation into capillary-like structures (i.e., tube formation) was affected by GjT. Since the angiogenic process is characterized by endothelial cell differentiation, *in vitro* angiogenesis assays were carried out to assess their capability with HUVECs for 24-48 h, with the GjT extract on matrigel. Dose-dependency at four different concentrations (0.1, 0.3, 1.0 or 3.0 μ g/ml) was also investigated. When non-treated cells were used as a control, the differentiation of HUVECs into capillary-like structures was observed. However, when the HUVEC cells were treated with the GjT extract, the number of capillary-like structures drastically decreased in a dose-dependent manner (Fig. 4A a-d). After 48 h of incubation, the control morphology was vivid in shape (Fig. 4B a), whereas the tube formation that was treated with GjT (0.1 μ g/ml) was significantly decreased (a comparison of the shapes of A-b

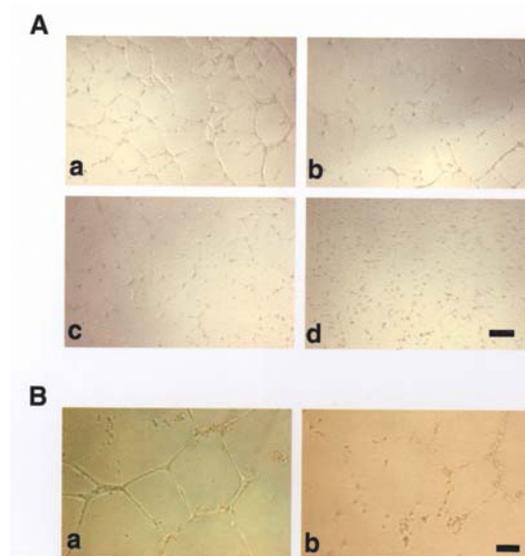


Figure 4. The differentiation of endothelial cells (HUVECs) into a capillary-like structure. (A) The effect of the GjT extract on the matrigel differentiation of HUVECs. No treatment cells made tube-like structures, but the treated HUVEC cells of the GjT-treated extract did not, and cellular extension was decreased after 24 h. (B) Tubes are already formed within 48 h of the tube collapsing; scale bar, 100 μ m.

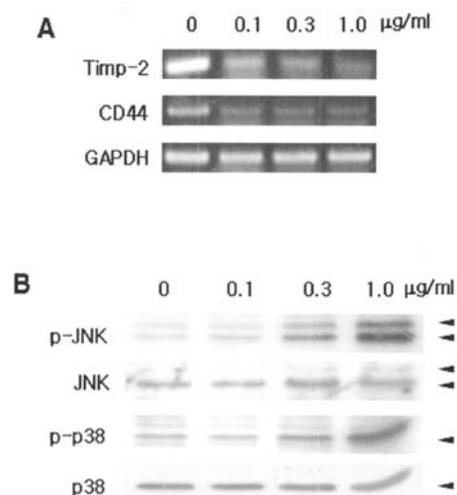


Figure 5. The expression of mRNA levels of MAPK kinases by the *Geum japonicum* Thunberg extract. (A) The GjT extract suppresses the total amount of CD44 and TIMP-2 RNA levels. (B) A Western blot analysis of the extract from B16 cells was conducted at concentrations of 0, 0.1, 0.3 and 1 g/ml, using antibodies which can detect the phosphorylated forms of ERK, JNK and p38. The GjT extract increased the phosphorylated forms of JNK in a dose-dependent manner.

and B-b). Together, these results suggest that tube formation was affected by the GjT extract in concentration- and time-dependent manner.

CD44 is an extra-cellular membrane protein that is involved in cell migration, tumorigenesis, metastasis and immune responses via its involvement with MMP-2 and -9 (21). MMP-2 is co-localized with MT-MMP-1 and TIMP-2, which are activators and activation-enhancing factors, respectively, for proMMP-2. Therefore, TIMP-2 was involved



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formation of fibrovascular tissues (22). However, fibroblasts produced angiogenic factor-induced endothelial cell proliferation *in vitro* and angiogenesis *in vivo* independently of MMP inhibition (23). When B16 melanoma cells were treated with 0.1, 0.3 and 1 $\mu\text{g/ml}$ of the GJT extract, the CD44 and TIMP-2 mRNA expressions were dramatically reduced in a concentration-dependent manner (Fig. 5A). These results strongly suggest that the GJT extract affects CD44 and TIMP-2 levels, showing that it may be involved in cell migration via altered signaling by the CD44 and TIMP-2 associated proteins.

The signal transduction pathways of the mitogen-activated protein kinases (MAPKs) are among the most widespread mechanisms of cellular regulation (24). All eukaryotic cells possess multiple MAPK pathways, each of which is preferentially recruited by a distinct set of stimuli, thereby allowing multiple divergent inputs, in parallel, to the cell response (25). Mammalian MAPK pathways can be recruited by a wide variety of different stimuli ranging from hormones, such as insulin and growth hormones, epidermal, platelet-derived and fibroblast growth factors, angiotensin-II, endothelin, inflammatory cytokines of the tumor necrosis factor (TNF) family and environmental stress (osmotic shock, ionizing radiation and ischemia injury, etc.). ERK 1 and 2 phosphorylate and activate the two transcription factors and other protein kinases, thereby influencing a large variety of cellular processes (26).

In order to prove the molecular mechanism of the metastatic and angiogenic potential in cancer cells, we carried out an analysis of the MAPK expression by treatment with the GJT extract. Cell lysates were probed for total and phospho-forms of ERK, JNK or p38 and the cells were detected by the distinct expression of the phosphorylated form of proteins. Expressions were detected by the form of phospho-JNK in a dose-dependent manner (Fig. 5B). The JNK kinase activity that was shown in cell-treated GJT for 1 h, was >3.3-fold that of the control. The expression of phospho-ERK did not change at a concentration of 1.0 $\mu\text{g/ml}$ (data not shown) (27), whereas that of p-JNK and p-p38 greatly increased more than that of the control. Collectively, these results suggest that the signaling induced by GJT is closely associated with the dual amplification of JNK and p38 phosphorylation. In order to find a novel tumor-suppressor agent, most of these functions require the modulation of different targets in angiogenesis, such as the growth factor VEGF, matrix metalloproteinase MMP-2 and -9, and integrin $\alpha_v\beta_3$. Herein, we demonstrated that the GJT extract inhibited the spreading and/or migration of B16 cells and blocked HUVEC differentiation on matrigel and actin as inhibitors of *in vitro* angiogenesis.

In this study, we proved that the inhibition of cell migration and motility by GJT was increased with phospho-JNK and -p38 expression in a dose-dependent manner (Fig. 5B). However, we anticipate the results of testing done using active compound(s) in GJT.

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