In vitro Anti-angiogenic Effects of Tea Saponin and Tea Aglucone on Human Umbilical Vein Endothelial Cells

Xiaohong Li[1,2], Baozhan Huang[1], Fan Fei[1,2], Hai Niu[1,3,*], Wen Huang[1,*]

[1]Laboratory of Ethnopharmacology, Regenerative Medicine Research Center, Institute for Nanobiomedical Technology and Membrane Biology, West China Hospital/West China Medical School, Sichuan University, Chengdu, P.R.China
[2]Department of Biopharmaceutics, Key Laboratory of Drug Targeting and Drug Delivery Systems, Ministry of Education, West China School of Pharmacy, Sichuan University, Chengdu, P.R. China
[3]College of Mathematics, Sichuan University, Chengdu, P.R. China
*Corresponding author: niuhai@scu.edu.cn; huangwen@scu.edu.cn

Abstract Green tea is a popular beverage world-wide, especially in Asian countries. Its health benefits, derived from the *Camellia sinensis* leaves, have been studied over the years. However, few reports are available about the health effects of tea saponin (TS), an important component of *Camellia sinensis* leaves, and tea aglucone (TA). In this present study, we investigated the effect of TS and TA on the proliferation, migration, invasion and tube formation of human umbilical vein endothelial cells (HUVECs). TS inhibited the proliferation of HUVECs in a dose-dependent manner, with an IC50 of 7.5 ± 0.6 μM. Inconsistent with the well-reported mechanisms of apoptotic induction and cell cycle arrest by steroidal saponins, effects of TS on apoptosis and cell cycle progression in HUVECs were not detected. The autophagic vacuoles in the TS-treated HUVECs, observed with transmission electron microscopy, suggested the involvement of autophagic induction in the growth inhibition of HUVECs. When treated with TS, cell migration, invasion and tube formation potency of HUVECs were markedly suppressed. TA also suppressed the proliferation of HUVECs, with an IC50 of 25.3 ± 1.2 μM. In comparison with TS, TA inhibited the migration, invasion, and tube formation of HUVECs less effectively. Our data about the anti-angiogenic effects of TS and TA, presented for the first time, would provide a new insight into the health potential for green tea.

Keywords: tea saponin, tea aglucone, anti-angiogenic effects, human umbilical vein endothelial cells

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1. Introduction

Green tea, originated in China as a medicinal drink, is a globally popular beverage made from cured leaves of the *Camellia sinensis*. It was claimed in book named The Divine Farmer's Herb-Root Classic that *Camellia sinensis* infusions were helpful for treating many different kinds of disease conditions. Modern scientific research also revealed that green tea may be effective as an antioxidant, anti-inflammatory, anti-carcinogenic, and as an antimicrobial [1,2].

Tea leaves contain diverse polyphenols (i.e. catechins), saponins and some protein nutrients [3,4,5]. Of these compounds, polyphenols are well studied as the active ingredients with antioxidant and anti-inflammatory properties [6]. Anti-carcinogenic property of polyphenols, probably caused by their antioxidant and anti-inflammatory effects, has also been found in many types of cancer [1]. The mechanisms may include inducing apoptosis in cancer cells and inhibiting angiogenesis [1], [7,8].

Carcinogenesis is a complex and multistep process in which distinct molecular and cellular modifications arise. Among these cellular processes, angiogenesis plays important roles by providing tumor with essential nutrients and oxygen, and a route for metastasis [9,10,11,12]. Thus, anti-angiogenesis has been considered as one of the most important anticancer therapies. In comparison with chemotherapy directed at cancer cells, which often rapidly mutate and acquire "drug resistance" to treatment, the antiangiogenic therapy is obviously advantageous [13].

Compared with well-studied tea polyphenols, less literature data is available about the pharmacological activities of tea saponin (TS). So, we are interested if the anticancer effect of tea leaf is associated with its saponin and its effect on angiogenesis. In this present study, we first investigated the in vitro anti-angiogenic properties of TS and tea aglucone (TA), by analyzing their effects on the proliferation, migration, invasion, and tube formation of human umbilical vein endothelial cells (HUVECs). It was found that both TS and TA could inhibit angiogenesis relevant processes of HUVECs, even TA acted at higher doses with lower effect.

2. Methods

2.1. Chemicals

TS and TA were obtained from Laboratory of Ethno-pharmacology, Regenerative Medicine Research Center, West China Hospital/West China Medical School, Sichuan University. The purities of TS and TA are above 95% by high-performance liquid chromatography analysis. Figure 1 shows the structure of TS and TA.

2.2. Cell Culture

HUVECs were isolated from human umbilical cord veins by a standard procedure, as previously described [14], and grown in EBM-2 medium with EGM-2 SingleQuots containing VEGF and other growth factors (Lonza, USA). HUVECs at passages 3 to 8 were used for all experiments.
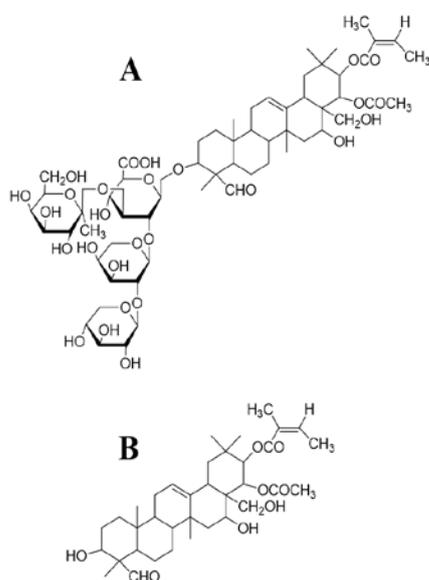

Figure 1. Chemical structures of TS (A) and TA (B)

2.3. Cell Proliferation Assay

Cell proliferation was analyzed using Cell Counting Assay Kit-8 (CCK-8) (Dojindo Molecular Technologies, Japan) according to the manufacturer's protocol. Briefly, 100 μl of cell suspension (5×10^3 cells/well) was dispensed into a 96-well plate and pre-incubated overnight, then exposed to various concentrations of TS or TA for 24 h. Ten microliters of CCK-8 solution was added to each well. After incubation for 1 h, the absorbance at 450 nm was measured using microplate reader. Experiments were performed at least three times with representative data presented. The inhibition percentage of TS or TA on cell proliferation was expressed using vehicle treated cells at 100%.

2.4. Cell Cycle Analysis

Cell cycle analysis was carried out with sub-confluent HUVECs. After incubation with vehicle, various concentrations of TS for 24 h, HUVECs were harvested, washed twice with cold PBS (pH 7.4), fixed with 70% ethanol overnight, washed twice again and then stained with propidium iodide (PI) using a Cycle TEST PLUS

DNA Reagent Kit (BD Pharmingen, USA), finally subjected to flow cytometry with a FACScan flow cytometer (Becton Dickinson, USA). Thirty thousand events were collected for each sample. The number of gated cells in the G1, G2/M or S-phase is presented as percentage.

2.5. Cell Apoptosis Detection

Cell apoptosis analysis was performed using Annexin V/PI apopotosis assay kit (KeyGEN Biotech, China) according to the manufacturer's protocol. Briefly, after incubation with vehicle, various concentrations of TS for 24 h, HUVECs were harvested, washed twice with cold PBS (pH 7.4), and stained with 3 μl of Annexin V and 3 μl of PI in 300 μl of binding buffer for 10 min in the dark. Cell apoptosis was then analyzed by Becton Dickinson FACScan Flow Cytometer (Becton Dickinson).

2.6. Transmission Electron Microscopy (TEM)

Cells were fixed in cold (4°C) 3% phosphate-buffered glutaraldehyde (pH 7.2) for 2 h, and post fixed for a further 2 h in 1% phosphate-buffered osmic acid. The sample was dehydrated in acetone and finally embedded in Epon812 (SPI). Ultrathin (50-70 nm) sections were cut and double-stained with uranyl acetate and lead citrate. Cells were examined on Hitachi transmission electron microscope system (Japan).

2.7. Wound Healing Migration Assay

HUVECs were allowed to grow to full confluence in 24-well plates, then wounded by scratching with pipette tips and washed with PBS. Serum-free EGM-2 medium containing vehicle, different concentrations of TS or TA were added to the scratched monolayers. Images were taken using an OLYMPUS digital camera after 16 h of cell migration. The migrated cells were quantified by manual counting, and the percentage of inhibition was expressed on the basis of vehicle treated cells.

2.8. Transwell Invasion Assay

Invasion assay was carried out as described previously [13]. In brief, the filter of the Transwell plate (Corning, USA) was coated with 50 μl Matrigel (BD Biosciences). After Matrigel polymerization, the bottom chambers were filled with EGM-2 medium containing various growth factors, and the top chambers containing 100 μl EBM-2 medium (without growth factors) were seeded with HUVECs (2×10^4 cells/well) with vehicle, various concentrations of TS or TA. After invading for 24 h, non-invaded cells were scraped with a cotton swab, and invaded cells were fixed with methanol and stained with 0.05% crystal violet. The cells were photographed under a light microscope and quantified by manual counting. The inhibition percentage of TS or TA on cell invasion was expressed using vehicle treated cells at 100%.

2.9. Tube Formation Assay

The tube formation assay was performed as described previously [15]. Assessment of in vitro capillary tube-like formation was carried out using a growth factor-reduced basement membrane Matrigel matrix (BD Biosciences).

Fifty microliters of Matrigel were distributed as a thin layer onto the bottom of 48-well cell plates and left for polymerization at 37°C for 30 min. HUVECs suspended in EGM-2 medium were seeded onto the Matrigel in the presence of vehicle or different concentrations of TS or TA. After 6 h, cells were photographed under a light microscope. Three microscopic fields were selected at random, and the length of tube-like structures per-field was measured using Image-pro Plus 6.0 System (Media Cybernetics, USA). The percentage of inhibition was expressed using vehicle treated cells at 100%.

2.10. Statistical Analysis

Data were expressed as *mean ± standard deviation (SD)*. Data were analyzed with one-way ANOVA using SPSS 16.0. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. TS and TA Inhibit HUVEC Proliferation

Angiogenesis, contributing to tumor growth and metastasis, is characterized by endothelial cell proliferation, migration and tube formation [16,17]. The endothelial cell proliferation induced by vascular endothelial growth factor (VEGF) leads on to the angiogenesis process. To assess the anti-angiogenic activity of TS and TA in vitro, their inhibitory effects on VEGF-induced HUVEC proliferation were first evaluated. The VEGF-induced HUVEC proliferation was obtained by treatment with 5 µg/ml VEGF-A.

It was found that treatment with TS induced morphologic changes in HUVECs at doses above 6 µM for 24 h (Figure 2A). TA induced morphologic changes at doses above 25 µM (data not presented). Cell viability was determined using CCK-8 assay. As shown in Figure 2B, treatment with TS resulted in the inhibition of VEGF-induced proliferation of HUVECs, with an IC50 value of 7.5 ± 0.6 µM. TA inhibited VEGF-induced proliferation at a much higher concentration with an IC50 value of 25.3 ± 1.2 µM (Figure 2B).

The inhibitory effects of both TS and TA on VEGF-induced growth of HUVECs suggested their potential as anti-angiogenic agents. Considering the toxic effects of both TS and TA on the cell morphology of HUVECs, we investigated the inhibitory effects of TS and TA on the migration, invasion and tube formation of HUVECs using non-toxic doses below the values of their respective IC50.
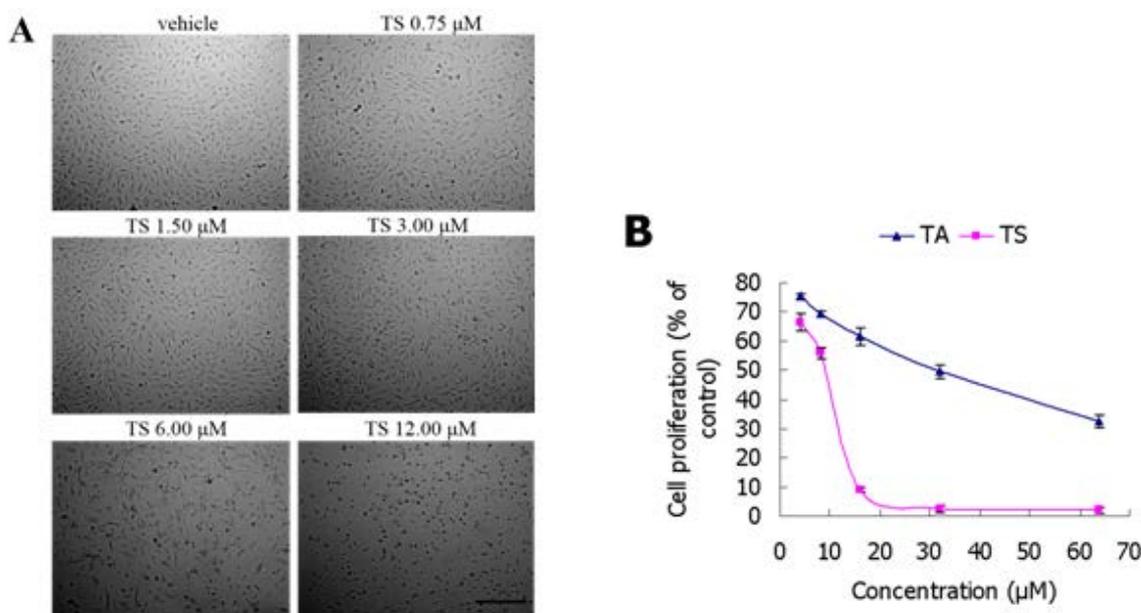

Figure 2. Effect of TS on HUVEC morphology (A) and VEGF-induced proliferation (B). (A) After treated with varying concentrations of TS for 24 h, cell morphology was observed under inverted light microscopy. (B) VEGF-induced HUVEC proliferation was obtained by treatment with 5 µg/ml VEGF-A. After treated with varying concentrations of TS or TA for 24 h with presence of 5 µg/ml VEGF-A, cell viability was quantified using CCK-8 kit. Scale bar, 10 µm

3.2. Effect of TS on HUVEC Cell Cycle Progression

To understand the anti-proliferation effect of TS, we examined the cellular responses associated with cell death under TS treatment. Owing to the fact that interference of cell cycle progression would definitely result in cell proliferation inhibition [18], cell cycle analysis was performed using PI staining and flow cytometry. HUVECs were treated with TS (3, 6, or 12 µM) for 24 h, further stained and examined. Significant modulation of HUVEC cell cycle progression by TS was not observed (Figure 3).
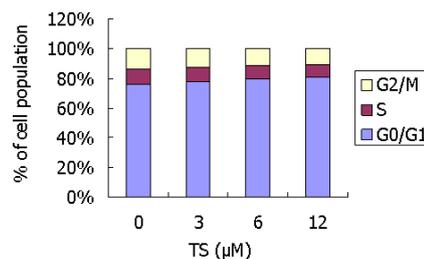

Figure 3. Graphical representation of cell cycle analysis of HUVECs. After treated with varying concentrations of TS for 24 h, cell cycle analysis was performed using PI staining and flow cytometry. The cell population is expressed as percentage of the total cells analyzed. One representative experiment of three is shown

3.3. Effect of TS on HUVEC Apoptosis

Cell death generally results from three different cellular processes: apoptosis, autophagy and necrosis [19]. To elucidate whether the growth-inhibitory effect of TS was related to the induction of apoptosis, flow cytometry analysis of Annexin V/PI stained HUVECs was performed. HUVECs were treated with TS (3, 6, or 12 μM) for 24 h, further stained and examined. Representative histograms of HUVECs after treatment with TS were presented in Figure 4. It was found that there was no significant difference in the proportion of apoptotic cells between vehicle and TS treated HUVECs.
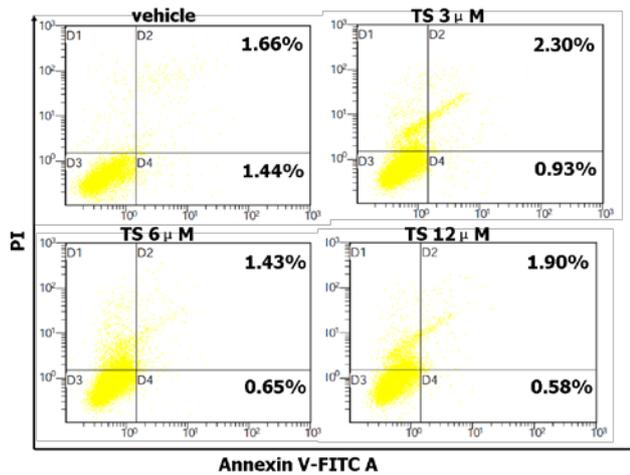

Figure 4. Flow cytometric evaluation of HUVEC apoptosis. Histograms derived from flow cytometry comparing apoptotic cells between vehicle and indicated concentrations of TS treated cells. After treatment for 24 h, the induction of apoptosis was determined using flow cytometric analysis of Annexin V-FITC and PI-stained HUVECs. Cells in the lower right quadrant indicate Annexin-positive, early apoptotic cells, and cells in the upper right quadrant indicate Annexin-positive/PI positive, late apoptotic cells. Data were the mean result from three parallel experiments

3.4. TS Induces Autophagic Vacuoles in HUVECs

Like apoptosis, autophagy is also controlled tightly and plays a critical role in cell growth [20,21]. Owing to the fact that prolonged autophagy can lead to non-apoptotic programmed cell death, autophagy of HUVECs was hence investigated. HUVECs were treated with TS (1.5, 3, or 6 μM) for 24 h and further examined using TEM. Representative morphologies of HUVECs after treatment with 3μM of TS were presented in Figure 5. As shown in Figure 5, in contrast to the control group, numerous autophagic vacuoles were observed in HUVECs treated with TS. The vehicle treated control cells exhibited an undifferentiated phenotype, whereas in the TS-treated cells, various kinds of autophagic vesicles (white arrowheads in Figure 5B) were observed within the cytoplasm. It may be noted that there were varying autophagosomes, such as typical autophagosomes (Au in Figure 5C) with intracellular components and organelles, early autolysosomes (Figure 5D) with residual autophagosomes (Au) and lysosomes (Ly), and late autolysosomes (Figure 5E) with degraded amorphous substances.

It is worth mentioning that characteristics of apoptosis, such as cell shrinkage, nuclear condensation and fragmentation were not observed in TS treated HUVECs (Figure 5A and Figure 5B). The result of morphological observation is consistent with that of the apoptotic analysis with Annexin V/PI staining. Both results revealed no apoptotic induction by TS.
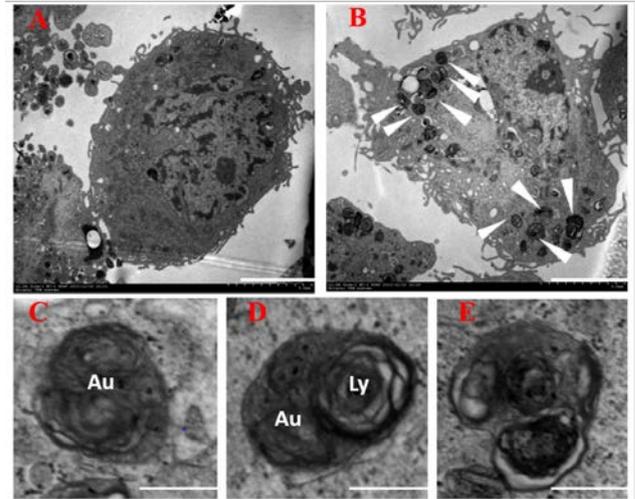

Figure 5. Transmission electron micrographs of HUVECs treated with vehicle (A) or 3 μM (B–E) of TS for 24 h. Control cells treated with vehicle exhibited a normal undifferentiated phenotype, showing absence of apoptotic bodies and nuclear condensation, whereas in the TS-treated cells, various kinds of autophagic vesicles (white arrowheads in B) are observed within the cytoplasm. These vesicles include the typical autophagosomes (Au in C), early autolysosomes (D) in which lysosomes (Ly) are still present, and late autolysosomes (E) with degraded amorphous substances. Scale bars represent 5 μm in A and B and 0.5 μm in C–E

3.5. TS and TA Inhibit HUVEC Migration

Endothelial cell migration is a key step for tube formation in tumor angiogenesis and tumor metastasis [16,17]. In this work, wound healing assays were carried out to investigate the effects of TS on HUVEC migration. The result revealed that TS inhibited the migration of HUVECs in a dose-dependent manner (Figure 6A). Treatment with TS at 1.5 and 3 μM reduced cell migration by 28.2% and 46.7%, respectively (Figure 6A). The results indicated that TS significantly ($P<0.001$) inhibited the migration of HUVECs.

For comparison, the inhibition effect of TA on HUVEC migration was also investigated. The result revealed that TA also inhibited the migration of HUVECs in a dose-dependent manner (Figure 6B). Treatment with 7.5 and 15 μM of TA reduced cell migration by 8.3% and 21.9%, respectively (Figure 6B). Compared with TS, TA acted at higher doses with much lower effect on HUVEC migration.

3.6. TS and TA Inhibit HUVEC Invasion

The invasion of HUVECs across the extracellular matrix is also a fundamental step during angiogenesis [16,17]. To examine the inhibitory effect of TS on the invasion of HUVECs, Transwell invasion assays were conducted to evaluate the ability of HUVECs, treated with varying concentrations of TS, on passing through the Matrigel and membrane barrier of the Transwell. The result showed that TS inhibited the invasion property of HUVECs in a dose-dependent manner (Figure 7A). Treatment with TS at 1.5 and 3 μM resulted in inhibition of cell invasion by 31.0% and 72.6% respectively (Figure 7A).

The results demonstrated that TS significantly inhibited migration of HUVECs ($P<0.001$).

For comparison, the inhibitory effect of TA on HUVEC invasion was also investigated. The result revealed that TA also inhibited the invasion of HUVECs in a dose-dependent manner (Figure 7B). Treatment with TA at 7.5 and 15 μM reduced cell invasion by 10.3% and 27.1%, respectively (Figure 7B). Compared with TS, TA acted at higher doses with lower effect on HUVEC invasion.
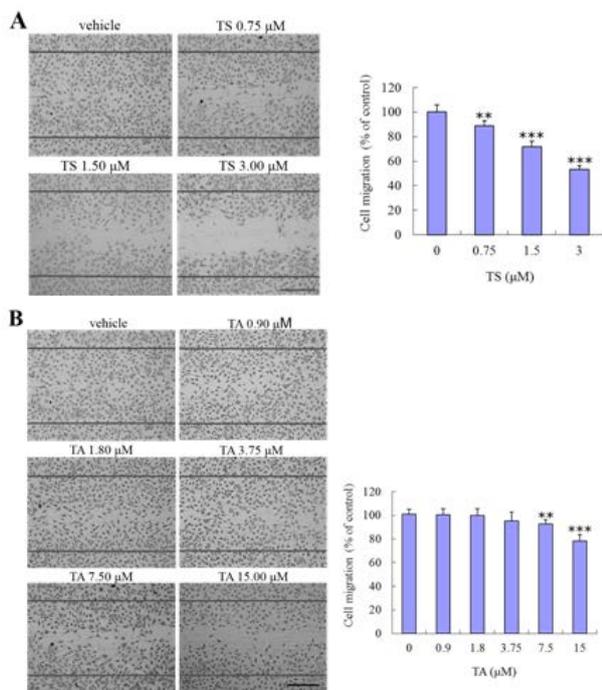

Figure 6. Effect of TS (A) and TA (B) on HUVEC migration in wound healing assay. Monolayer HUVECs were wounded by scratching with pipette tips and treated with ethanol vehicle or different concentrations of TS or TA in serum-free EGM-2 medium. After 16 h of incubation, the migrated cells were quantified by manual counting. The percentage of inhibition was expressed using vehicle treated cells at 100%. The results are expressed as *mean±SD* ($n = 5$). Columns, mean; bars, *SD*. **, $P<0.01$; ***, $P<0.001$ versus vehicle-treated group. Scale bar, 10 μm

3.7. TS and TA Inhibit HUVEC Tube Formation

Although angiogenesis is a complex and multistep process with several kinds of cells involved, tube formation of endothelial cells is one of the crucial steps [16,17]. To investigate the inhibitory effect of TS on angiogenesis, we carried out in vitro tube formation assay of HUVECs on the surface of Matrigel. The results revealed that HUVECs formed capillary-like structures on the surface of Matrigel in the control group within 6 h. Treatment with TS dose-responsively inhibited the tube formation (Figure 8A). Treatment with TS at 1.5 and 3 μM resulted in tube length reduction by 54.1% and 65.3% respectively (Figure 8A). The results demonstrated that TS significantly inhibited tube formation property of HUVECs ($P<0.001$).

For comparison, the inhibitory effect of TA on HUVEC tube formation was also investigated. The result revealed that TA also inhibited the tube formation of HUVECs in a dose-dependent manner (Figure 8B). Treatment with TA at 7.5 and 15 μM reduced tube length by 16.0% and 27.5%, respectively (Figure 8B). Compared with TS, TA

acted at higher doses with lower effect on HUVEC tube formation property.
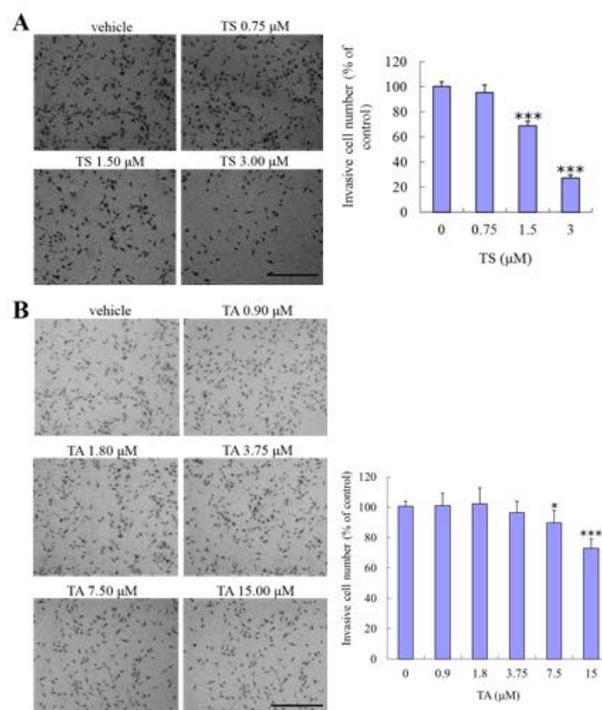

Figure 7. Effect of TS (A) and TA (B) on HUVEC invasion in Transwell assay. A total of 2×10^4 HUVECs were seeded in the top chamber and treated with ethanol vehicle or varying concentrations of TS or TA. After 24 h, HUVECs that invaded through the membrane were stained with crystal violet and quantified. The percentage of inhibition was expressed using vehicle treated cells at 100%. The results are expressed as *mean ± SD* ($n = 5$). Columns, mean; bars, *SD*. *, $P<0.05$; ***, $P<0.001$ versus vehicle-treated group. Scale bar, 10 μm

4. Discussion

Green tea is an extremely popular drink in the world. Its habitual consumption has long been associated with health benefits including antioxidant, anti-inflammatory and anti-carcinogenic efficacy [1]. The effects of green tea on cancer, mostly chemo-preventive effects, are generally thought to be mediated by its polyphenols known as catechins [22,23]. Compared with the well-studied tea polyphenols, less attention was paid to the health effects of TS, which is an important component of *Camellia sinensis*.

In green tea leaves, several saponins have already been identified, including theasaponin B1 [24], assamsaponin J [25], isotheasaponin B1-B3 [26], foliatheasaponin I-IV and floratheasaponin A [27]. Pharmacological studies have revealed that tea-leaf saponin might have antimicrobial [28], anti-inflammatory [28], anti-allergic [29] and antihypercholesterolemic effects [30]. In this work, we evaluated the in vitro anti-angiogenic effect of TS and TA for the first time. Our data indicated the concentration-dependently inhibitory effects of TS and TA on HUVEC proliferation, migration, invasion and tube formation.

For endothelial cells that undergo angiogenesis, the involved cellular functions are defined as proliferation, migration and invasion [16,17]. The well-defined mechanisms of existing anti-angiogenic agents would involve the interferences of those cellular processes of

HUVECs [31,32]. For angiogenesis inhibitors, interference of VEGF-induced HUVECs proliferation is a common phenomenon [31]. In this work, both TS and TA inhibited the VEGF-induced growth of HUVECs in a dose-responsive manner (Figure 2). Notwithstanding the drug induced morphologic changes of HUVECs revealed the cell toxic effects of both TS and TA, their inhibitory effects on VEGF-induced growth of HUVECs suggested their potential as antiangiogenic agents.

To avoid the toxic effects, the inhibitory effects of TS and TA on migration, invasion and tube formation of HUVECs were investigated using non-toxic doses below the values of their respective IC50. Notably, the inhibition by TS and TA occurs at concentrations below those that show significant direct effects on the normal growth of endothelial cell. One and a half or 3 µM of TS were sufficient to obviously block migration, invasion and capillary-like structure formation in vitro. Seven and a half or 15 µM of TA also resulted in the inhibition of migration, invasion and tube formation of HUVECs with relatively lower effect. The inhibitory properties of TS and TA on these cellular processes have shown promise of anti-angiogenic potency for tea leaf.
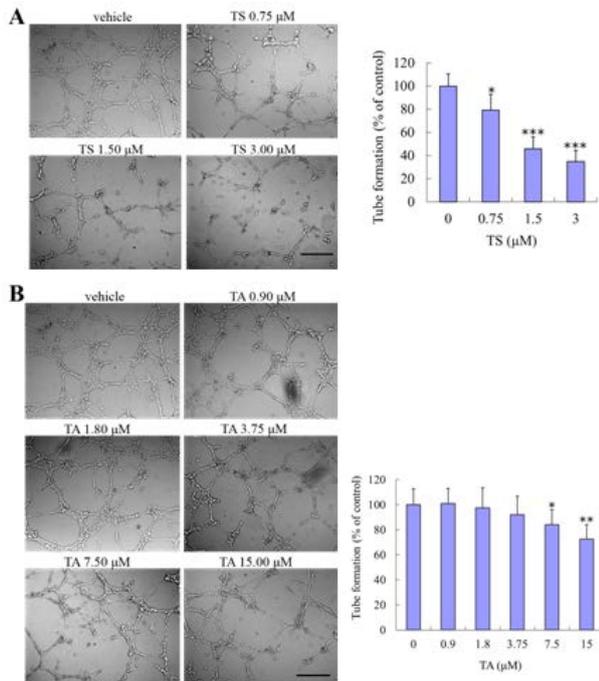

Figure 8. Effect of TS (A) and TA (B) on HUVEC tube formation. After treated with vehicle or varying concentrations of TS or TA for 6 h, tubular structure in each group was measured using Image-pro Plus 6.0 System. The percentage of inhibition was expressed using vehicle treated cells at 100%. The results are expressed as *mean ± SD* (n = 3). Columns, mean; bars, *SD*. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ versus vehicle-treated group. Scale bar, 10 µm

The differences in anti-angiogenic potencies between TS and TA might result from their bioavailability variances. Owing to the well-known fact that low availability of a medication is a great barrier to its efficacy, in order for any of the components of green tea to be a health benefit they have to have bioavailability. Compared with TA, the relatively higher efficiency of TS at low dosage might be attributed to improvements in solubility and tight junction opening [33], or higher ability of membrane transportation owing to the presence of glycosyl moieties [34].

Cell death and growth inhibition generally results from three different cellular processes: apoptosis, autophagy and necrosis [18]. In addition, interference of cell cycle progression would definitely result in cell proliferation inhibition. Presently, many kinds of steroidal saponinshave been reported to induce apoptosis in a wide variety of tumor cells through cell cycle arrest [35], activation of p53 and caspase-3 [36,37], *etc*. Here, TS induced no apoptosis, but obvious autophagic process in HUVECs, with no effect on its cell cycle. When TS-treated HUVECs were investigated under a transmission electron microscope, characteristic autophagosomes and autolysosomes were present (Figure 5.B–E).

Like apoptosis, autophagy is also controlled tightly and plays a critical role in tissue homeostasis, development and disease [20,21]. It is a cellular defense process that involves the sequestration and delivery of cytoplasmic material by autophagosomes to the lysosome where it is degraded and recycled [38,39]. Although autophagy is initiated as a protective response to stress, persistent autophagy can lead to growth inhibition and cell death. The autophagic vacuoles in the TS-treated HUVECs might suggest the involvement of autophagic induction in the growth inhibition of HUVECs.

Recently, VEGF receptor 2 (VEGFR2) - mediated anti-angiogenic effects of two steroid saponins, Theasaponin E1 (present in tea seed) and deltonin, have been reported separately [40,41]. Consistent with deltonin and Theasaponin E1, TS inhibited the cellular processes of angiogenesis in vitro, including HUVEC migration, invasion, and tube formation. Its underlying mechanism might also be related with suppression of VEGF/VEGFR2 signal pathway. It`s interesting that we found TS inhibited angiogenesis related processes at the same lower dose (3 µM) as that induced autophagy, suggesting that the anti-angiogenic effects of TS might be related with its ability to induce autophagy. The growth inhibition of HUVECs induced by persistent autophagy would further induce the inhibition of HUVEC migration, invasion, and tube formation. Taken together, our results suggest that TS may have multi-target action as an anti-angiogenic agent.

All together, the findings of this study indicated that TS and TA, derived from green tea, were effective in inhibiting HUVEC proliferation, migration, invasion and tube formation. The mechanism might be associated with the induction of autophagic process. In future work, the utilization of autophagy inhibitors would help us investigate the mechanism by which TS inhibits angiogenesis either through directly modulating angiogenic process, or activating autophagy which would then lead to inhibited angiogenesis. Our data about the anti-angiogenic effects of TS and TA, presented for the first time, would provide a new insight into the health potential for green tea.

Statement of Conflicts of Interest

The authors disclose no potential conflicts of interest.

Acknowledgement

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