Cytochalasin H, an Active Anti-angiogenic Constituent of the Ethanol Extract of *Gleditsia sinensis* Thorns

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Angiogenesis, the process of new vessel formation from the pre-existing blood vasculature, is critical for continuous tumor growth and is considered to be a validated antitumor target. The results of our previous study demonstrate the anti-angiogenic potential of an extract of *Gleditsia sinensis* thorns, which has been traditionally used in Korean medicine to remedy diverse diseases, including tumors. In the present study, we attempted to identify the active anti-angiogenic constituents of the ethanol extract of *G. sinensis* thorns (EEGS). By virtue of *in vitro* activity-guided fractionation using human umbilical vein endothelial cells (HUVEC) primary endothelial cells, chromatographic separation, and NMR spectral analyses, we isolated and identified the potent active constituent, cytochalasin H, a biologically active secondary metabolite of fungi. This unexpected active constituent may have originated from the endophytic fungi, *Chaetomium globosum*, which naturally populate *G. sinensis*, the identity of which was determined by analysis of fungal community. Cytochalasin H isolated from the EEGS showed *in vitro* anti-angiogenic activities such as suppressed cell growth and mobility in HUVEC, and inhibited the pro-angiogenic protein-induced formation of new blood vessels *in vivo*. The anti-angiogenic effect of cytochalasin H was in part due to reduced expression of pro-angiogenic factor, such as endothelin-1. This is the first report regarding the isolation and identification of cytochalasin H, as an active anti-angiogenic constituent of *G. sinensis* thorns.

**Key words** cytochalasin H; *Gleditsia sinensis* thorn; angiogenesis; cancer; human umbilical vein endothelial cell (HUVEC)

A continuous supply of oxygen and nutrients through the blood vessel system is critical for sustained tumor growth. When the size of the tumor is relatively small, tumor cells can take up oxygen and nutrients by diffusion from the environment surrounding the tumor. However, as tumors grow larger, the inner tumor cells are inevitably subjected to nutrient and oxygen limitation without a proper blood supply. In fact, it is known that tumor size cannot exceed >1 mm³ in an avascular state.¹ Since first hypothesized by Folkman in 1971,² angiogenesis, the process of new vessels sprouting from the pre-existing vasculature, has been considered to be one of the potential targets of anticancer therapy. To date, only bevacizumab (trade name Avastin), a humanized monoclonal antibody targeting vascular endothelial growth factor (VEGF), has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of certain types of cancer, including colon, lung, kidney, and brain cancer.³ However, adverse effects of bevacizumab have been reported, such as hypertension, and the FDA removed the breast cancer indication from bevacizumab in 2010 due to its safety issues and ineffectiveness in breast cancer patients.

*Gleditsia sinensis* LAM. (Leguminosae) is a perennial shrub that is widely distributed in Eastern Asia, including the Chinese mainland and the Korean peninsula. Different parts of *G. sinensis*, such as the normal and anomalous fruits, seeds, radix cortex, and thorns, have been prescribed for the treatment of diverse diseases, including suppuration, carbuncle, obesity, thrombosis, and tumors in traditional oriental medicine in general and Korean Medicine (KM) specifically.⁵–⁸ Previous studies have demonstrated that the extract of *G. sinensis* thorns exerts anticancer effects *in vitro* and *in vivo* in diverse cancer models, including gastric,³ colon,⁹,¹⁰ and cervical¹¹ cancers, by suppressing cell cycle progression⁵,⁹,¹⁰ as well as inhibiting proliferating cell nuclear antigen (PCNA) and mutant tumor protein 53 (p53) expression.¹¹ The Tang research group demonstrated that the extract of the anomalous fruits of *G. sinensis* which is rich in saponins, exerts anticancer effects in a panel of solid and non-solid tumor cells by generating reactive oxygen species,²¹ inhibiting proteasome activity,³¹ inhibiting telomerase activity and oncogene expression,¹⁴ and suppressing cyclooxygenase-2 (COX-2) expression.¹⁵ The anti-angiogenic potential of *G. sinensis* has also been reported by various research groups, including us. Chow et al. demonstrated that the extract of the anomalous fruits of *G. sinensis* can reduce the expression of VEGF mRNA in a dose-dependent manner in MDA-MB-231 breast cancer and HepG2 hepatoma cell lines, and reduce fibroblast growth factor (FGF)-induced angiogenesis in the chick chorioallantoic membrane (CAM) assay.²⁰ Dai’s research group demonstrated that the saponin fraction and its active constituent, Gleditisioside B, which were isolated from the anomalous fruits of *G. sinensis*, inhibit FGF-induced mobility of human umbilical vein endothelial cells (HUVEC) by inhibiting matrix metalloproteinase 2 (MMP2) and focal adhesion kinase (FAK) signaling.¹⁷,¹⁸ The results of our previous study demonstrated that the ethanol extract of *G. sinensis* thorns (EEGS) efficiently inhibits the angiogenic activity of HUVEC and reduces pro-angiogenic factor-induced new vessel formation in a matrigel plug assay.¹⁹ To date, there have been no scientific reports identifying the active anti-angiogenic constituents of *G. sinensis* thorns. In the present study, we isolated and identified cytochalasin H.

The authors declare no conflict of interest.

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as an active anti-angiogenic constituent of the extract of *G. sinensis* thorns, using activity-guided fractionation.

**MATERIALS AND METHODS**

**Plant Materials** *G. sinensis* thorns were purchased from the Kwangmyungdang Medicinal Herbs Co. (Batch no. K136201010, Ulsan, Republic of Korea) and identified by Dr. Go Ya Choi, Basic Herbal Medicine Research Group, Herbal Medicine Research Division, Korea Institute of Oriental Medicine, Republic of Korea. A voucher specimen (KIOM-CRC-1) was deposited at the Cancer Research Center, Herbal Medicine Research Division, Korea Institute of Oriental Medicine, Republic of Korea.

**Isolation of the Active Constituent** Dried *G. sinensis* thorns (9.0 kg) were ground and extracted with 80% EtOH (40 L × 2 d × 3 times) by maceration at room temperature. The extracts were concentrated in vacuo to produce a dried extract (315.3 g). This EtOH extract (364.0 g) was suspended in distilled water and sequentially partitioned with *n*-hexane and EtOAc to generate the *n*-hexane- (32.3 g), EtOAc- (94.9 g), and water-soluble (236.0 g) fractions. Activity-guided fractionation was performed to isolate the active anti-angiogenic constituents from the subfractions using a HUVEC-mediated wound healing assay. Schematic descriptions of the chromatographic separation procedure and the chemical structure of cytochalasin H are shown in Supplementary Fig. 1.

**Chromatography, NMR, and Mass Spectroscopy** Flash chromatography was performed using the Isolera One flash purification system (Biotage, Uppsala, Sweden). Pre-packed SNAP HP-Sil (39 × 157 mm, Biotage) and SNAP KP-C18-HS (30 × 72 mm, Biotage) cartridges were used for flash chromatography. Dry load cartridges (71 × 168 mm or 39 × 157 mm scales, Biotage) packed with Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, U.S.A.), Diaion HP-20 (Supelco, Bellefonte, PA, U.S.A.), and ODS-A gel (YMC, Kyoto, Japan) were also used for flash chromatography. The high resolution-electrospray ionization-mass spectrum (HR-ESI-MS) result was measured on a hybrid quadruple orthogonal time-of-flight (Q-TOF) mass spectrometer (SYNAPT G2, Waters, Millipore, Bedford, MA, U.S.A.), and ODS-A gel (YMC, Kyoto, Japan) were used along with the Isolera One purification system (Biotage) with a mobile phase consisting of acetonitrile and 0.1% acetic acid in water. The mobile phase gradient elution was programmed as follows: acetonitrile: 5–30% (0–20 min), 30–70% (20–35 min), and 70–100% (35–40 min). The flow rate of the mobile phase was set at 0.3 mL/min. The sample injection volume was set at 2.0 μL. The column temperature was maintained at 40°C and the UV detector was set at 220 and 254 nm. All the sample solutions (2000 μg/mL, 100% methanol) were filtered (Millex-FG 0.2 μm, Millipore) prior to injection.

**Cell Culture and Viability** HUVECs from Lonza (Walkersvill, MD, U.S.A.) were grown in EGM-2 endothelial growth medium (Lonza). They were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The cells were used for experiments between passage numbers 5 and 10. A total of 5 × 10⁴ cells were inoculated into each well of a 24-well tissue culture plate. After 24 h, the cells were exposed to increasing concentrations of the drug and were maintained for 24 h. The numbers of total (viable and dead) cells and dead cells were determined based on cell membrane integrity using an ADAM-MC automatic cell counter (NanoEnTek, Seoul, Republic of Korea), as described previously.¹⁶ The total (viable and dead) cell numbers were determined by mixing equal volumes of the cell suspension with AccuStain T cell lysis solution (total) or AccuStain N (dead) buffer solution, respectively. Cell viability was automatically calculated by ADAM-MC software.

**Wound Healing Assay** When the HUVEC had reached approximately 90% confluence in a 24-well tissue culture plate, the cells were scratched with yellow tips. The wounds were photographed using an inverted microscope (Olympus IX71, Tokyo, Japan). After a brief wash with fresh EGM-2 medium, the cells were exposed to fresh EGM-2 containing increasing concentrations of the drug. After 12 h, the wounded area was photographed and digitally quantified using MetaMorph image analysis software (Molecular Devices, Downingtown, PA, U.S.A.). The healed area (%) was calculated according to the following formula: healing area (%) = [1 − wounded area (t=12 h)/wounded area (t=0 h)] × 100.

**Tube Formation Assay** The effects of the test drugs on 3-dimensional tube formation by the HUVEC were tested using a Cultrex *in vitro* angiogenesis assay kit (Trevgien, Gaithersburg, MD, U.S.A.), as described in the manufacturer’s user guide. A total of 1.5 × 10⁴ cells were inoculated into a 96-well tissue culture plate that had been pre-coated with basement membrane extracts (BME). After 12 h drug treatments, tubes were observed and photographed under the microscope. The degree of tube formation by the HUVEC was performed by quantifying the tube length and branch numbers using image analysis software (MetaMorph).

**Endothelin 1 (EDN1) Immunoassay** The levels of EDN1 that were released by the HUVEC into the culture media were quantified using the human EDN1 immunoassay kit (R&D Systems, Minneapolis, MN, U.S.A.). After 24 h of drug treatment, the culture media were cleared by centrifugation and loaded into an immune-microplate that had been pre-coated with EDN1 monoclonal antibodies. Standards consisting of serially diluted recombinant EDN1 were loaded in parallel with the samples. The EDN1 in the supernatants was captured for 1 h at room temperature (rt). After washing, enzymelinked EDN1 monoclonal antibody was added and incubated for 3 h at rt. After washing, color development was initiated by add-
ing a substrate solution. The absorption was determined at 450 nm using a microplate reader ($E_{max}$, Molecular Devices). The concentration of EDN1 in the culture medium was determined by interpolating it from the standard curve.

**Semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)** Gene expression in HUVEC following cytochalasin H treatment was determined by semi-quantitative RT-PCR. Total RNA was isolated from the HUVEC treated with cytochalasin H for 24 h using the Easy-spin<sup>TM</sup> total RNA extraction kit (iNtRON Biotechnology, Seoul, Republic of Korea). Single-stranded cDNAs were synthesized from 1 µg of total RNA using the SuperScript<sup>™</sup> III first-strand synthesis system (Invitrogen, Carlsbad, CA, U.S.A.). PCR was carried out using 100 ng of cDNA as a template and gene specific primer sets. The PCR primers were as follows: for EDN1, 5'-TAT TTG CTC ATG ATT TTC TCT CTC G-3', 5'-TTG ACC AAA ATG ATG TCC AG-3'; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-AAG GCT GAG AAC GGG AAG-3'; for 18S rRNA, 5'-CCT GAG AAC GGG AAG-3'; and 5'-GGA CTC CAC GAC GTA CTC-3'.

**In Vivo Angiogenesis Assay** A directed in vivo angiogenesis assay kit (Trevigen) was used to investigate the effects of the drugs on in vivo angiogenesis induced by pro-angiogenic factors (VEGF and FGF2), as previously described.<sup>19</sup> In brief, angioreactors that had been pre-filled with growth factor-reduced BME with combinations of VEGF, FGF2, and increasing concentrations of the drug were subcutaneously implanted into the dorsal flanks of 6 week old C57BL/6 nude mice. After 12 d, the implanted angioreactors were harvested, and the degree of new vessel formation was determined by FITC-lectin mediated quantification of the vascular endothelial cells that had infiltrated into the angioreactors. The intensities of fluorescence were determined at an excitation 485 nm and an emission 510 nm (SPECTRA MAX GEMINI EM, Molecular Devices). The animal studies were approved by the Institutional Animal Care and Use Committee at the Korea Institute of Oriental Medicine (Protocol #12-058).

**Statistics** The results are represented as mean±standard deviation (S.D.). The differences between the control and treated groups were determined using Student’s t-test. Values were considered significant at $p<0.05$.

**RESULTS**

**Anti-angiogenic Potential of EEGS** We previously revealed that EEGS has potential anti-angiogenic activity both in vitro and in vivo.<sup>19</sup> EEGS efficiently inhibited cellular mobility and 3-dimensional vascular tube formation by HUVEC primary cells, which led to the suppression of vessel formation in the matrigels that had been premixed with pro-angiogenic factors. Consequently, these UHPLC data demonstrated that cytochalasin H has the 80% EtOH extract, its solvent fractions (n-hexane-, EtOAc-, and water-soluble), and the activity-guided chromatographic subfractions (F06, F0604, and F060405) from the active EtOAc-soluble fraction were analyzed and monitored at UV 254 and 220 nm using a UHPLC system (Supplementary Fig. 2). No cytochalasin H peak was observed in the 80% EtOH extract and its solvent fractions at both UV 254 and 220 nm (Supplementary Figs. 2A, B). Although the EtOAc-soluble fraction was active, it showed no peak of cytochalasin H at UV 220 nm (Supplementary Fig. 2B), which may be attributable to the fact that its concentrations in the crude extracts were low (i.e., its concentrations were below the limit of detection). The chromatograms of F0604 and F060405, the chromatographic subfractions, showed smaller and larger target peaks ($t_R$ 24.5 min), respectively, at UV 220 nm (Supplementary Fig. 2D), as expected. The UV spectrum of cytochalasin H showed an absorption maximum at 220 nm. Supplementary Fig. 2C showed that the sensitivity of cytochalasin H at UV 254 nm was lower than it was at UV 220 nm. Consequently, these UHPLC data demonstrated that cytochalasin H was successfully isolated and purified from the 80% EtOH extract of the thorns of *G. sinensis* using activity-guided chromatographic separation. The structure of cytochalasin H was unambiguously determined by NMR (1D- and 2D-NMR) and HRESIMS data<sup>20</sup> (Supplementary Fig. 1). A white powder; 1H-NMR (500 MHz, CDCl$_3$): δ: 7.33 (2H, t, $J$=7.0 Hz, H-28/H-30), 7.25 (1H, t, $J$=7.5 Hz, H-29), 7.15 (2H, d, $J$=7.0 Hz, H-27/H-31), 5.87 (1H, dd, $J$=2.5, 16.5 Hz, H-20), 5.74 (1H, dd, $J$=9.5, 15.5 Hz, H-13), 5.56 (1H, m, H-21), 5.55
(1H, s, NH), 5.54 (1H, br d, J = 16.5 Hz, H-19), 5.41 (1H, m, H-14), 5.36 (1H, s, H-12a), 5.12 (1H, s, H-12b), 3.83 (1H, d, J = 10.5 Hz, H-7), 3.26 (1H, m, H-3), 2.95 (1H, t, J = 10.5 Hz, H-8), 2.86 (1H, dd, J = 4.5, 13.5 Hz, H-10a), 2.79 (1H, m, H-5), 2.65 (1H, dd, J = 9.5, 13.5 Hz, H-10b), 2.25 (3H, s, H-25), 2.13 (1H, t, J = 4.0 Hz, H-4), 2.05 (1H, m, H-15a), 1.88 (1H, brd, J = 14.0 Hz, H-17a), 1.82 (1H, m, H-15b), 1.81 (1H, m, H-16), 1.57 (1H, brd, J = 14.5 Hz, H-17b), 1.35 (3H, s, H-23), 1.05 (3H, d, J = 6.0 Hz, H-22), 1.00 (3H, d, J = 6.5 Hz, H-11). 13C-NMR (125 MHz, CDCl3) δ: 174.4 (C-1, s), 170.3 (C-24, s), 148.1 (C-6, s), 138.9 (C-14, d), 138.3 (C-19, d), 137.6 (C-26, s), 129.2 (C-27-C31, d), 129.2 (C-28-C30, d), 127.31 (C-13, d), 127.29 (C-29, d), 126.2 (C-20, d), 114.3 (C-12, t), 77.7 (C-21, d), 74.5 (C-18, s), 69.9 (C-7, d), 54.0 (C-3, d), 53.9 (C-17, t), 52.0 (C-9, s), 50.6 (C-4, d), 47.4 (C-8, d), 45.8 (C-10, t), 42.9 (C-15, t), 33.1 (C-5, d), 31.4 (C-23, q), 28.6 (C-16, d), 26.7 (C-22, q), 21.1 (C-25, q), 14.3 (C-11, q). HR-ESI-MS m/z: 516.2719 [M+Na]+ (Calcd for C30H39NO5Na: 516.2726).

In Vitro Anti-angiogenic Potential of Cytochalasin H

First, we determined the viability of HUVEC in the presence of increasing concentrations of cytochalasin H (0–2000 nmol/L). Cell viabilities were assessed by measuring cytoplasmic membrane integrity as described in Materials and Methods. As shown in Fig. 2A, cytochalasin H efficiently inhibited HUVEC proliferation in a dose-dependent manner. A significant inhibitory effect of cytochalasin H on HUVEC proliferation was observed at concentrations ≥ 125 nmol/L. However, the anti-proliferative effect of cytochalasin H was not related to cytotoxicity during the 24h drug treatment. Although a slight decrease in cell viability was observed in the cytochalasin H-treated HUVEC, greater than 80% cell viability was maintained at concentrations up to 2000 nmol/L.

The anti-angiogenic potential of cytochalasin H in HUVEC was evaluated using an in vitro wound healing assay. In the absence of cytochalasin H, HUVEC residing at the borders of the scratches migrated toward the wounded area and covered it within 12h (Supplementary Fig. 3). A significant, and concentration-dependent decrease in cell mobility was observed at concentrations ≥ 125 nmol/L and only 6.3% of the wound was recovered at 2000 nmol/L (Fig. 2B). In addition, we evaluated the effects of cytochalasin H on 3-dimensional vessel formation by HUVEC on matrix-coated surfaces. In
the absence of cytochalasin H, the HUVEC constructed vessel tubes by connecting to neighboring cells (Supplementary Fig. 4). However, in the presence of cytochalasin H, the intercellular connections were limited and the HUVEC cells failed to form tubes. To quantify the degree of tube formation, photographs were taken at the end of the experiments under the microscope and the tube length and branch numbers were calculated using image analysis software (Fig. 2C). Both parameters decreased by cytochalasin H in a dose-dependent manner. Statistical analyses revealed that the tube length and branch number were significantly reduced by the treatment with 125 nmol/L of cytochalasin H. In our previous study, proteomic and immunoassay studies revealed that both the intracellular and extracellular expression of the pro-angiogenic protein EDN1 was reduced by treatment with EEGS in a dose-dependent manner.19 In an effort to verify whether cytochalasin H, an active anti-angiogenic constituent isolated from the EEGS, could also down-regulate EDN1 expression, the extracellular levels of EDN1 that had been released by HUVEC were quantified using an ELISA. As shown in Fig. 2D, EDN1 expression by HUVEC was decreased by cytochalasin H treatment in a dose-dependent manner, and 2000 nmol/L of cytochalasin H inhibited EDN1 expression by 83.8% as compared to vehicle treatment. Down-regulation of EDN1 expression by cytochalasin H was due to decrease in intracellular EDN1 mRNA level by cytochalasin H, which was demonstrated by semi-quantitative RT-PCR (Fig. 2D, inlet).

**In Vivo Anti-angiogenic Potential of Cytochalasin H**

Because cytochalasin H inhibited in vitro angiogenic properties, such as cell migration and tube formation, we next investigated its effects on the formation of new blood vessels in vivo using a commercially available direct in vivo angiogenesis kit. Vessel formation was not observed in the absence of the pro-angiogenic inducers VEGF/FGF2 (negative control). Massive vessel ingrowth from the open ends of the angioreactors was induced by VEGF/FGF2. Vessel ingrowth was suppressed by cytochalasin H treatment in a dose-dependent manner (Fig. 3, top panel). To quantify vessel ingrowth, the endothelial cells constructing the blood vessels were labeled using FITC-lectin. Anti-angiogenic effects of cytochalasin H were observed at concentrations \( \geq 250 \text{nmol/L} \), and vessel formation was completely inhibited at concentrations \( \geq 1000 \text{nmol/L} \) (Fig. 3, bottom panel).

**DISCUSSION**

In our previous study, we demonstrated that EEGS has a cytostatic and anti-angiogenic potential in HUVEC primary cells in vitro and pro-angiogenic proteins-induced angiogenesis in vivo by suppressing pro-angiogenic proteins such as EDN1 and MMP2.17 In the present study, we attempted to identify the active constituents of EEGS that display these cytostatic and anti-angiogenic properties. Unexpectedly, activity-guided fractionation and spectral studies identified cytochalasin H, a fungal secondary metabolite, as an active constituent of *G. sinensis* thorns. To our knowledge, the present study is the first to report cytochalasin H is an active constituent of EEGS.

Cytochalasin H is a member of the cytochalasin family, which are biologically active fungal secondary metabolites. Cytochalasin H was first isolated from crude extracts of endophytic *Phomopsis paspali*, which were isolated from weevil-damaged pecans.21 Since then, cytochalasin H has been isolated from solid cultures of other endophytic fungi, such as *Endothia gyrosa*, mangrove endophytic fungus *Phomopsis sp.*, and culture fluids of the ascomycete *Hymenoscyphus episiphillus*.22 A group of cytochalasins has also been isolated from *Phomopsis, Chaetomium, Zygosporium spp.*, and *Hyphoxylon sp.*, but cytochalasins have not been found in bacteria and plants.24-25 Therefore, the cytochalasin H that was isolated as an active anti-proliferative and anti-angiogenic constituent of EEGS may have originated from naturally populating endophytic fungi. To demonstrate our hypothesis, we investigated fungal populations in the *G. sinensis* thorn using high-throughput pyrosequencing (ChunLab, Inc., Seoul, Republic of Korea, http://www.chunlab.com). The dried *G. sinensis* thorns supplied by two different suppliers (supplier #2 and #3 in Fig. 1) were used for analysis. As a result, we could identify cytochalasin-producing endophytic fungus, *Chaetomium globosum*, from both samples (Supplementary Fig. 5). The *C. globosum* has been isolated from diverse medicinal plants, such as *Terminalia arjuna*, *Crataeva magna*, *Azadirachta indica*, and *Holarrhena antidysenterica*.26 Based on this observation, we here suggest that cytochalasin H isolated from the *G. sinensis* thorn may originate from the *C. globosum* which was endophytic associated with *G. sinensis* thorn. Other research group has suggested that Gleditsioside B, which was isolated from the saponin fractions of the anomalous fruits of *G. sinensis*, is an active anti-angiogenic constituent in vitro,23 which differed from the results of the present study. Our unpublished data
showed that the in vitro anti-angiogenic potential of *G. sinensis* thorns was replicated by the extracts of seeds and twigs, but was replicated by the extracts of leaves, barks, and pods of *G. sinensis* (data not shown). However, the anti-angiogenic potential of *G. sinensis* thorns was more potent than the extracts of any other parts. We have not yet confirmed whether the active constituents of the seeds and twigs are cytochalasin H.

Until now, cytochalasins had not been under investigation in clinical trials due to their broad spectrum of cytotoxicity. However, computer-assisted phase-contrast microscopy performed by Van Goitsenoven et al. revealed that cytochalasins can be grouped into two categories, i.e., cytotoxic versus cytostatic, indicating that it may be possible to develop novel, non-toxic cytostatic anticancer agents by tuning cytochalasin targets.27) Our results showed that cytochalasin H isolated from *G. sinensis* thorns can successfully inhibit the angiogenic properties, such as cell growth, mobility, and 3-dimensional-tube formation (≥125 nmol/L), of HUVEC in vitro, and blood vessel formation by mouse endothelial cells in vivo (≥250 nmol/L) without seriously altering cell viability. The effective doses in our study are relatively low doses when compared with the LD50 of 12.5 mg/kg that was observed in day-old cockerels.20)

The cytostatic mechanism of cytochalasin including cytochalasin H is well known. After permeating cell membrane, cytochalasins bind to actin and inhibit its polymerization.28) The cytostatic mechanism of cytochalasin including cytochalasin H analogues that lack anti-actin activity.

In conclusion, we report for the first time that cytochalasin H, a biologically active fungal secondary metabolite, is an active anti-angiogenic constituent of the extract of *G. sinensis* thorns. Although cytochalasin H was isolated as an active constituent of the *G. sinensis* thorns, further studies should be conducted to understand the pharmaceutical mechanism of cytochalasin H and its medicinal plant source, *G. sinensis* thorns, are good candidates for anticancer drug development.

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