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CHINA MEDICAL UNIVERSITY

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TAICHUNG, TAIWAN, R.O.C

Journal of Ethnopharmacology *Nov 16, 2010*

Dear editor:

Enclosed is of "*Toona sinensis* (leaf extracts) inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis in vascular endothelial cells" by Hseu YC, Chang CS, Lin WC, Hung DZ, Lin MK, Kuo YH, Wang MT, Cho HJ, Wang L, Hsin-Ling Yang. The paper is submitted to be considered for publication in "**Journal of Ethnopharmacology**".

A lot of herbs used in traditional Chinese medicine may be of great treasure. *Toona sinensis* is conventionally used in traditional Chinese medicine. The edible leaves of *Toona sinensis* have been used as an oriental medicine for treating rheumatoid arthritis, cervicitis, urethritis, tympanitis, gastric ulcers, enteritis, dysentery, itchiness, and cancer (Edmonds and Staniforth, 1998; Xien, 1996). Angiogenesis, the formation of new vessels from preexisting vasculature, is an essential process in a variety of physiological and pathological conditions, including inflammatory diseases, atherosclerosis, diabetic retinopathy, rheumatoid arthritis, cancer and metastasis (Risau, 1997; Folkman, 2002). Because of the critical dependence of human cancer and inflammatory diseases on angiogenesis, therapeutic strategies have been developed targeting various aspects of the angiogenic processes, many with promising results. Because tumor epithelial cells *in vivo* depend on angiogenesis to provide nutrients for their growth and survival, it is plausible that this anti-angiogenic effect may play a primary role in mediating the cancer chemopreventive activity (Felmeden et al., 2003; Folkman, 2002). Moreover, the pharmacology of many anti-inflammatory drugs has revealed that at least part of their efficacy is attributable to their anti-angiogenic effects (Jackson et al., 1997). Hence, we hypothesized that the anticancer and anti-inflammatory activity of *T. sinensis* could be

partly attributable to its anti-angiogenic activity, and that *T. sinensis* could be a promising candidate drug for the treatment of diseases with impaired angiogenesis.

ABSTRACT :

"*Objective: Toona sinensis* is well known as a traditional Chinese medicine; also, it has been shown to exhibit anticancer and anti-inflamatory effects. This study was aimed to evaluating the anti-angiogenesis effect of the aqueous extracts of *T. sinensis* (TS extracts) or gallic acid, a major component of TS extracts, against VEGF-induced both EA.hy 926 and human umbilical vein endothelial cells (HUVECs).

Methods: Anti-proliferative activity of TS extracts or gallic acid, was determined against EA.hy 926 and HUVECs by trypan blue exclusion method. Invasion, tube formation and chick chorioallantoic membrane assay were carried out to determine the *in vitro* and *in vivo* anti-angiogenic effects.

Results: Non-cytotoxic concentration of TS extracts (50–100 μg/mL) and gallic acid (5 μg/mL) inhibited the proliferation of VEGF-stimulated EA.hy 926 and HUVECs. Inhibitory effects of TS extracts and gallic acid on angiogenesis were assessed by VEGF-induced migration/invasion and capillary-like tube formation by EA.hy 926 and HUVECs. Additionally, gelatin zymography assays showed that TS extracts and gallic acid suppressed the activity of metalloproteinase (MMP)-9 and MMP-2 activated by VEGF. *In vivo*, TS extracts and gallic acid strongly suppressed neovessel formation in the chorioallantoic membrane of chick embryos. Flow cytometry analyses and Western blot demonstrated that treatment with TS extracts and gallic acid induced G_0/G_1 arrest in VEGF-stimulated EA.hy 926 cells via a reduction in the amounts of cyclin D1, cyclin E, CDK4, hyperphosphorylated retinoblastoma protein (pRb), VEGFR-2, and eNOS.

Conclusion: These results support an anti-angiogenic activity of *Toona sinensis* that may contribute critically to its cancer and inflammation chemopreventive potential."

The study was performed according to the international, national and institutional rules considering animal experiments, clinical studies and biodiversity rights and a clear explanation of the functional/nutraceutical importance. Neither the entire paper nor any part of its content has been published or has been accepted by another journal. We believe the paper may be of particular interest to your reader. Correspondence about the paper should be directed to me at the following address:

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Thank you for your attention to our paper.

Sincerely yours,
Hsin-Ling Yang.

Journal of Ethnopharmacology
AUTHOR CHECKLIST

Dear Author,

It frequently happens that on receipt of an article for publication, we find that certain elements of the manuscript, or related information, is missing. This is regrettable of course since it means there must be a delay in processing the article while we obtain the missing details.

In order to avoid such delays in the publication of your article, if accepted, could you please run through the list of items below and check each box. **Please enclose a copy of this list with the manuscript submission.**

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- Do you declare that this manuscript/data, or parts thereof, has not been submitted or published elsewhere for publication? ■
- Do you declare that all the listed authors have read and approved the submitted manuscript? ■
- Do you declare that the present study was performed according to international, national and institutional rules considering animal experiments, clinical studies and biodiversity rights? ■

Revised manuscripts

- **Have you addressed each remark from the referees?** ☐

Ref.: Ms. No. JEP-D-10-01531

Toona sinensis (leaf extracts) inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis in vascular endothelial cells Journal of Ethnopharmacology

Dear Dr.Yang,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision. For your guidance, reviewers' comments are appended below. If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript. To submit a revision, go to http://ees.elsevier.com/jep/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there. You can submit your revised manuscript within a period of three weeks. Please note: If we don't receive your revised manuscript within that time, we assume that the changes requested were too extensive and will reject the article. In that case we invite you, however, to resubmit your revised manuscript as a new submission at a later stage.

Reply: We provided a point by point response to reviewer comments.

Reviewers' comments:

Reviewer #2: Manuscript - JEP-D-10-01531

Title - Toona sinensis (leaf extracts) inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis in vascular endothelial cells

Author - You-Cheng Hseua, Wen-Hsin Linb, Chih-Shiang Chang et al.

General comments：

The above cited manuscript is a complete study of the anti-angiogenic activity of an aqueous extract and a purified compound obtained from this extract (gallic acid). The authors report very interesting results supported by a complete set of experimental procedures which revealed different aspects of the mechanism of action of both sample preparation. The study highlights the ethnopharmacological use of the plant. The paper is well justified, results are clear, sound and discussion is precise, informative and of high quality level supported by the results and literature. The manuscript is clear; language is nice and scientifically relevant. I recommend this paper for publication. A schedule of few suggestions is given below to revise minor points.

Title - Clear and informative.

Abstract - good.

Key words - adequate

Minor corrections

"Our data demonstrated that TS extracts, and gallic acid, effectively blocks VEGF-induced proliferation,.. [block]

.and inhibits neo-angiogenesis in the chick chorioallantoic membrane (CAM) in vivo [inhibit]

Reply: The paper has been deleted these paragraphs as reviewer 3 suggestion**.**

[Delete-Our data demonstrated that TS extracts, and gallic acid, effectively block VEGF-induced proliferation, migration/invasion, and capillary-like tube formation by EA.hy 926 cells or HUVECs *in vitro* (*in vitro* angiogenesis), and inhibit neo-angiogenesis in the chick chorioallantoic membrane (CAM) *in vivo*.]

.freeze dried to form powder; the stock (2 mg/mL in PBS buffer) was.. [pH of buffer

must to be informed]

Reply: The paper has been revised as reviewer's suggestion**.** (Please see page 6, line 21).

"The crude extracts (50 g) were concentrated in a vacuum and freeze dried to form powder; the stock (2 mg/mL in PBS buffer, [pH 7.4]) was subsequently stored at -20 °C until analyzed for anti-angiogenic properties."

..reaction buffer (50 mM Tris-base, 200 mM NaCl, 5 mM CaCl2 and 0.02% Brij 35)... [pH of buffer must to be informed]

Reply: The paper has been revised as reviewer's suggestion**.** (Please see page 11, line 4).

"After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in a reaction buffer (50 mM Tris-base [pH 7.5], 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij 35) at 37 °C for 24 h."

buffer (0.062 M Tris-HCl, 2% SDS, 10% glycerol and 5% <beta>-mercaptoethanol). [pH of buffer must to be informed]

Reply: The paper has been revised as reviewer's suggestion**.** (Please see page 12, line 6).

"Total protein content was determined using a Bio-Rad protein assay reagent, with bovine serum albumin as the standard, protein extracts were reconstituted in sample buffer (0.062 M Tris-HCl [pH 6.8], 2% SDS, 10% glycerol and 5% β-mercaptoethanol), and the mixture was boiled for 5 min."

EA.hy 926 cells, a human vascular endothelial cell line, ...

human umbilical vein endothelial cells were prepared from human umbilical veins as described previously (Hseu et al., 2002). The origin of both cell lines used in the study was not cited. In the case of non commercial source, all the procedure to obtain them should be detailed and reference to the institutional concordance and ethic matter should be reported.

Reply: The paper has been revised as reviewer's suggestion**.** (Please see page 7, line 14 and page 8, line 1).

"EA.hy 926 cells, a human vascular endothelial cell line (Edgell et al., 1983), .."

"…human umbilical vein endothelial cells were prepared from human umbilical veins as described previously (Jaffe et al., 1973)."

Since TS extracts or gallic acid does not have a cytotoxic effect at up to a.. [do not]

Reply: The paper has been revised as reviewer's suggestion**.** (Please see page 19, line 1).

"In conclusion, our observations indicate that T. sinensis exerts an inhibitory effect on.."

[In conclusion, our observations indicate that leaf aqueous extract of T. sinensis exerts an inhibitory effect on]

Reply: The paper has been revised as reviewer's suggestion**.** (Please see page 22, line 1).

Reviewer #3: The main finding of the current study was the demonstration of the antiangiogenic property of Toona sinesis aqueous extract and one of its isolated compounds, gallic acid in cultured vascular endothelial cells. However, there are number of issues in this manuscript that require attention before it can be considered for publication.

The issues are listed as follows:

1) In abstract section:

-Objective/s of the study needs to be stated correctly and precisely.

-Methods should be described briefly.

-Results section: the authors used extract and gallic acid to study their antiangogenic property, however only results from the extract was described in the result section.

-Perhaps the whole abstract section needs to be rewritten.

Reply: We had already revised it as reviewer's suggestion**.** (Please see Abstract).

2) Introduction section: The authors should not include their present results in the introduction (page 5, line 53)

Reply: We had already deleted it as reviewer's suggestion**.**

Delete: "Our data demonstrated that TS extracts, and gallic acid, effectively block VEGF-induced proliferation, migration/invasion, and capillary-like tube formation by EA.hy 926 or HUVECs *in vitro* (*in vitro* angiogenesis), and inhibit neo-angiogenesis in the chick chorioallantoic membrane (CAM) *in vivo*."

3) Materials & methods

-Location, identification and voucher no of the plant should be included.

Reply: The paper has been revised as reviewer's suggestion**.** (Please see page 6, line 12-16).

"The leaves of *T. sinensis* were sourced from Fooyin University, Kaohsiung Hsien, Taiwan. A voucher specimen (FY-001) was characterized by Dr. Horng-Liang Lay, Graduate Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung County, Taiwan, and deposited at Fooyin University, Kaohsiung Hsien, Taiwan (Hsieh et al., 2004)."

-What was the basis of dosage selection for the extract and gallic acid?

Reply: "For all subsequent experiments, we employed the non-cytotoxic concentration of TS extracts (i.e., $\leq$ 100 μg/mL) and gallic acid (i.e., 5 μg/mL), and focused on the effect of TS extracts or gallic acid on VEGF-induced angiogenesis." (Please see page 13, line 20-22). The concentration of TS extracts and gallic acid that were used in these studies were not cytotoxic to either EA.hy 926 cells or HUVECs without VEGF stimulation.

-Why there was only one dose for gallic acid and 4 different doses for extract in all experiments?

Reply: Recent reports indicate that gallic acid treatment of carcinoma cells resulted in a strong cell growth inhibition, cell cycle arrest and apoptotic death in a dose-and time dependent manner (Agarwal et al., 2006; Indap et al., 2006). Besides, over the concentration of 10 μg/mL of gallic acid showed cytotoxic effect to HUVECs (You et al., 2010). Thereby, the present study non-cytotoxic concentration of gallic acid (5 μg/mL) was used to monitor the anti-angiogenic effect against VEGF-induced HUVECs. Our initial screening resulted that below the concentration of 5 μg/mL of gallic acid do not showed any modulation in HUVECs. Thereby we have chosen 5 μg/mL of gallic acid for our further investigations. In addition, gallic acid is one of the active compound isolated from the aqueous leaf extracts of *Toona Sinensis*.

4) Results & Discussion sections

-Percentage of the compounds that present in the extract should be included.

Reply: The paper has been revised as reviewer's suggestion**.** (Please see page 17, line 19-22).

"In the present study, we demonstrate that TS extracts or gallic acid, a major component of TS extracts at a yield of 6% from the total extracts, potentially inhibit the proliferation, migration/invasion and tube formation, an *in vitro* marker of angiogenesis, by VEGF-activated vascular endothelial cells, both EA.hy 926 cells and HUVECs."

-The authors did mention that there were number of other bioactive compounds isolated from the plant extract, and gallic acid was chosen along with the aqueous extract in the study. Since in has been reported that gallic acid has the antiangiogenic property (page 21, line 27), thus the authors should also included/test other bioactive compounds isolated in the extract that may also contribute to this activity

Reply: We will test the other bioactive compounds isolated in the TS extract in the next study.

The authors should also clarify the main reason/s to study the antiangiogenic activity of the plant aqueous extract having knowing that gallic acid which was one of the main bioactive compounds present and has been shown to possess this activity.

Reply: The paper has been revised as reviewer's suggestion. (Please see page 21, line 23-25).

"Moreover, in future we have planned to investigate antiangiogenic effect of other bioactive compounds isolated from the aqueous leaf extracts of *Toona sinensis*."

Graphical abstract

The aqueous leaf extracts of *Toona sinensis* (TS extracts) or gallic acid (GA), a major component of TS extracts, have the potential for anti-angiogenic effects through the inhibition of neo-angiogenesis in the chick chorioallantoic membrane *in vivo*.

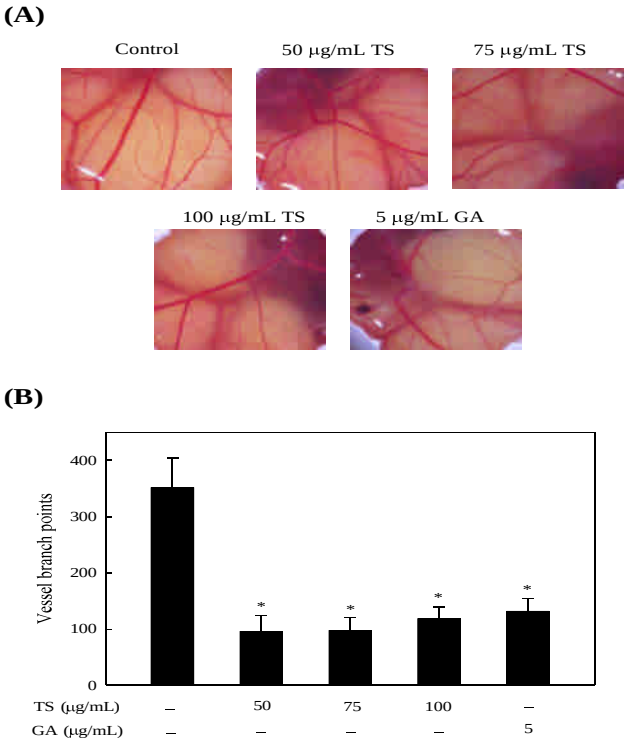

Fig. 6

ABSTRACT

Objective: Toona sinensis is well known as a traditional Chinese medicine; also, it has been shown to exhibit anticancer and anti-inflmmatory effects. This study was aimed to evaluating the anti-angiogenesis effect of the aqueous extracts of *T. sinensis* (TS extracts) or gallic acid, a major component of TS extracts, against VEGF-induced both EA.hy 926 and human umbilical vein endothelial cells (HUVECs).

Methods: Anti-proliferative activity of TS extracts or gallic acid, was determined against EA.hy 926 and HUVECs by trypan blue exclusion method. Invasion, tube formation and chick chorioallantoic membrane assay were carried out to determine the *in vitro* and *in vivo* anti-angiogenic effects.

Results: Non-cytotoxic concentration of TS extracts (50–100 μg/mL) and gallic acid (5 μg/mL) inhibited the proliferation of VEGF-stimulated EA.hy 926 and HUVECs. Inhibitory effects of TS extracts and gallic acid on angiogenesis were assessed by VEGF-induced migration/invasion and capillary-like tube formation by EA.hy 926 and HUVECs. Additionally, gelatin zymography assays showed that TS extracts and gallic acid suppressed the activity of metalloproteinase (MMP)-9 and MMP-2 activated by VEGF. *In vivo*, TS extracts and gallic acid strongly suppressed neovessel formation in the chorioallantoic membrane of chick embryos. Flow cytometry analyses and Western blot demonstrated that treatment with TS extracts and gallic acid induced G_0/G_1 arrest in VEGF-stimulated EA.hy 926 cells via a reduction in the amounts of cyclin D1, cyclin E, CDK4, hyperphosphorylated retinoblastoma protein (pRb), VEGFR-2, and eNOS.

Conclusion: These results support an anti-angiogenic activity of *Toona sinensis* that may contribute critically to its cancer and inflammation chemopreventive potential.

Keywords:

Traditional Chinese medicine

Toona sinensis

Gallic acid

Angiogenesis

EA.hy 926 cells

HUVECs

Toona sinensis (leaf extracts) inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis in vascular endothelial cells

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Running title: *Toona sinensis* extracts inhibit VEGF-induced angiogenesis

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ABSTRACT

Objective: Toona sinensis is well known as a traditional Chinese medicine; also, it has been shown to exhibit anticancer and anti-inflmmatory effects. This study was aimed to evaluating the anti-angiogenesis effect of the aqueous extracts of *T. sinensis* (TS extracts) or gallic acid, a major component of TS extracts, against VEGF-induced both EA.hy 926 and human umbilical vein endothelial cells (HUVECs).

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Conclusion: These results support an anti-angiogenic activity of *Toona sinensis* that may contribute critically to its cancer and inflammation chemopreventive potential.

Keywords:

Traditional Chinese medicine

Toona sinensis

Gallic acid

Angiogenesis

EA.hy 926 cells

HUVECs

1. Introduction

Toona sinensis Roem. (Meliaceae; *T. sinensis*) is a type of arbor that is widely distributed in Asia. It has long been used as a traditional Chinese medicine for a wide variety of conditions in Chinese society and is very popular in vegetarian cuisine in Taiwan. The edible leaves have been used as an oriental medicine for treating rheumatoid arthritis, cervicitis, urethritis, tympanitis, gastric ulcers, enteritis, dysentery, itchiness, and cancer (Edmonds and Staniforth, 1998; Xien, 1996). While the underlying pharmacological mechanisms of this new drug are still a matter of debate, various biological activities of *T. sinensis* leaf extracts have been reported, including anti-cancer (Chang et al., 1998; Chang et al., 2002; Chang et al., 2006), anti-inflammatory (Yang et al., 2006), anti-diabetes (Yu, 2002; Hsu et al., 2003), and antioxidant (Hseu et al., 2008; Cho et al., 2003) effects, as well as inhibiting Leydig cell steroidogenesis (Poon et al., 2005) and improving the dynamic activity of human sperm (Yang, 2003). Moreover, the safety levels and nontoxic characteristics of aqueous extracts of *T. sinensis* were evaluated using acute and sub-acute toxicity studies in mice (Liao et al., 2006).

Angiogenesis, the formation of new vessels from preexisting vasculature, is an essential process in a variety of physiological and pathological conditions, including inflammatory diseases, atherosclerosis, diabetic retinopathy, rheumatoid arthritis, cancer and metastasis (Risau, 1997; Folkman, 2002). Complex sequential steps are involved in angiogenesis, such as basement membrane degradation by proteases, endothelial cell proliferation and migration/invasion, formation of capillary tubes and survival of newly formed blood vessels (Bussolino et al., 1997). Angiogenesis critically depends on several conditions such as endothelial cell proliferation, endothelial cells secretion of matrix metalloproteinases (MMP) required to break down surrounding tissue matrix and the endothelial cell movement/migration. Angiogenesis is tightly regulated by an intricate balance between

stimulators and inhibitors (Bussolino et al., 1997). Among these, vascular endothelial growth factor (VEGF), a soluble angiogenic factor produced by many tumors as well as normal cell lines, plays a key role in regulating normal and pathologic angiogenesis (Ferrara and Davis-Smyth, 1997). VEGF contributes to the angiogenic process by stimulating proliferation, migration/invasion, and the formation of new blood vessels by endothelial cells (Ferrara and Davis-Smyth, 1997). Because of the critical dependence of human cancer and inflammatory diseases on angiogenesis, therapeutic strategies have been developed targeting various aspects of the angiogenic processes, many with promising results (Felmeden et al., 2003; Folkman, 2002; Jackson et al., 1997).

Many medicinal herbs have been shown to be rich sources of phytochemicals with chemoprevention potential for various types of human cancer and inflammatory diseases. Because tumor epithelial cells *in vivo* depend on angiogenesis to provide nutrients for their growth and survival, it is plausible that this anti-angiogenic effect may play a primary role in mediating the cancer chemopreventive activity (Felmeden et al., 2003; Folkman, 2002). Moreover, the pharmacology of many anti-inflammatory drugs has revealed that at least part of their efficacy is attributable to their anti-angiogenic effects (Jackson et al., 1997). Hence, we hypothesized that the anticancer and anti-inflammatory activity of *T. sinensis* could be partly attributable to its anti-angiogenic activity, and that *T. sinensis* could be a promising candidate drug for the treatment of diseases with impaired angiogenesis. In this study, the ability of the aqueous leaf extracts of *T. sinensis* (TS extracts) (50–100 μg/mL) and gallic acid (3,4,5-trihydroxybenoic acid) (5 μg/mL), a major component of TS extracts, to inhibit VEGF-induced angiogenesis in culture using EA.hy 926 cells and human umbilical vein endothelial cells (HUVECs) was investigated. The levels of angiogenic control and related molecules were assayed to determine the *T. sinensis*-mediated anti-angiogenic mechanism.

2. Materials and methods

2.1 Chemicals

Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), M-199 medium, glutamine, and penicillin-streptomycin-neomycin (GIBCO BRL, Grand Island, NY), anti-cyclin E antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany), anti-β-actin (Sigma Chemical Co., St. Louis, MO), anti-cyclin D1, anti-CDK4, anti-pRb, anti-VEGFR-2, and anti-eNOS antibodies (Cell Signaling Technology Inc., Danvers, MA) were obtained from their respective suppliers. All other chemicals were of the highest grade commercially available and supplied either by Merck (Darmstadt, Germany) or Sigma.

2.2. T. sinensis preparation and extraction

The leaves of *T. sinensis* were sourced from Fooyin University, Kaohsiung Hsien, Taiwan. A voucher specimen (FY-001) was characterized by Dr. Horng-Liang Lay, Graduate Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung County, Taiwan, and deposited at Fooyin University, Kaohsiung Hsien, Taiwan (Hsieh et al., 2004). The aqueous extracts of *T. sinensis* were prepared by adding 1000 mL water to 1000 g fresh *T. sinensis* leaves and boiling until it was reduced to 100 mL, as previously described (Hsu et al., 2003). The crude extracts were centrifuged at 3000 rpm for 12 min and the supernatant was used for this study. The crude extracts (50 g) were concentrated in a vacuum and freeze dried to form powder; the stock (2 mg/mL in PBS buffer, [pH 7.4]) was subsequently stored at -20 °C until analyzed for anti-angiogenic properties. The crude TS extracts separated from fresh *T. sinensis* leaves had a yield of 5%, which was based on the initial weight of the crude extracts (Yang et al., 2006).

2.3. Isolation of gallic acid from TS extracts

The TS extracts were dissolved in a mobile phase consisting of methanol-water (50:50, v/v) before high performance liquid chromatography (HPLC) analysis and separation. Eight compounds (gallic acid, methyl gallate, ethyl gallate, kaempferol, kaempferol-3-O-β-D-glucoside, quercetin, quercitrin, quercetin-3-O-β-D-glucoside and rutin) were isolated from the TS extracts, as previously described (Yang et al., 2006). The identity of the compounds was fully characterized by comparison of their spectral data (IR, NMR and mass) with the analogous information reported in the literature (Yang et al., 2006; Hsu et al., 2003). Gallic acid, the natural phenolic component purified from the TS extracts, was collected for use in this study at a yield of 6% (Yang et al., 2006). The stock of gallic acid (2 mg/mL in PBS buffer [pH 7.4]) was subsequently stored at -20 °C until analyzed for anti-angiogenic properties.

2.4. Endothelial cell culture

EA.hy 926 cells, a human vascular endothelial cell line (Edgell et al., 1983), was grown in DMEM supplemented with 15% FBS, HAT (100 mM sodium hypoxanthine, 0.4 mM aminopterin, and 16 mM thymidine), 1% glutamine, and 1% penicillin-streptomycin-neomycin at 37 °C in a 5% CO_2 humidified incubator. This EA.hy 926 cell line was used because it possessed endothelial characteristics including the formation of tube-like structures (Bauer et al., 1992). The use of a cell line also allowed us to overcome the difficulty of obtaining larger numbers of uncontaminated primary cells as well as the requirement of expensive growth factors associated with the use of primary endothelial cells. In an effort to avoid interspecies variation, a number of reports has used human umbilical vein endothelial cells (HUVECs) in proliferation assays, migration/invasion assays, and tube formation assays, or as sources of metalloproteinases (MMPs) in gelatin zymography assays. HUVECs were prepared from human umbilical

veins as described previously (Jaffe et al., 1973). In brief, the umbilical cord was infused with 0.05% collagenase solution containing the ECs and flushed from the cord by perfusion with cord buffer and centrifuged. The resulting cell suspension was divided equally between several 10-cm petri dishes, and grown to confluence in M-199 medium and supplemented with 20% FBS at 37 °C in 5% CO_2. Upon confluence, the cells of primary cultures were detached using trypsin–EDTA, and sub-cultured to confluence in tissue culture wells at 37 °C. EA.hy 926 (1×10^5 cells/well) or HUVECs (4×10^5 cells/well) were grown to confluence on tissue culture plates, pre-incubated with TS extracts (0, 50, 75, or 100 μg/mL) or gallic acid (5 μg/mL) for 1 h followed by incubation with or without VEGF (20 ng/mL) and allowed to proliferate for 24 h. Cultures were harvested and the cell number was determined using a hemocytometer. Cell viability was checked before and after treatment using trypan blue exclusion and examined using phase contrast microscopy. For all experiments, the supernatant was removed following TS extracts or gallic acid supplementation for 1 h, the cells were washed with PBS, and the culture media was replaced with new medium containing 20 ng/mL VEGF for the indicated time.

2.5. In vitro cell wound healing repair assay

To determine the effects of TS extracts or gallic acid on cell migration, an *in vitro* wound healing repair assay was performed. EA.hy 926 (1×10^5 cells/well) or HUVECs (4×10^5 cells/well) were cultured on a 1% gelatin-coated 12-well plate and incubated with the indicated concentration of TS extracts or gallic acid for 1 h in 1% FBS-medium. At confluence, monolayers were wounded using a 200 μL micropipette tip, washed twice with PBS and incubated for 12 or 24 h in 1% FBS medium with VEGF (20 ng/mL). Next, cells were washed twice with PBS, fixed with 100% methanol, and stained with Giemsa Stain solution. The cultures were photographed (200× magnification) to monitor the migration of

cells into the wounded area, and the closure of wounded area was calculated.

2.6. Endothelial cell invasion assay

Invasion assays were performed using BD Matrigel invasion chambers (Bedford, MA, USA). For the invasion assay, 10 μL Matrigel (25 mg/50 mL) was applied to 8-μm polycarbonate membrane filters, and the bottom chamber of the apparatus contained standard medium. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins. Briefly, the top chambers were seeded with EA.hy 926 cells (1×10^5 cells/well) or HUVECs (4×10^5 cells/well) in 500 μL serum-free medium, and the cells were incubated with TS extracts (0, 50, 75, and 100 μg/mL) or gallic acid (5 μg/mL) for 1 h prior to the addition of 20 ng/mL VEGF. Cells were placed in the bottom chambers (750 μL), which were filled with serum-free medium. Cells were allowed to migrate for 12 h or 24 h at 37 °C. After the incubation period, non-migrated cells on the top surface of the membrane were removed with a cotton swab. The migrated cells on the bottom side of the membrane were fixed in cold 100% methanol for 8 min and washed twice with PBS. The cells were stained with Giemsa stain solution and then de-stained with PBS. Images were obtained using an optical microscope (200 × magnification); invading cells were quantified by manual counting. Percent inhibition of invading cells was quantified and expressed with untreated cells (control) representing 100%.

2.7. Endothelial cell tube formation assay

To determine whether TS extracts or gallic acid affected the angiogenic process, tube formation was evaluated using the BD BioCoat Angiogenesis System: Endothelial Cell Tube Formation Assay kit (BD Biosciences, Bedford, MA). In brief, after a treatment with

TS extracts (0, 50, 75, and 100 μg/mL) or gallic acid (5 μg/mL), cells were harvested and seeded in a BD Matrigel Matrix coated 96-well plates with EA.hy 926 cells (1×10^5 cells/well) or HUVECs (4×10^5 cells/well) in serum-free medium, for 30 min followed by incubating with or without VEGF (20 ng/mL) at 37 °C. After 18 h, the capillary networks were photographed using a phase-contrast microscope, and a 200 × magnification; the number of tubes was quantified from three random fields (Ashton et al., 1999). The percent inhibition was expressed with untreated cells (control) representing 100%.

2.8. In vivo chick chorioallantoic membrane (CAM) assay

For the CAM assay (Kim et al., 2002), fertilized chick embryos were pre-incubated for 8 days at 37.5 °C in 85% humidity. A hole was drilled over the air sac at the end of the eggs and an avascular zone was identified in the CAM. A 1 x 1 cm window in the shell was sectioned to expose the CAM. Filter-paper disks were sterilized and loaded with TS extracts (0, 50, 75 and 100 μg/mL) or gallic acid (5 μg/mL) and applied to the CAM surface. Windows were sealed with clear tape and eggs were incubated for 48 h. Blood vessels were viewed and photographed. The anti-angiogenic effects of TS extracts or gallic acid on CAMs was quantified by counting the number of blood vessel branch points.

2.9. Determination of MMP-9 and -2 by zymography

The activities of MMP-9 and -2 released from cells were measured by gelatin zymography protease assays. EA.hy 926 cells (1×10^5/well) or HUVECs (4×10^5/well) were seeded into 12-well culture dishes and grown in medium with 15% or 20% FBS to a nearly confluent monolayer. The cells were resuspended in medium, and then incubated with up to 100 μg/mL TS extracts or 5 μg/mL gallic acid 1 h prior to the addition of VEGF (20 ng/mL). After 24 h, collected media of an appropriate volume (adjusted by vital cell

number) were prepared using SDS sample buffer, without boiling or reduction, and were subjected to 1 mg/mL gelatin-8% SDS-PAGE electrophoresis. After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in a reaction buffer (50 mM Tris-base [pH 7.5], 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij 35) at 37 °C for 24 h. Then, the gels were stained with Coomassie brilliant blue R-250.

2.10. Cell cycle distribution analysis (Flow cytometric analysis)

Cellular DNA content was determined by flow cytometric analysis of propidium iodide (PI)-labeled cells. EA.hy 926 cells (5×10^5 cells/60-mm dish) were incubated with TS extracts (0, 50, 75 and 100 μg/mL) or gallic acid (5 μg/mL) for 1 h, and then stimulated with VEGF (20 ng/mL) for 24 h. Cells were then collected by trypsinization, and fixed in ice-cold 70% ethanol at -20 °C overnight. The cells were resuspended in PBS containing 1% Triton X-100, 0.5 mg/mL of RNase and 4 μg/mL of PI at 37 °C for 30 min. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a single argon-ion laser (488 nm) was used for flow cytometric analysis. Forward and right angle light scatter, which correlated with the size of the cell and the cytoplasmic complexity, respectively, were used to establish size gates and exclude cellular debris from the analysis. The DNA content of 10,000 cells per analysis was monitored using the FACSCalibur system. The cell cycle was determined and analyzed using ModFit software (Verity Software House, Topsham, ME).

2.11. Preparation of cell extracts and immunoblot analysis

EA.hy 926 cells (5×10^5 cells/60-mm dish) were incubated with various concentrations of TS extracts (0, 50, 75, and 100 μg/mL) or gallic acid (5 μg/mL) for 1 h prior to the addition of VEGF (20 ng/mL). After 24 h, the cells were detached and washed once in cold

PBS and suspended in 100 μL lysis buffer (10 mM Tris-HCl [pH 8], 0.32 M sucrose, 1% Triton X-100, 5 mM EDTA, 2 mM DTT, and 1 mM phenylmethyl sulfony flouride). The suspension was put on ice for 20 min and then centrifuged at 16000 × *g* for 20 min at 4 °C. Total protein content was determined using a Bio-Rad protein assay reagent, with bovine serum albumin as the standard, protein extracts were reconstituted in sample buffer (0.062 M Tris-HCl [pH 6.8], 2% SDS, 10% glycerol and 5% β-mercaptoethanol), and the mixture was boiled for 5 min. Equal amounts (50 μg) of the denatured proteins were loaded into each lane, separated on 8-15% SDS polyacrylamide gel, followed by transfer of the proteins to PVDF membranes overnight. Membranes were blocked with 0.1% Tween-20 in Tris-buffered saline containing 5% non-fat dry milk for 20 min at room temperature, and the membranes were reacted with primary antibodies for 2 h. They were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody for 2 h before being developed using the SuperSignal ULTRA chemiluminescence substrate (Pierce, Rockford, IL). Band intensities were quantified by densitometry with absorbance of the mixture at 540 nm determined using an ELISA plate reader. Western blot analysis, with antibodies against cyclin D1, cyclin E, CDK4, pRb, VEGFR-2, and eNOS was performed as described previously (Hseu et al., 2008).

2.12. Statistical Analysis

Experiments are presented as mean ± standard deviation (mean ± SD). All study data were analyzed using an analysis of variance (ANOVA), followed by Dunnett's test for pair-wise comparison. Statistical significance was defined as $p < .05$ for all tests.

3. Results

In this study, the human endothelial cell lines, EA.hy 926 cells and HUVECs, were used

to investigate the ability of the aqueous leaf extracts of *T. sinensis* (TS extracts) and the natural phenoic components purified from TS extracts, gallic acid, to inhibit angiogenesis, and to elaborate the molecular mechanisms involved in the inhibition EA.hy 926 cells and HUVECs were incubated with the indicated concentrations of TS extracts or gallic acid for 1 h prior to the addition of 20 ng/mL VEGF.

3.1. Effects of TS extracts on the viability of EA.hy 926 cells or HUVECs

Angiogenesis involves the local proliferation of endothelial cells. To determine if TS extracts or gallic acid altered cultured endothelial cells, the effect of either TS extracts or gallic acid on the viability of EA.hy 926 cells and HUVECs stimulated with or without VEGF were examined. After 24 h, both cell lines displayed a very high rate of proliferation in response to VEGF, as demonstrated by trypan blue exclusion (Fig 1A). Exposure to 50–100 μg/mL TS extracts or 5 μg/mL gallic acid significantly ($p < .05$) inhibited VEGF-induced proliferation of EA.hy 926 cells or HUVECs (Fig. 1A). The concentration of TS extracts and gallic acid that were used in these studies were not cytotoxic to either EA.hy 926 cells or HUVECs without VEGF stimulation (Fig. 1B). No distinct cellular or morphological changes that are typically associated with apoptosis, such as cell detachment, rounding or chromosomal fragmentation, were detected after a 24 h incubation with TS extracts or gallic acid at a concentration below 100 μg/mL or of 5 μg/mL, respectively. Therefore, for all subsequent experiments, we employed the non-cytotoxic concentration of TS extracts (i.e., $\leq$ 100 μg/mL) and gallic acid (i.e., 5 μg/mL), and focused on the effect of TS extracts or gallic acid on VEGF-induced angiogenesis.

3.2. Effect of TS extracts on in vitro migration of EA.hy 926 cells or HUVECs

The migration of endothelial cells through the basement membrane is a crucial step in

the establishment of new blood vessels (Bussolino et al., 1997). To determine the effects of TS extracts on endothelial cell migration *in vitro*, confluent monolayers of EA.hy 926 cells or HUVECs were wounded and cultured with control buffer plus 50–100 μg/mL TS extracts or 5 μg/mL gallic acid. As shown in Fig. 2A–C, treatment with VEGF significantly ($p < .05$) induced the migration of EA.hy 926 cells after 12 h and, in 24 h, HUVECs; the addition of TS extracts or gallic acid significantly ($p < .05$) decreased VEGF-induced migration of both EC cells (Fig. 2C).

3.3. Effects of TS extracts on the invasive potential of EA.hy 926 cells

Next, the effect of either TS extracts or gallic acid on the invasiveness of EA.hy 926 cells or HUVECs was evaluated using the Boyden chamber assay, which allowed us to determine the ability of cells to pass through a layer of extracellular matrix on a Matrigel-coated filter. As shown in Fig. 3A–C, VEGF significantly induced the invasiveness of EA.hy 926 cells after 12 h and, after 24 h, the invasive potential of HUVECs; the addition of TS extracts or gallic acid (5 μg/mL) significantly ($p < .05$) decreased VEGF-induced invasion of EA.hy 926 cells or HUVECs (Fig. 3C).

3.4. Effects of TS extracts on tube formation by EA.hy 926 cells or HUVECs

Since vascular maturation during angiogenesis is characterized by the formation of tubular structures by capillary endothelial cells, we performed a tube formation assay to investigate the effect of exposure to TS extracts or gallic acid for 18 h on VEGF-induced capillary-like structure formation by EA.hy 926 cells or HUVECs. Following stimulation by VEGF, EA.hy 926 cells or HUVECs became aligned into cords on the Matrigel, and a tube-like structure was formed (Fig. 4 A and B). Treatment of cells with either TS extracts or gallic acid resulted in a significant ($p < .05$) inhibition of VEGF-stimulated tube

formation (Fig. 4 A–C). The results clearly demonstrate that exposure to TS extracts or gallic acid is effective at controlling the VEGF-stimulated tube formation by endothelial cells.

3.5. Effect of TS extracts on MMP-9 and MMP-2 activity in EA.hy 926 and HUVECs

Since gelatinase and collagenase MMPs are also involved in the angiogenic process (Egeblad and Werb, 2002), we used a gelatin zymography assay to determine the activity of secreted MMP-2 and MMP-9. To examine the effect of exposure to TS extracts or gallic acid on MMPs, EA.hy 926 cells or HUVECs were treated with TS extracts (50–100 μg/mL) or gallic acid (5 μg/mL) for 24 h in serum-free medium. The conditioned medium was collected and examined for MMP-9 and MMP-2 activity using gelatin zymography assays. Results from the zymography assay revealed that EA.hy 926 cells or HUVECs constitutively secreted MMP-9 and MMP-2 (Fig. 5A and B). VEGF treatment increased the level of protease secretion, and treatment with TS extracts or gallic acid inhibited MMP-9 and MMP-2 secretion by these cells (Fig. 5A and B) ($p < .05$).

3.6. TS extracts inhibit angiogenesis in vivo

To determine if treatment with TS extracts or gallic acid could suppress blood vessel formation *in vivo*, we employed an *in vivo* angiogenesis model, the chick chorioallantoic membrane (CAM) assay. As shown in Fig 6 A and B, a filter paper coverslip containing TS extracts (50–100 μg/mL) or gallic acid (5 μg/mL) for 48 h effectively ($p < .05$) inhibited the formation of capillary vessels in the CAM compared with the coverslip containing PBS alone, which had no visible effect on the preexisting blood vessels. The results indicate that TS extracts or gallic acid are capable of inhibiting neovessel formation *in vivo* under natural conditions.

3.7. Effect of TS extracts on cell cycle in EA.hy 926 cells

To determine whether the anti-proliferative activity of TS extracts or gallic acid involves cell cycle regulation, the profile of the DNA content in TS extracts- or gallic acid-treated EA.hy 926 cells was evaluated using flow cytometry to measure the fluorescence of PI-DNA binding. The point during cell cycle progression at which 75 μg/mL TS extracts or 5 μg/mL gallic acid induced growth inhibition in VEGF-stimulated EA.hy 926 was determined, with cellular distribution in the different phases of the treatment. Treatment of EA.hy 926 with VEGF for 24 h triggered the transition of cells from the G_0/G_1-phase to S-phase and pretreatment of cells with TS extracts or gallic acid significantly arrested cells in the G_0/G_1-phase (Fig. 7A and B) ($p < .05$). Additionally, the percentage of cells in the G_0/G_1-phase increased, while those in the S- and G_2/M phases decreased, after treatment with TS extracts or gallic acid **(**Fig. 7A and B) *(*$p < .05$). Our findings suggested that both TS extracts and gallic acid specifically induced G_0/G_1 arrest of VEGF-stimulated EA.hy 926 cells at a nontoxic dose; this may be responsible for the inhibition of VEGF-stimulated EA.hy 926 cell proliferation.

3.8. Effect of TS extracts on the G_0/G_1-related protein expression

In order to examine the molecular mechanism(s) and underlying changes in cell cycle patterns, we investigated the effects of G_0/G_1 (cell cycle)-related proteins of EA.hy 926 cells activated by VEGF. We treated EA.hy 926 cells with TS extracts (75 μg/mL) or gallic acid (5 μg/mL) for 24 h. Western blot analysis revealed that, in VEGF-stimulated EA.hy 926 cells, reductions in cyclin D1, cyclin E, CDK4, and hyperphosphorylated retinoblastoma protein (pRb) occurred after treatment with TS extracts, and cyclin E expression decreased in response to gallic acid (Fig. 8A). These results implied that the

G_0/G_1 arrest of VEGF-stimulated EA.hy 926 cells exposed to TS extracts was associated with reduced amounts of cyclin D1, cyclin E, CDK4, and pRb, and with reduced amounts of cyclin E in gallic acid-treated cells.

Additionally, because VEGF-induced activation of EA.hy 926 cells is mediated by the binding of VEGF to VEGFRs (Rahimi et al. 2000), the expression of membrane VEGFR-2 was examined by Western blot analysis. As shown in Fig. 8B, both TS extracts (75 μg/mL) and gallic acid (5 μg/mL) decreased the expression of membrane VEGFR-2 protein in VEGF-stimulated EA.hy 926 cells. Furthermore, VEGF is an important mediator of NO and eNOS catalyzed NO production, and this is believed to occur through the VEGFR-2 receptor (Shizukuda et al., 1999). Thus, we investigated the TS extracts or gallic acid-mediated eNOS protein levels in cultured EA.hy 926 cells. Western blot analysis of eNOS exposed to TS extracts (75 μg/mL) or gallic acid (5 μg/mL) for 24 h was resolved on SDS-PAGE. Incubation of cells with VEGF increased eNOS protein levels and addition of TS extracts or gallic acid inhibits the expression of eNOS induced by VEGF (Fig. 8B).

4. Discussion

A lot of herbs used in traditional Chinese medicine may be of great treasure. Recent studies have identified a number of pharmacological and toxicological properties of *T. sinensis*. In the present study, we demonstrate that TS extracts or gallic acid, a major component of TS extracts at a yield of 6% from the total extracts, potentially inhibit the proliferation, migration/invasion and tube formation, an *in vitro* marker of angiogenesis, by VEGF-activated vascular endothelial cells, both EA.hy 926 cells and HUVECs. Moreover, we show that TS extracts or gallic acid exert a potent anti-angiogenic effect *in vivo*, as verified from its inhibition of naturally induced neovascularization by capillary vessel formation in the CAM. To our knowledge, this is the first scientific demonstrating the

inhibitory effect of *T. sinensis* leaf extracts on vascular endothelial angiogenesis activated by growth factor VEGF.

Matrix MMPs play a major regulatory role in matrix reorganization and are clearly implicated in the initiation of angiogenesis and endothelial cell differentiation and spreading during angiogenesis (Egeblad and Werb, 2002; Kahari and Saarialho-Kere, 1999). In particular, the release of MMP-2 and MMP-9 from endothelial cells represents an important step in neovascularization, because this major extracelluar matrix proteolytic enzyme is secreted when endothelial sprouting takes place, thus enhancing cell migration across the extracellular matrix and tube-like structure formation (Mignatti and Rifkin, 1993). Further, MMP-2 and MMP-9 are both type IV collagenases that have been shown to be important in tumor migration/invasion *in vitro* because of their ability to break down basement membrane components, in particular collagen IV (Sato et al., 1994; Waas et al., 2002). It has been shown that a reduction in tube formation by endothelial cells was associated with a decrease in gelatinolytic activities of both MMP-2 and MMP-9, whereas an enhancement of activity increased tube formation (Egeblad and Werb, 2002). The importance of MMPs in cancer, in particular the contribution of MMP-2 and MMP-9 to cancer metastasis and angiogenesis, promoted the development of inhibitors capable of targeting gelatinase activity in tumors (Foda and Zucker, 2001). Our results demonstrate that TS extracts or gallic acid inhibited VEGF-stimulated activities of MMP-2 and MMP-9. Because increased MMP activity is closely associated with the angiogenic pathway, the inhibition of migration/invasion and tube formation by TS extracts or gallic acid may also be due to decreased MMP activity.

A critical step in angiogenesis involves the local proliferation of endothelial cells (Risau, 1997). VEGF mediates the mitogenic activities of EA.hy 926 cells and HUVECs above the basal level. TS extracts or gallic acid effectively inhibits proliferation of EA.hy 926 cells

and HUVECs. Since TS extracts or gallic acid do not have a cytotoxic effect at up to a concentration of 100 μg/mL or 5 μg/mL, respectively, the anti-proliferative effect may not be due to apoptosis or necrosis of endothelial cells, but rather to the inhibition of cell cycle progression induced by VEGF. To confirm this theory, we examined the effect of TS extracts or gallic acid on the cell cycle progression of EA.hy 926 cells stimulated by VEGF. Accumulating evidence indicates that cyclin is an essential component of the cyclin-dependent kinase (CDK) complex and plays a crucial role in regulating the activity of CDK (Sherr, 1996). Cyclin D1 and E are critical mediators of the progression of endothelial cells through the G_1/S phase of the cell cycle, forming cyclin D1-CDK4 and cyclin E-CDK2 complex that phosphorylates and inactivates the retinoblastoma (Rb) protein, thereby releasing E2F to mediate the G_1 to S transition (Sherr, 1995). D-type cyclins, like cyclin D1, are active in mid-G_1 phase, whereas E-type cyclins, like cyclin E, are active in late-G_1 phase prior to the transition of cell cycle from G_1 to S phase (Sherr, 1996). Thus, TS extracts specifically inhibit the G_0/G_1 arrest of VEGF-stimulated EA.hy 926 cells through downregulation of cyclin D1, cyclin E, and CDK4 expression and blocking Rb phosphorylation, which may be responsible for the inhibition of EA.hy 926 cell proliferation. On the contrary, our results indicate that gallic acid treatment of VEGF-stimulated EA.hy 926 cells reduced the amounts of cyclin E. Therefore, it is possible that the antiproliferative effect of gallic acid on VEGF-stimulated endothelial cells is accomplished by arresting cells at the G_0/G_1 phase of the cell cycle by suppressing cyclin E expression.

VEGF is one of the most potent and extensively studied angiogenic stimulators (Ferrara and Davis-Smyth, 1997). After being released, VEGF binds to the VEGFR on the endothelial cell membrane and initiates a cascade of signals for vessel formation (Hicklin and Ellis, 2005). Binding of VEGF to VEGFR-2, one of the VEGFRs involved in

angiogenic responses, leads to receptor autophosphorylation and then to a cascade of angiogenic signals (Hicklin and Ellis, 2005). VEGFR-2 is a receptor tyrosine kinase composed of seven extracellular immunoglobulin domains, a transmembrane domain and a tyrosine kinase active cytoplasmic domain (de Vries et al., 1992; Terman et al., 1992). It is generally accepted that activation of the VEGFR-2, which is relatively specific for vascular endothelial cells, promotes migration and, during angiogenesis (Rahimi et al. 2000). Thus, in culture studies, VEGFR-2 knockout cells block VEGF stimulation of cell migration and proliferation (Koolwijk et al., 2001). The results are consistent with the finding that inhibition of VEGFR-2 expression decreases proliferation and migration/invasion by EA.hy 926 cells, suggesting that TS extracts or gallic acid induced a decrease in VEGFR-2, leading to the inhibition of endothelial cell angiogenesis.

The earliest stages of angiogenesis are defined by vasodilation mediated by NO and increased vascular permeability of pre-existing capillaries or post-capillary venules in response to VEGF (Fukumura et al., 2001). Furthermore, VEGF is an important mediator of NO production and eNOS-catalyzed NO production, and this is believed to occur through the VEGFR-2 receptor (Shizukuda et al., 1999). NOS converts L-arginine to citrulline and NO in the presence of oxygen and NADPH. NOS can occur in one of three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Vasodilation by smooth muscle relaxation, mediated by NO, is a prerequisite for the endothelial cell to enter the angiogenic cascade and increased NO production correlates positively with increase of vascular density and tumor growth (Fukumura et al., 2001). Because of their prime role in angiogenesis, the inhibition of the expression of eNOS by TS extracts or gallic acid resulted in significant reduction of angiogenesis.

In our previous study, a number of compounds, including gallic acid, methyl gallate, ethyl gallate, kaempferol, kaempferol-3-O-β-D-glucoside, quercetin, quercitrin,

quercetin-3-O-β-D-glucoside, and rutin, were isolated from the leaves of *T. sinensis*; identity of the compounds was determined by HPLC and based on the analogous information reported in the literature (Yang et al., 2006; Hsu et al., 2003). The total phenolic content of the TS extract was estimated to be 130 ± 26 mg of gallic acid equivalent/g of plant extracts (Yang et al., 2006). The yield of gallic acid, the natural phenolic component purified from TS extracts, was about 6%. Although it remains unclear which of the components of *T. sinensis* are active compounds, gallic acid has received increased attention recently because of some interesting new findings regarding its biological activities (Chen et al., 2009). Gallic acid is widely distributed in various plants and fruits, such as gallnuts, sumac, oak bark, green tea, apple peels, grapes, strawberries, pineapples, bananas, lemons and in red and white wine (Beer et al., 2003; Sun et al., 2002; Wolfe et al., 2003). It is a polyhydroxyphenolic compound and is one of the major bioactive compounds isolated and purified from *T. sinensis*. Further, gallic acid was elucidated as one of the active angiogenesis inhibitors in a human (tissue-based fibrin–thrombin clot) angiogenesis assay (Liu et al., 2006). Even though the therapeutic utility of gallic acid in this regard is unknown, its common occurrence in fruits and food as well as its small molecular weight (170 Da) might be an advantage in terms of safety and dosing design. Studies have demonstrated that gallic acid selectively induces cancer cell death by apoptosis; however, gallic acid shows no cytotoxicity against normal cells (Isuzugawa et al., 2001; Inoue et al., 1994; Inoue et al., 1995). Therefore, gallic acid may be a useful phytochemical for cancer chemoprevention (Surh, 2003). These results imply that gallic acid is one of the active compounds responsible for the antiangiogenic activities of *T. sinensis* leaf extracts. Moreover, in future we have planned to investigate antiangiogenic effect of other bioactive compounds isolated from the aqueous leaf extracts of *T. sinensis*.

In conclusion, our observations indicate that aqueous leaf extracts of *T. sinensis* exert an inhibitory effect on several essential steps of angiogenesis, including proliferation, migration/invasion, and tube formation of vascular endothelial cells *in vitro*, and on neo-angiogenesis of the chick CAM *in vivo*. In addition, *T. sinensis* could regulate the activities of migration and invasion-associated proteinases (MMP-2 and MMP-9); it could also be effective at suppressing the proliferation of endothelial cells as shown by cell cycle arrest (G_0/G_1) and expression cell cycle-related proteins. The anti-angiogenic activity reported in this paper support the merit of further investigations to assess and define its cancer and inflammation chemopreventive and/or therapeutic potential for humans.

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Figure legend

Fig. 1. Anti-proliferative activity of TS extracts and gallic acid purified from TS extracts. (A) EA.hy 926 cells and HUVECs were pre-treated with 0, 50, 75, or 100 μg/mL TS extracts or 5 μg/mL gallic acid for 1 h followed by incubation with (A) or without (B) VEGF (20 ng/mL) and allowed to proliferate for 24 h. Cell numbers were obtained by counting cell suspensions with a hemocytometer. Results are presented as mean ± SD of three assays; * indicates significant difference in comparison to the VEGF alone group, $p < .05$. GA, gallic acid.

Fig. 2. TS extracts and gallic acid inhibit VEGF-induced migration of EA.hy 926 cells and HUVECs in an *in vitro* wound healing repair assay. Cells were pretreated with 0, 50, 75, or 100 μg/mL TS extracts or 5 μg/mL gallic acid for 1 h. Subsequently, EA.hy 926 cells (A) or HUVECs (B) were scratched and then stimulated with VEGF (20 ng/mL) for 12 or 24 h. (C) Migration was observed using a phase-contrast microscope, at a 40× magnification, and the closure of area was calculated; * indicates significant difference in comparison to VEGF alone group, $p < .05$. GA, gallic acid.

Fig. 3. TS extracts and gallic acid inhibit VEGF-induced invasion by EA.hy926 cells and HUVECs in a transwell assay. Cells were pretreated with 0, 50, 75, or 100 μg/mL TS extracts or 5 μg/mL gallic acid for 1 h and then stimulated with VEGF (20 ng/mL). Photomicrographs of EA.hy 926 cells (A) or HUVECs (B) invading under the membrane for 12 or 24 h. (C) The percentage inhibition of invading cells was quantified and expressed with untreated cells (control) representing 100%. Invasiveness was determined by counting cells in three microscopic fields per sample. Results are presented as mean ± SD of three assays; * indicates significant difference in comparison to VEGF alone group,

$p < .05$. GA, gallic acid.

Fig. 4. TS extracts and gallic acid inhibit VEGF-induced tube formation by EA.hy 926 cells and HUVECs. Cells were pre-treated with 0, 50, 75, or 100 μg/mL TS extracts or 5 μg/mL gallic acid for 1 h. EA.hy 926 cells (A) and HUVECs (B) were then collected and replated on Matrigel-coated plates at a density of 1×10^5 or 4×10^5 cells/well and incubated in the absence (Control) or presence of VEGF (20 ng/mL). After 18 h, the presence or absence of tube formation was determined using a phase-contrast microscope (×40). (C) The capillary networks were photographed using a phase-contrast microscope, and the number of tubes was quantified from three random fields. Results are presented as mean ± SD of three assays; * indicates significant difference in comparison to VEGF alone group, $p < .05$. GA, gallic acid.

Fig. 5. TS extracts and gallic acid inhibit the activity of MMP-9 and MMP-2, respectively, in VEGF-stimulated EA.hy 926 cells (A) and HUVECs (B). Cells were pretreated with 0, 50, 75, or 100 μg/mL TS extracts or 5 μg/mL gallic acid for 1 h and then stimulated with VEGF (20 ng/mL) for 24 h. The conditioned media and cells were subjected to gelatin zymography to analyze the activities of MMP-9 and MMP-2. Determined activities of these proteins were subsequently quantified by densitometric analysis. Representative results from three independent experiments are shown. Results are presented as mean ± SD of three assays; * indicates significant difference in comparison to VEGF alone group, $p < .05$. GA, gallic acid.

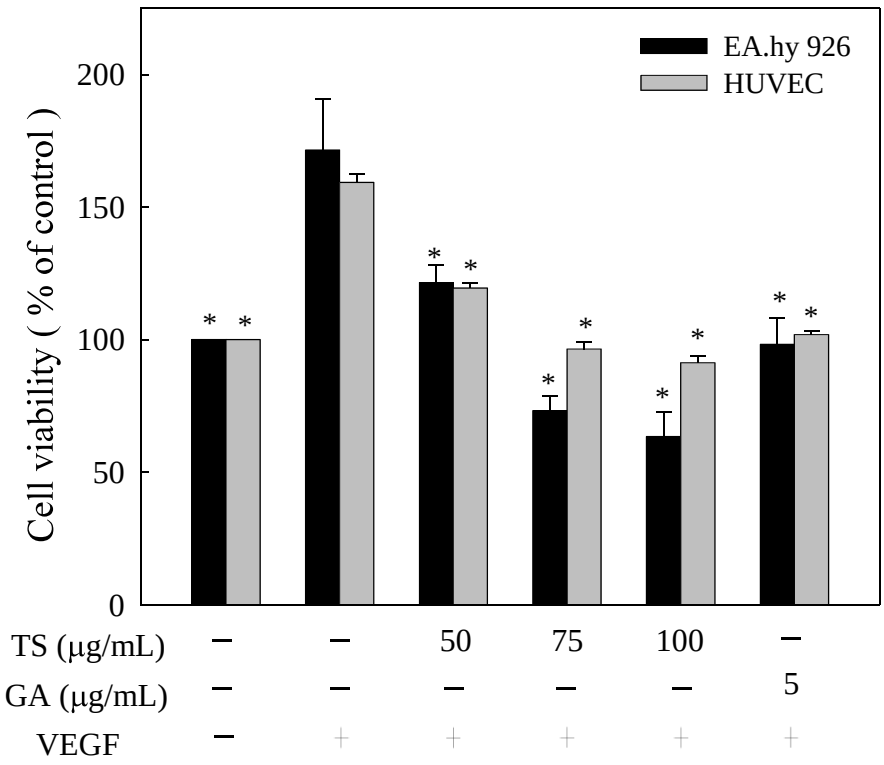
Fig. 6. TS extracts and gallic acid inhibit angiogenesis *in vivo*. A filter-paper disk with 0, 50, 75, or 100 μg/mL TS extracts or 5 μg/mL gallic acid was placed in to chick CAMs.

After 48 h, CAMs were peeled off and photographed; blood vessels were viewed and photographed. (A) Representative photographs of chick CAM assays. (B) Quantitative analysis of neovascularization from the photographs. Results are presented as mean ± SD of three assays; * indicates significant difference in comparison to control group, $p < .05$. GA, gallic acid.

Fig. 7. TS extracts and gallic acid induced VEGF-stimulated cell cycle (G_0/G_1) arrest in EA.hy 926 cells. Cells were pretreated without or with 75 μg/mL TS extracts or 5 μg/mL gallic acid for 1 h and then stimulated with VEGF (20 ng/mL) for 24 h, stained with PI, and analyzed for cell cycle phase using flow cytometry. (A) Representative flow cytometry patterns are shown. (B) Cellular distribution (percentage) in different phases of the cell cycle (G_0/G_1, G_1, S and G_2/M) after TS extract and gallic acid treatment is shown. Results are presented as mean ± SD of three assays; * indicates significant difference in comparison to VEGF alone group, $p < .05$. GA, gallic acid.

Fig. 8. Effects of TS extracts and gallic acid on VEGF-induced expression of cyclin D1, cyclin E, CDK4, pRb (A), VEGFR2 and eNOS (B) in EA.hy 926 cells. Cells were pretreated without or with 75 μg/mL TS extracts or 5 μg/mL gallic acid for 1 h and then stimulated with VEGF (20 ng/mL) for 24 h. Protein (50 μg) from each sample was resolved on a 8-15% SDS-PAGE and Western blotting was performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. Representative results from three independent experiments are shown. GA, gallic acid.

(A)


EA.hy 926
HUVEC
Cell viability (% of control)
200
150
100
50
0
TS (μg/mL) – – 50 75 100 –
GA (μg/mL) – – – – – 5
VEGF – + + + + +

(B)

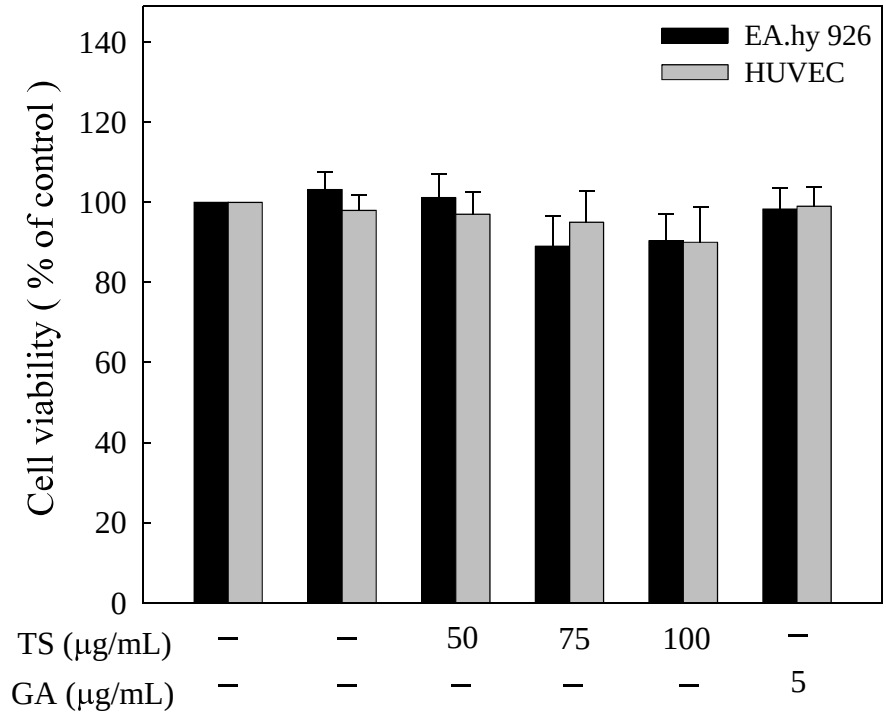
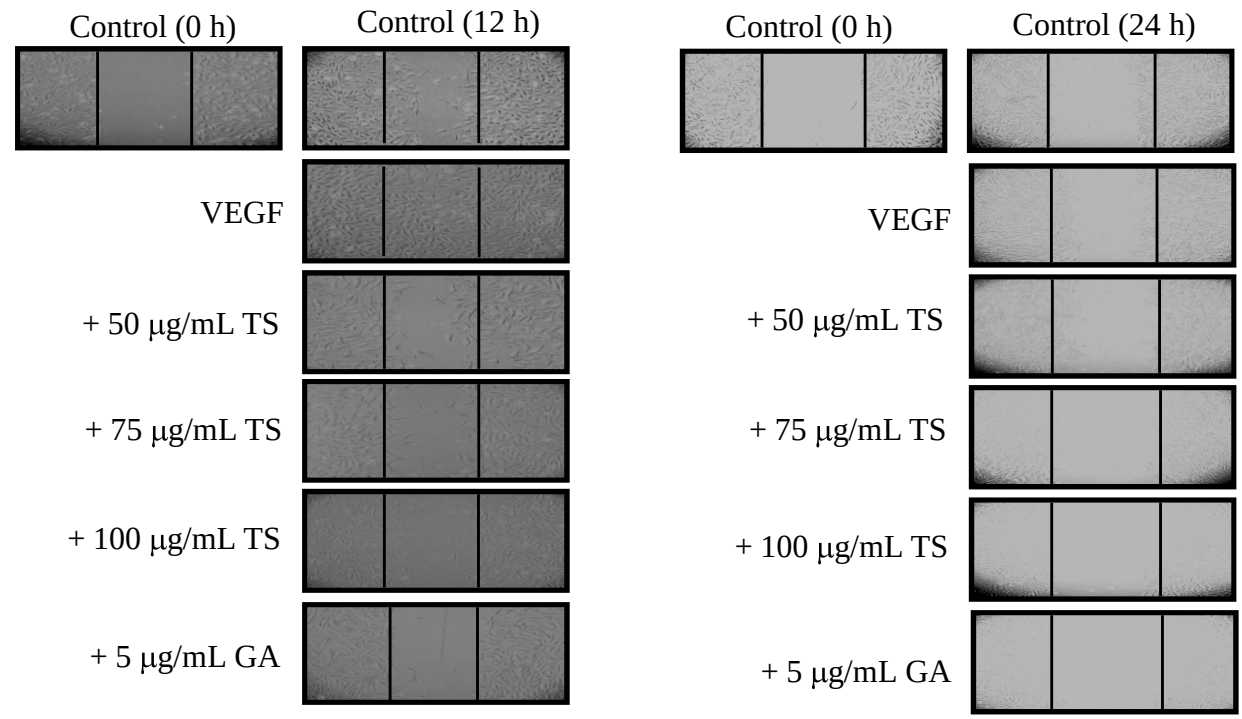

EA.hy 926
HUVEC
Cell viability (% of control)
140
120
100
80
60
40
20
0
TS (μg/mL) – – 50 75 100 –
GA (μg/mL) – – – – – 5

Fig.1


(A) EA.hy 926
(B) HUVEC
Control (0 h)
Control (12 h)
Control (24 h)
VEGF
+ 50 μg/mL TS
+ 75 μg/mL TS
+ 100 μg/mL TS
+ 5 μg/mL GA

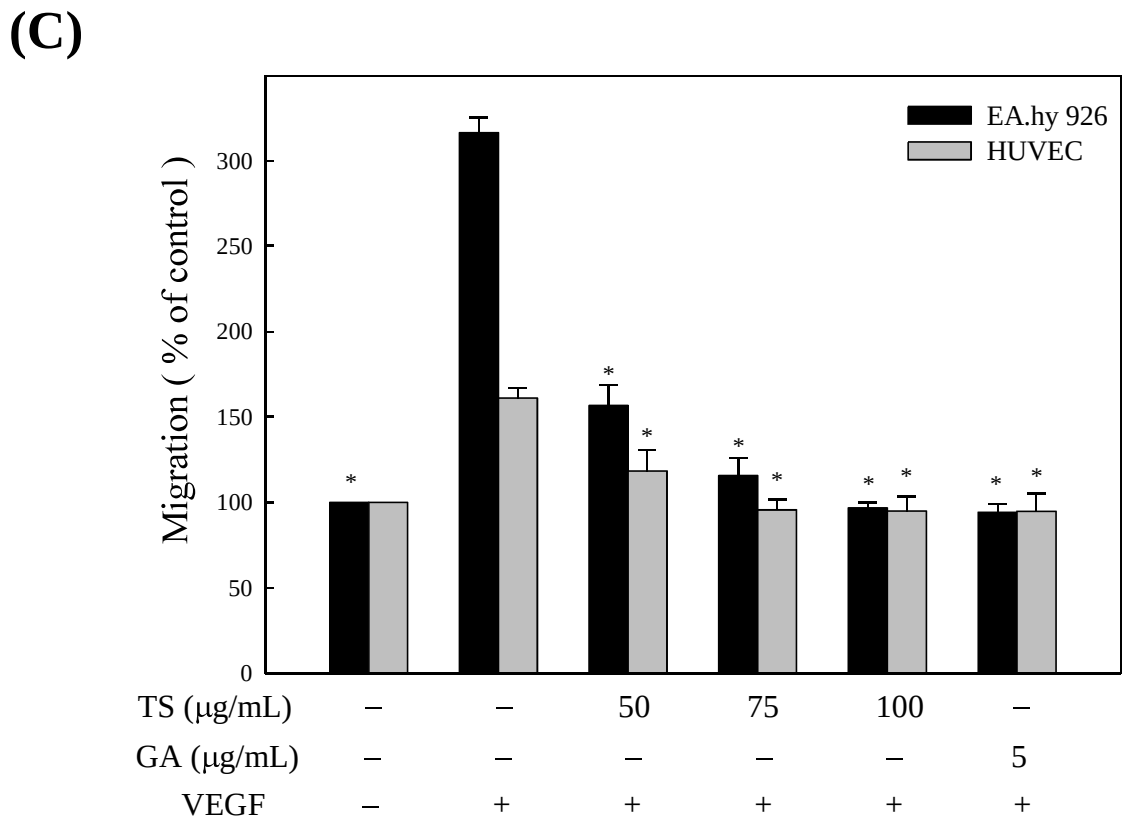
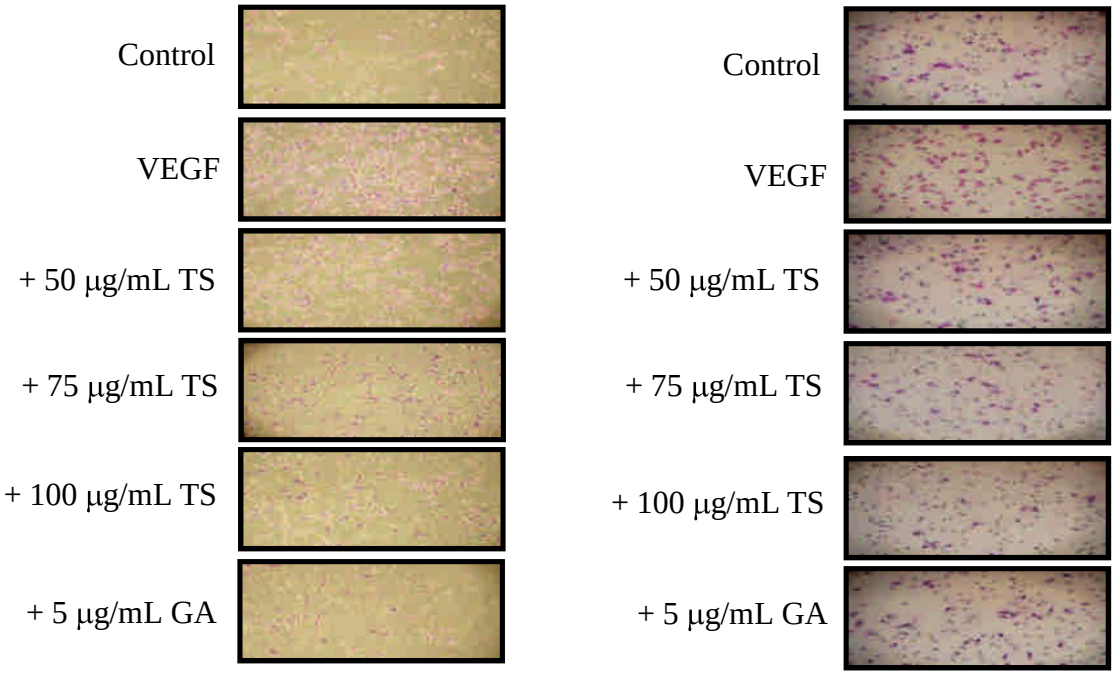

(C)
EA.hy 926
HUVEC
Migration (% of control)
0
50
100
150
200
250
300
TS (μg/mL) – – 50 75 100 –
GA (μg/mL) – – – – – 5
VEGF – + + + + +

Fig.2

(A) EA.hy 926

(B) HUVEC



(C)

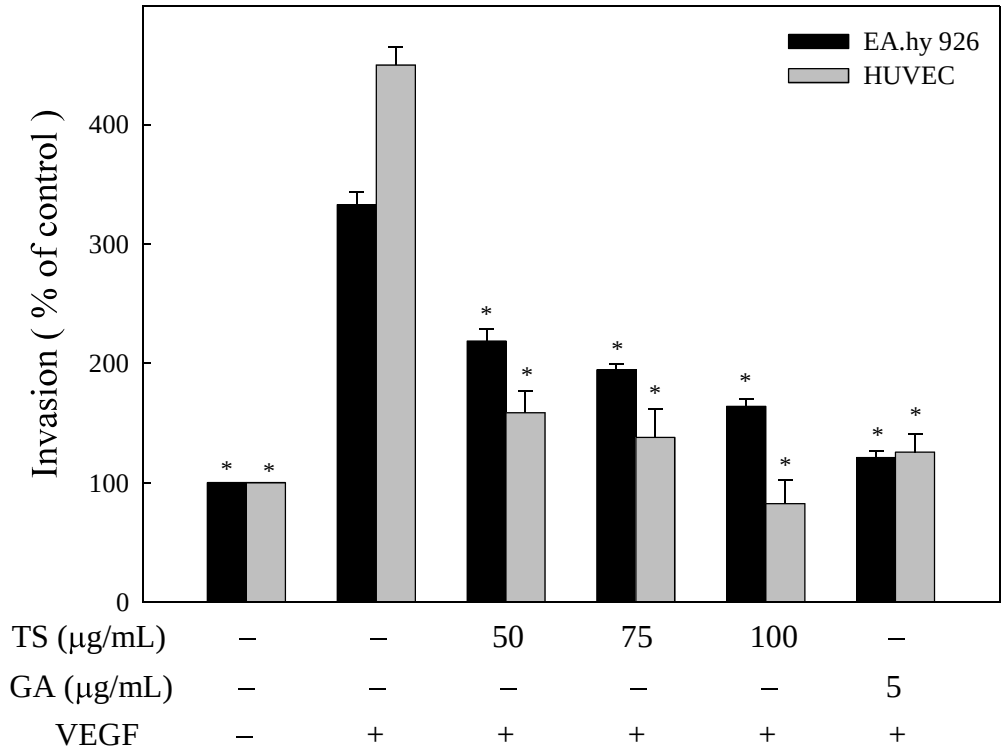
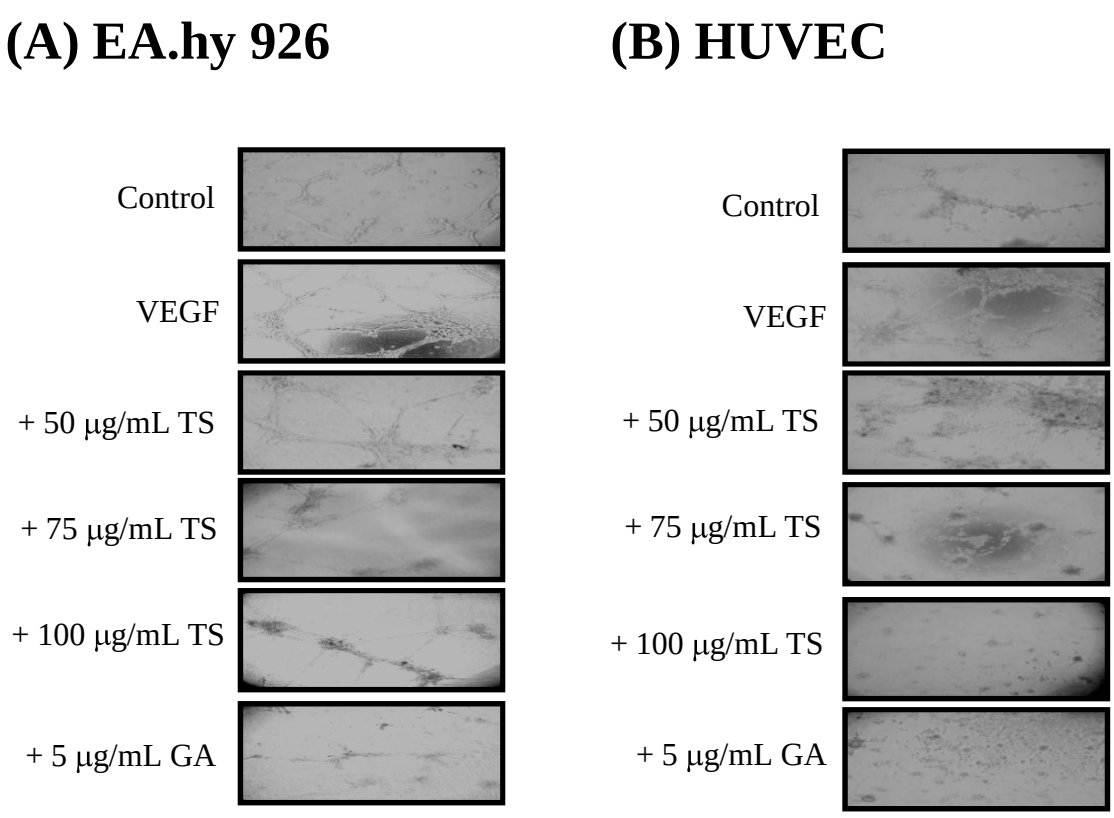


Fig.3


(A) EA.hy 926
(B) HUVEC
Control
VEGF
+ 50 μg/mL TS
+ 75 μg/mL TS
+ 100 μg/mL TS
+ 5 μg/mL GA
Control
VEGF
+ 50 μg/mL TS
+ 75 μg/mL TS
+ 100 μg/mL TS
+ 5 μg/mL GA

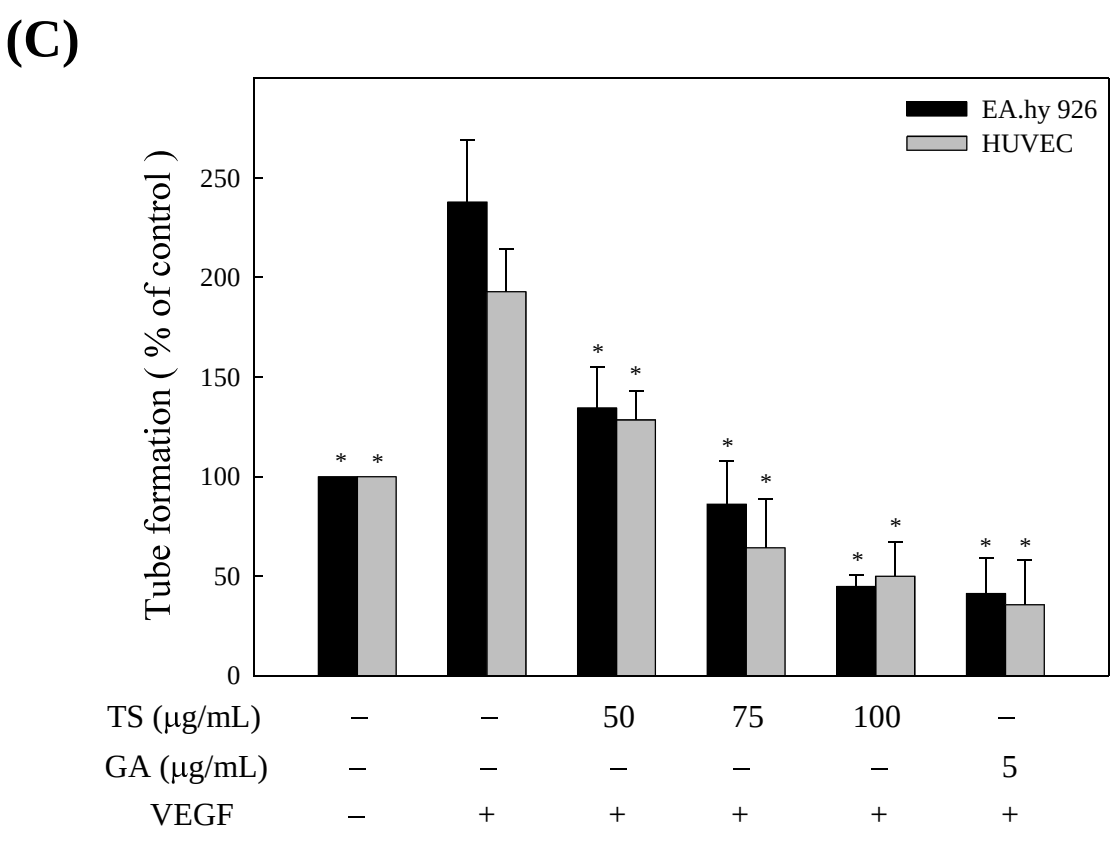

(C)
EA.hy 926
HUVEC
Tube formation (% of control)
0
50
100
150
200
250
TS (μg/mL) – – 50 75 100 –
GA (μg/mL) – – – – – 5
VEGF – + + + + +

Fig.4

Figure 5

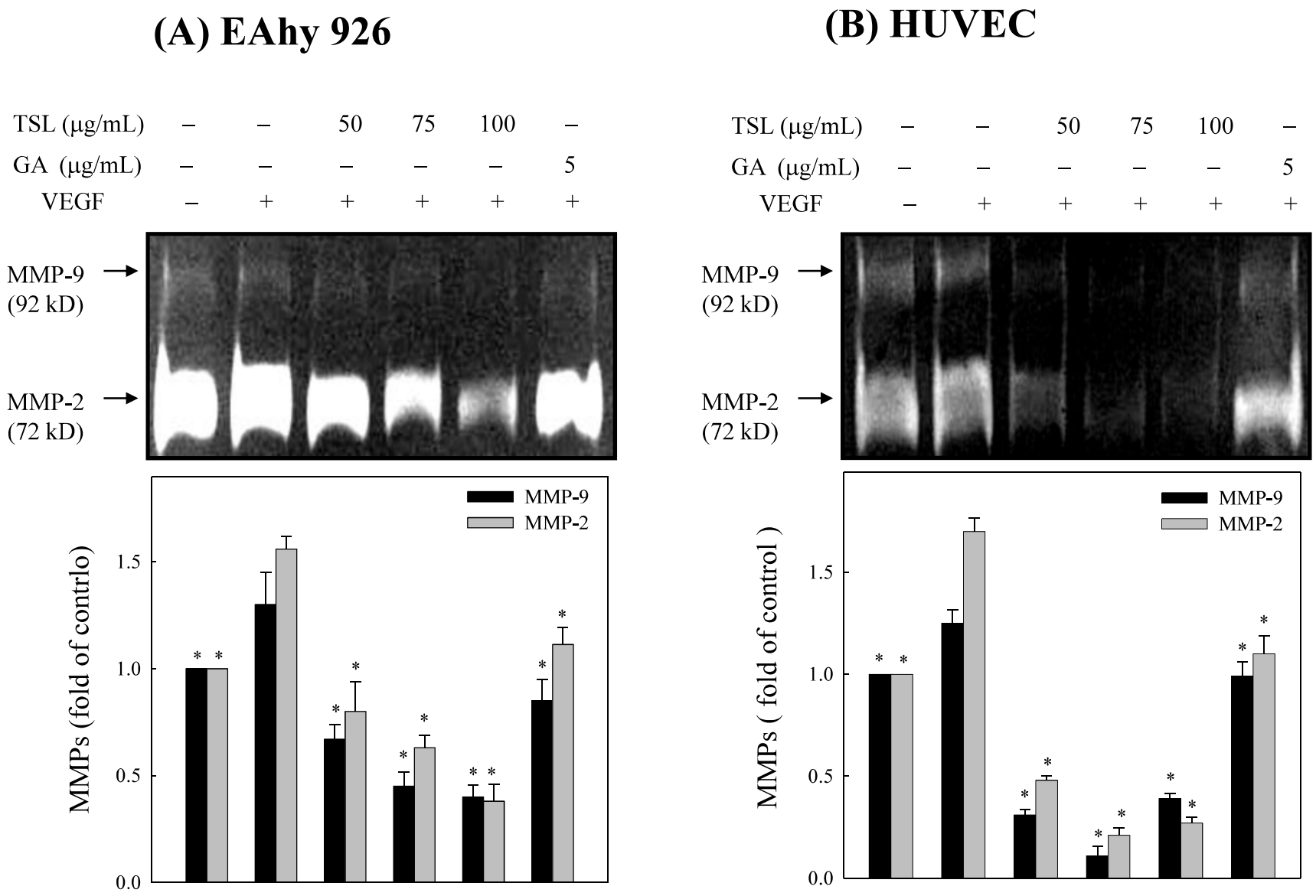
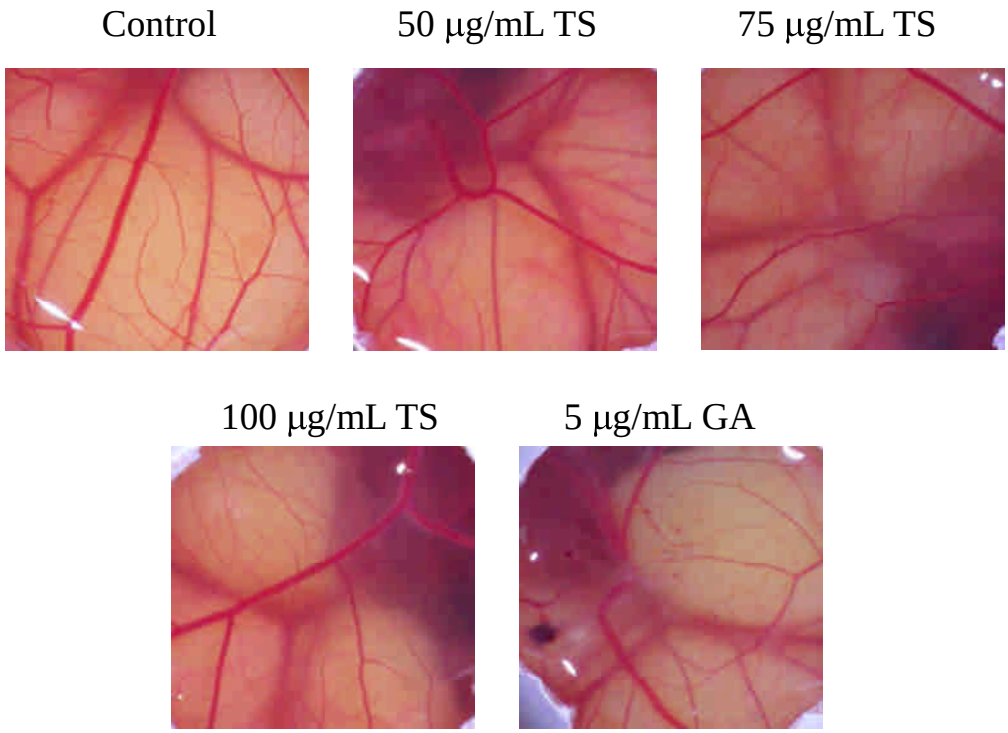


Fig.5

(A)


Control
50 μg/mL TS
75 μg/mL TS
100 μg/mL TS
5 μg/mL GA

(B)

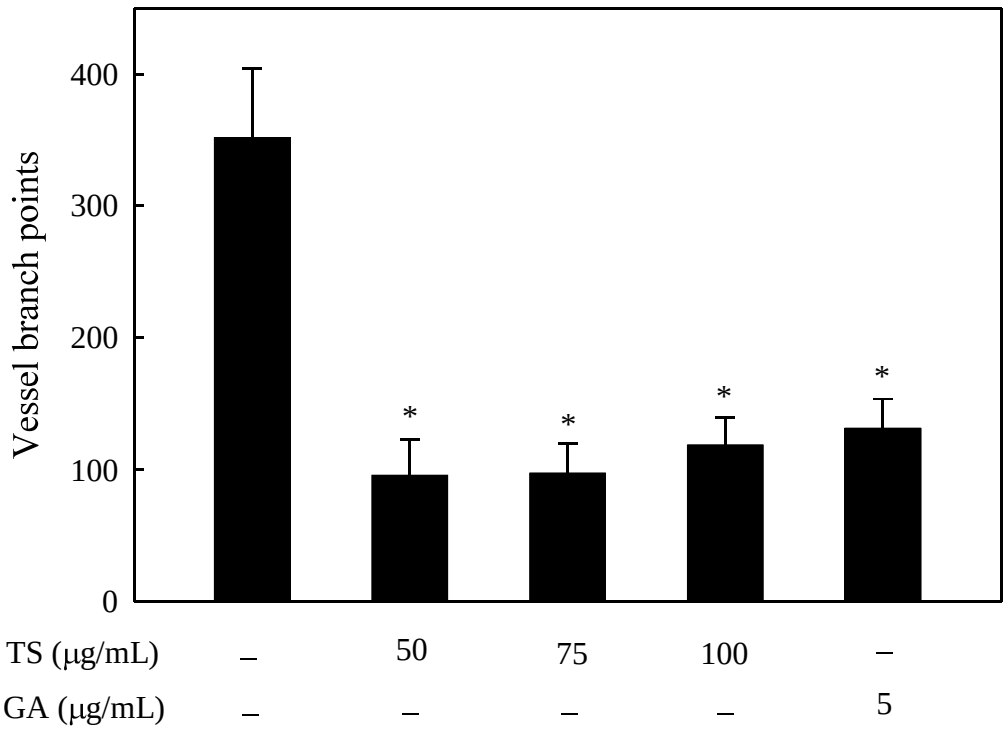

Vessel branch points
400
300
200
100
0
*
*
*
*
TS (μg/mL) – 50 75 100 –
GA (μg/mL) – – – – 5

Fig. 6

Figure 7

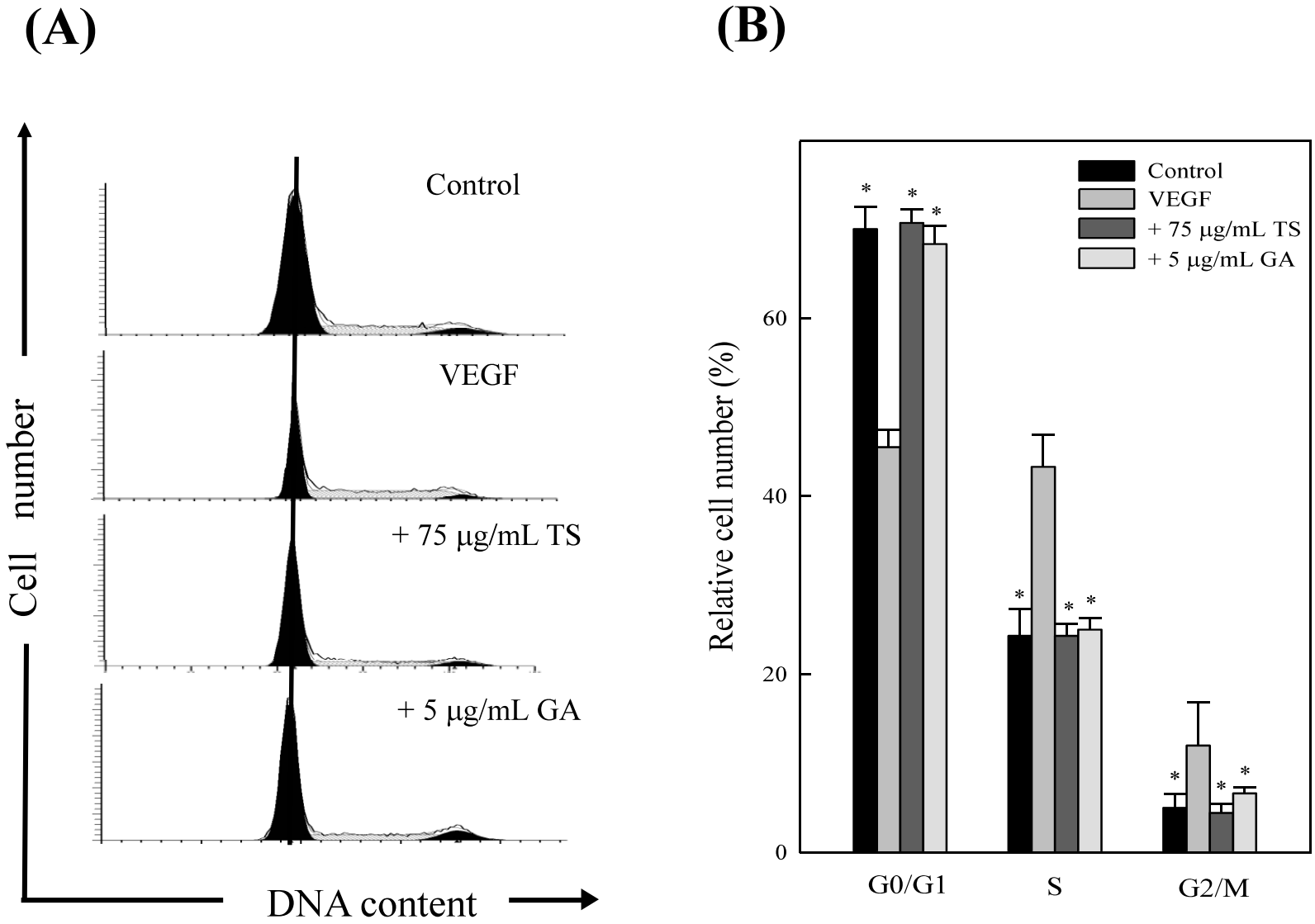


Fig. 7

Figure 8

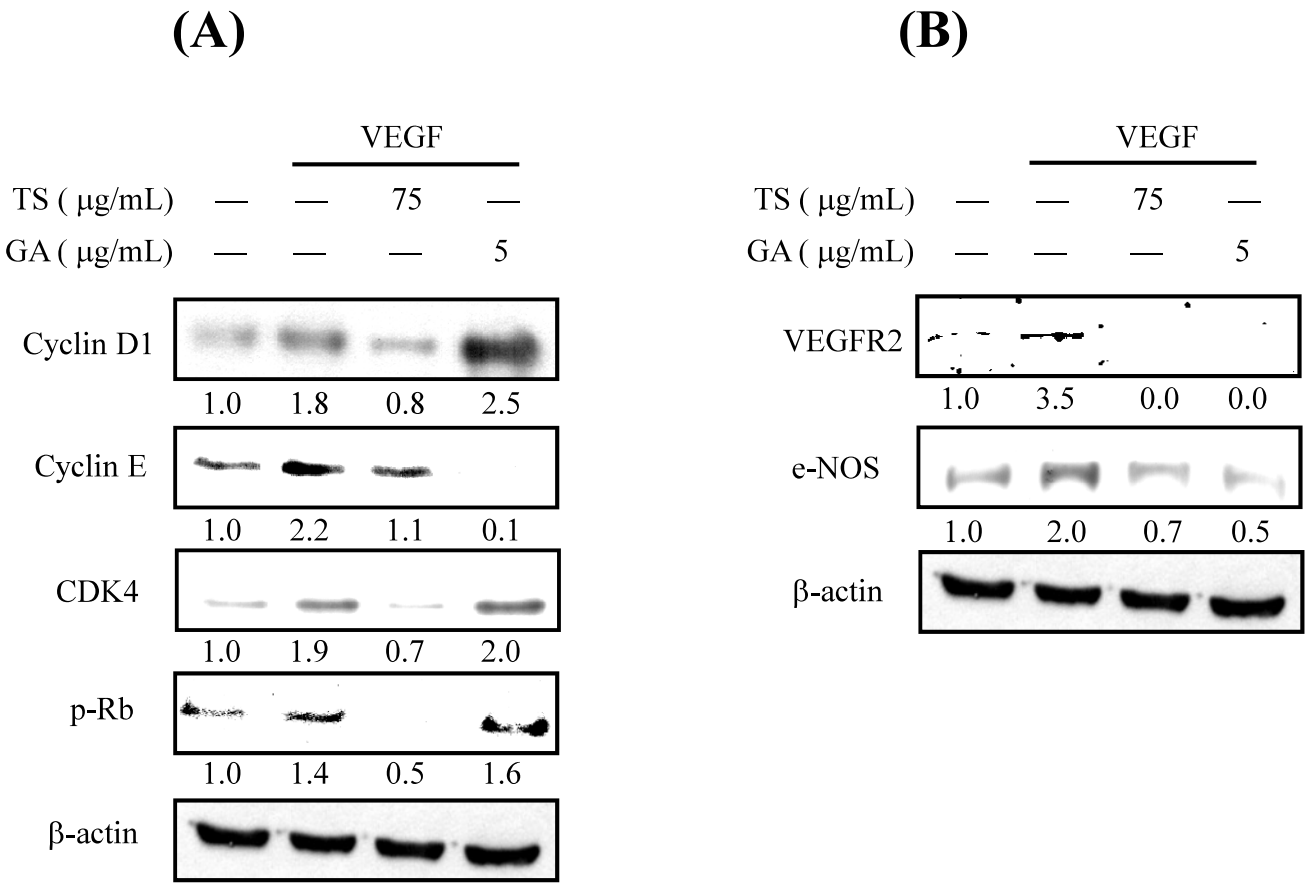


Fig. 8