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Original Article

Andrographis paniculata elicits anti-invasion activities by suppressing TM4SF3 gene expression and by anoikis-sensitization in esophageal cancer cells

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Abstract: Esophageal cancer is the sixth most common cancer in male causing death worldwide. It is usually diagnosed at advanced stage with high postoperative recurrence and systemic metastasis, which leads to poor prognosis. The potential inhibitory effect of herbal medicines on metastasis of esophageal cancer has drawn researchers' great attention. In the present study, the anti-invasion activities of *Andrographis paniculata* (AP) have been evaluated in two esophageal cancer cell lines, EC-109 and KYSE-520, as well as human microvascular endothelial cells (HMEC-1). The anti-tumor and anti-metastatic activities of AP were also evaluated in human esophageal xenograft-bearing mouse models. Our results demonstrated for the first time that aqueous extract of AP inhibited the motility and invasion of esophageal cancer cells, which is the initial step of metastasis, without cytotoxicity. Anoikis resistance has also been reversed in AP-treated cancer cells. Besides, the expression of metastasis-related gene *TM4SF3* in EC-109 cells was significantly decreased in AP extract-treated cells in a concentration-dependent manner. Furthermore, the anti-tumor and anti-metastatic efficacies in subcutaneous and intraperitoneal esophageal xenograft-bearing mice were demonstrated after oral administration of AP aqueous extract for 3 weeks. Last but not least, the active component, isoandrographolide, responsible for the anti-migratory activity was firstly revealed here. In conclusion, the AP aqueous extract exerted inhibitory activities on the migration and anoikis resistance of esophageal cancer cells EC-109 and KYSE-520, as well as suppressed the proliferation and motility of endothelial cells. Combining the mentioned effects may account for the anti-tumor and anti-metastasis effects of AP aqueous extract in xenograft-bearing mice. The findings in the present study further enhance the understanding of the therapeutic mechanisms of the herb AP, which may lead to clinical applications.

Keywords: Esophageal cancer, *Andrographis paniculata*, anoikis, metastasis, *TM4SF3*, isoandrographolide

Introduction

Esophageal cancer (EC) is a highly aggressive cancer and is the sixth most common cause of death from cancer worldwide [1]. The highest mortality rates occur in Eastern Asia and incidence rates worldwide in men are more than double than those in women [2]. Life style risk factors such as smoking, alcohol drinking and dietary exposures (e.g. red meat, hot foods/liquids, pickles) have been reported to account for the majority of EC cases in Chinese population [3] and in other countries [4]. Surgical resection remains the most important part of curative treatment for EC. However, the high postoperative recurrence rate (around 50%) leads to low long-term survival rate, e.g. a median survival of 3-10 months [5, 6]. A study reported that survival after recurrent EC in patients after esophagectomy has not improved over the past 18 years in Netherlands [7]. EC is often accompanied by extensive metastasis to lymph nodes in the cervical, thoracic, and abdominal regions as well as distal organs (lung

and liver) [8]. Prognosis of EC is poor as the disease is usually diagnosed at advanced stage with high frequency of metastasis.

Surgery followed by neoadjuvant chemotherapy and concurrent radiation has been shown to be beneficial in treatment of esophageal cancers [9, 10]. Combinations of cisplatin and 5-FU are commonly used in chemotherapy for patients with unresectable locally advanced or metastatic EC [10, 11]. New regimens with addition of capecitabine and paclitaxel have shown anti-tumor activities in phase II clinical trials [12, 13]. Nevertheless, chemotherapy related adverse effects including hematological toxicity especially neutropenia, fatigue, nausea, vomiting remain as major reasons for incomplete treatment among these patients [6]. Chemotherapy-related haematological toxicity results in a substantial economic burden on patients and society [14]. Besides, there are increasing use of complementary and alternative medicines by cancer patients and survivors [15]. Recent surveys revealed that the overall prevalence of complementary and alternative medicines use among cancer patients in US and European countries was ranged from 32 to 77% [16, 17]. In Chinese population, over 50% cancer patients have been using Traditional Chinese Medicines, including herbal medicines concurrently or after conventional therapies [18, 19]. Natural products derived from Chinese herbal medicines (CHM) are gaining increasing importance for the treatment of cancer.

The use of CHM for treating EC has been demonstrated of the efficacies in clinical studies. The tested CHM formulae were shown to reduce cancer development in esophageal dysplasia patients and to increase survival rate and quality of life. Systematic reviews on the CHM for EC have also shown that CHM treatment resulted in improvement of immune system, extension of survival, reduction of adverse reactions of chemo- and radiotherapy, and the holistic function of the patients [20]. Extracts or isolated compounds from CHM, such as *Andrographis paniculata* [21], *Coptidis rhizoma* [22], *Curcuma aromatic* [23], *Isodon xerophilus* [24] have been demonstrated to induce apoptosis in esophageal carcinoma cells *in vitro*. Among the commonly used Chinese herbal medicines prescribed for cancer patients in local clinics, *Andrographis paniculata* (AP) has drawn our attention due to its multi-function properties being reported. According to the Chinese Pharmacopeia, the actions of AP are to remove heat, counteract toxicity, and reduce swelling. AP is prescribed in influenza with fever, sore throat, acute or chronic cough, carbuncles ... etc. [25]. In modern pharmacological studies, AP was shown to have *in vitro* and *in vivo* immunomodulatory and anti-tumor activities [26]. The inhibitory effects of andrographolide, a major component of AP, on the proliferation of esophageal cancer cells [21], as well as the migration and invasion of lung [27] and colon cancer cells [28] have also been reported. On the other hand, the AP ethanolic extract has been shown to enhance cell-mediated immune response in metastatic melanoma tumor bearing mice [29]. Nonetheless, the anti-metastatic effect of the aqueous extract of AP on esophageal cancer cells was seldom reported.

In the present study, the effects of aqueous extract of AP (APW) on proliferation, migration, anoikis-resistance of esophageal cancer cells have been demonstrated. The anti-tumor and anti-metastatic activities of APW, which is the traditional preparation of herbal medicine, were confirmed in human esophageal xenograft-bearing mouse models.

Materials and methods

Aqueous extract preparation and chemical analysis

The dried herb of *Andrographis paniculata* (AP) with origin of Mainland China, was purchased from the herbal supplier of Hong Kong. Morphological and chemical authentications of the raw herb have been accomplished in accordance with the Chinese Pharmacopoeia 2010 [25]. The chemical profiles have been compared qualitatively using thin layer chromatography with the reference herb provided by National Institute for the Control of Pharmaceutical and Biological Products. The quantification of these two chemical markers in AP raw herb was achieved by UPLC. The contents of andrographolide and dehydroandrographolide were 0.621% and 0.097% (w/w), respectively. Authenticated voucher specimen (Number: 3435) was deposited in the museum of the Institute of Chinese Medicine, The Chinese University of Hong Kong. To prepare the aqueous extract, dried AP (1 kg) was cut into pieces (**Figure 1A**)

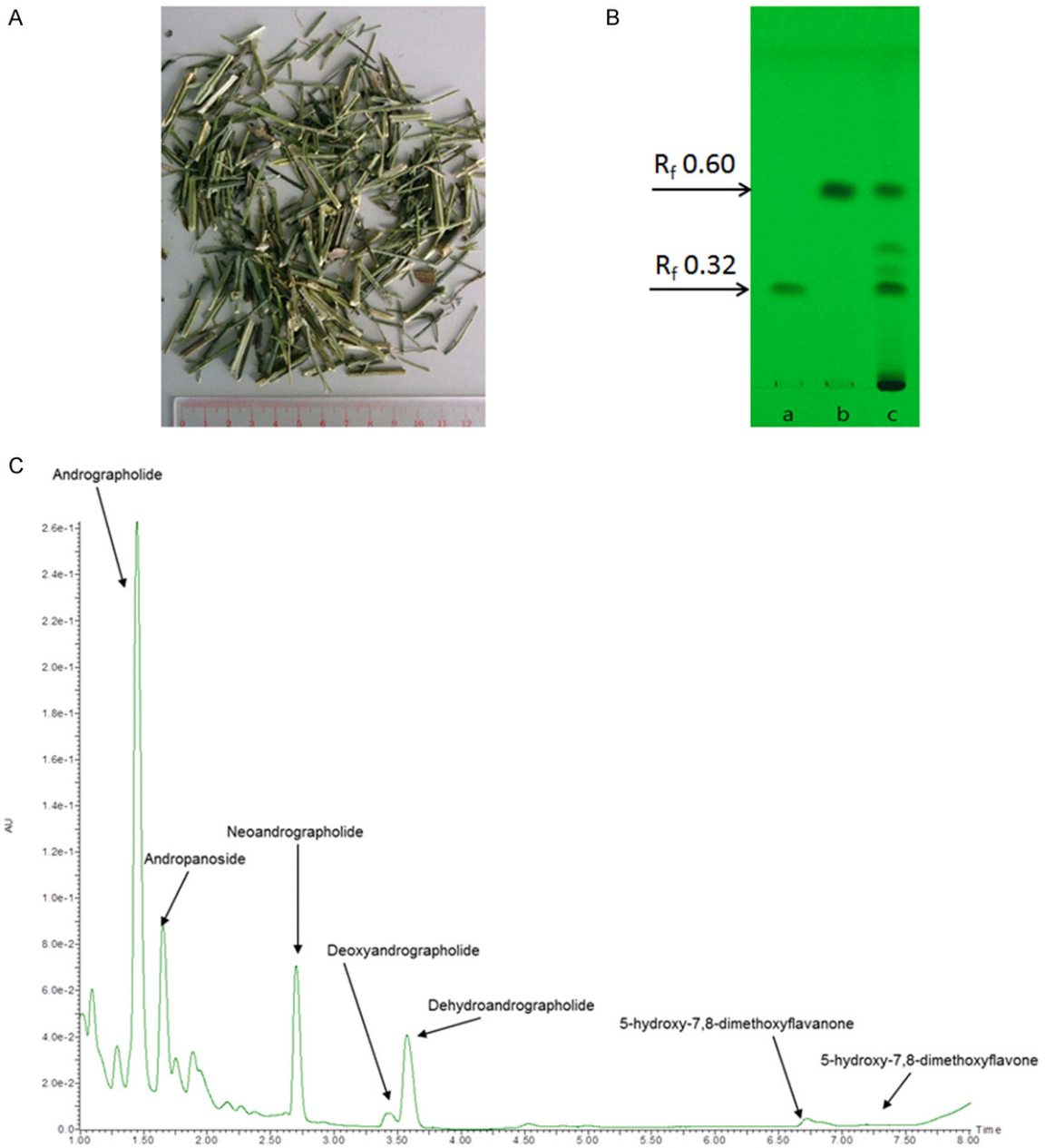


Figure 1. A. Dried herb of Andrographis paniculata. B. The TLC chromatogram of AP aqueous extract and chemical markers, using silica gel 60 F_{254} (20 × 10 cm, Merck, Germany). TLC plate was observed under UV 254 nm. The mobile phase was composed of chloroform:ethyl acetate:methanol = (4:3:0.4). Lane a: Andrographolide; Lane b: Dehydroandrographolide; Lane c: AP aqueous extract. C. Representative UPLC chromatogram of AP aqueous extract showing 7 identified chemical constituents.

and soaked in distilled water for 1 h, and then extracted with boiling water under reflux for another hour. The extract was filtered, and the extraction was repeated once. Subsequently, the filtrates were combined and evaporated under vacuum (Tokyo Riakikai Tokyo, Japan) and then lyophilized with a freeze drier (Dongduchun, Korea) into powder. The percentage yield of AP aqueous extract (APW) was 15.7% (w/w). The APW powder was stored in desiccators at room temperature and was dissolved in distilled water in animal studies. In cell studies, APW was dissolved in culture medium and filtered before use.

UPLC analysis of AP aqueous extract

The dried extract was dissolved in water and filtered with 0.2 μm filter. The sample was then analyzed with a Waters Acquity UPLC system (MA, USA). The column was Thermo Scientific Accucore C18, 3.0 mm × 150 mm packed with 2.6 μm hydrophobic bonded C18 phase, accompanied with a guard column of 3.0 mm × 10 mm, 2.6 μm (Thermo Scientific Accucore C18 Defender guard). The mobile phase consisted of acetonitrile (A) and distilled water (B). A gradient was used as 0-1 min, 29% A; 1-2 min, 35% A; 2-3.5 min, 35% A; 3.5-4 min, 41% A; 4-7 min, 47% A; 7-8 min, 95% A; 8-9 min, 95% A; 9-10 min, 29% A; 10-11 min, 29% A. The flow rate was 1.2 mL/min. The injection volume for all samples was 5 mL. Detection wavelength was set at 200 nm for neoandrographolide and deoxyandrographolide; 226 nm for andrographolide; 252 nm for andropanoside and dehydroandrographolide; 275 nm for 5-hydroxy-7,8-dimethoxyflavone; and 291 nm for 5-hydroxy-7,8-dimethoxyflavanone.

Chemicals and reagents for biological assays

Dulbecco's modified Eagle's medium/F12 (DMEM/F12), DMEM medium, RPMI medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-ethylenediaminetetraacetic acid (EDTA), TRIzol were obtained from Life Technologies (NY, USA). Gelatin, MCDB 131 medium, L-glutamine, hydrocortisone and epidermal growth factor, heparin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma (Saint Louis, MO, USA). [Methyl-^{3}H]-thymidine and unifilters were from PerkinElmer (Waltham, MA, USA). Primary antibodies for Western blot were purchased from Abcam (Cambridge, UK), while secondary horseradish peroxidase-conjugated antibodies were from Life Technologies. QuantiFast SYBR Green RT-PCR kit from Qiagen (Hilden, Germany).

Cell culture

Human esophageal squamous carcinoma cell line (EC-109) was purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Beijing, China) and KYSE-520 squamous carcinoma cell line was obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were maintained in RPMI1640 medium containing 10% (v/v) heat-inactivated FBS, 100 units/mL penicillin-streptomycin. Human microvascular endothelial cells (HMEC-1) were purchased from American Type Culture Collection (MD, USA) and were maintained in MCDB 131 medium containing 2 mM L-glutamine, 1 μg/mL hydrocortisone and 10 ng/mL epidermal growth factor.

The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. When the cells reached 80% confluence in culture flasks, trypsin-EDTA was used to remove the cells and the cells were used in experiments or reseeded in flask.

Cell proliferation and cytotoxicity assays

The esophageal cancer cells (5 × 10^4/mL) or HMEC-1 cells (3 × 10^4/mL) were seeded in 96-well flat-bottom culture plates with 100 μL culture medium and incubated overnight. Subsequently, 100 μL culture media containing various concentrations (100-1600 μg/mL) of AP aqueous extract (APW) were added into the wells. Then the plates were incubated at 37°C for 48 h. Plain medium containing vehicle solvent was added to the control wells. The effects of APW on the proliferation were determined by [methyl-^{3}H]-thymidine incorporation. The cytotoxicities of APW in cells were assessed by MTT assay. The procedures of thymidine incorporation and MTT assay were described in details in our previous study [30].

Cell migration assay

The cell motility of the endothelial cells and cancer cells was evaluated using scratch wound assay. Cells (1 × 10^5 in 1 mL medium) were added into each well of 24-well plate and incubated overnight. The cells were starved with 1% v/v FBS medium for 24 h. The cell monolayers were then scraped with a cross in the middle of well with 200 μL pipette tips. After scrapping the cells, the medium was changed to fresh medium with 50-1600 μg/mL of APW. The cells were incubated for 16 h (endothelial cells) or 24 h (cancer cells) and each well was photographed under microscope (Olympus IX-71, Japan). The percentages of open wound area were measured and calculated. Motility was determined by the decrease in closed wound area.

In the transwell migration assay, cancer cells (5 × 10^4 in 100 μL culture medium) were added into transwell chambers. At the same time, 100 μL medium containing various concentrations of APW (with 1% v/v FBS) was added to the upper chambers. Then, 500 μL of complete culture medium (with 10% v/v FBS), served as chemoattractant media, was added in the lower chambers. After incubation for 4-5 h at 37°C, cells were fixed with methanol and stained with haematoxylin. The non-migrated cells on the top surface of the filter membrane were scraped with cotton swab. Stained filters were photographed under microscope (Olympus IX-71). The migrated cells were quantified by manual counting in a blinded manner. Changes in cell numbers were represented as a percentage of control values (as 100%).

Anoikis resistance assay

Esophageal cancer cells grew or survived in suspension culture. EC109 cells and KYSE-520 cells were trypsinized and plated in ultra-low attachment cell culture plates covalently bounded with hydrogel (Corning Life Sciences, USA), in concentrations of 2.5 × 10^4 cells/well and 2.0 × 10^4 cells/well, respectively, in the culture media with or without the presence of different concentrations of APW or andrographolide (ADE). After 48 h, cells were grown as suspension spheroids and photos were taken by microscope (Olympus IX71, Japan) at 100 × magnification. Anoikis resistance was investigated by i) MTT assay for 3D culture: assessing the cell proliferation in suspension culture using a modified MTT assay in accordance with our previous studies [31, 32]. ii) Cell Death ELISA for suspension culture: Esophageal cancer cells in suspension spheroids were collected for apoptosis analysis after 48 h using the Cell Death Detection ELISA PLUS kit (Roche Applied Science, Basel, Switzerland), according to the manufacturer's instructions. Briefly, cancer cells/spheroids were collected by centrifuge at 200 ×*g* and lysed with 100 μL lysis buffer. After lysis, the intact nuclei were pelleted by centrifugation at 200 ×*g* for 10 min. Then, 20-μL aliquots of the supernatant were transferred to the streptavidin-coated wells with immunoreagent (biotinized anti-histone and peroxidase-conjugated anti-DNA) for 2 h at room temperature. Each well was then washed three times with incubation buffer and incubated with ABTS (2,2'-Azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) solution for 5 min. ABTS stop solution was then added to the wells immediately. Finally, absorbance was measured at 405 and 490 nm using a microplate spectrophotometer to determine the amount of colored product, using a mixture of ABTS and ABTS stop solution as a blank control.

Gelatin zymography

EC-109 cells (1 × 10^5/mL culture medium) were seeded in 24-well plates and incubated with various concentrations of APW (400, 600 or 800 μg/mL) at 37°C for 24 h. The supernatant was collected and stored at -80°C. Protein sample (20 μg) from the supernatant was fractionated in 10% SDS-polyacrylamide gel with 0.1% gelatin substrate. Following electrophoresis, the gels were washed three times in 2.5% Triton X-100 in PBS for 30 min at room temperature. The gels were then incubated overnight at room temperature in developing buffer (50 mM Tris base, 200 mM NaCl, 0.005 mM $ZnCl_2$, 5 mM $CaCl_2$□$2H_2O$, and 0.02% NaN_3, pH 7.5) and then stained with 0.125% (w/v) Coomassie brilliant blue for 20-30 min and destained in destain buffer (10% acetic acid and 5% ethanol in distilled water) for 1-2 days. Visualization of bands was performed on a Bio-RAD, XBS+ imaging system (Bio-Rad, Hercules, USA).

Real time-PCR analysis

EC-109 cells (1 × 10^6/mL) were seeded in 6-well culture dish and incubated for 24 h to allow attachment. Various concentrations (200-800 μg/mL) of APW were added to the wells and incubated for 24 h. After treatment, cells were harvested with TRIzol Reagent. Total RNA was extracted from cells using TRIzol Reagent according to the manufacturer's protocols. The RNA concentration was spectrophotometrically determined using a BioPhotometer (Eppendorf, NY, USA). To quantify the amount of mRNA of *TM4SF3*, RT-PCR were performed in Bio-Rad CFX96™ Real-time system C1000 Thermal cycler using the QuantiFast SYBR Green RT-PCR kit from Qiagen as described in previous studies [33]. Each 10 μL PCR reaction mix contained 100 ng RNA, 5 μL 2X QuantiFast SYBR Green RT-PCR Master Mix, 0.08 μL QuantiFast RT Mix, RNase-free water and 1 μL of both the specific primers (20 μM), forward primer

5'-ATAGATATCGACAAGCCTGTAACGAA-3' and reverseprimer5'-GATCTCGAGGTTCCCGATCTGG-3' [34], which were synthesized by Life Technologies (NY, USA). Reactions were performed in triplicate using the following protocol: pre-incubation at 50°C for 10 min, then 95°C for 5 min followed by 40 PCR cycles at 95°C for 10 s and 60°C for 30 s. A melt curve analysis was performed at the end of the reaction to assess the specificity of the amplification. Relative quantification was obtained by the comparative threshold cycle (ΔΔCt) method (CFX Manager Software, version 1.6, Bio-Rad, Hong Kong). The specific gene mRNA levels were normalized to that of the internal control *GAPDH* and then expressed as the fold change compared to the control group. All the experiments were performed in triplicates.

Esophageal tumor-bearing mouse models

Male nude mice (6-8 weeks of age) were provided by Laboratory Animal Services Center, The Chinese University of Hong Kong, and were housed under pathogen-free conditions. The experiments were approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong (Ref. No. 12-078-MIS).

EC-109 cells (5×10^6 cells per 200 μL PBS) were inoculated subcutaneously into the flank of nude mice on day 1. When the tumor reached the volume of 70 mm^3, the mice were randomly assigned into 3 groups (n = 8): control group (distilled water, oral gavage everyday), APW-L group (320 mg/kg, oral gavage everyday) and APW-H group (1600 mg/kg, oral gavage everyday). All treatments lasted for 3 weeks. Body weights were monitored and the size of tumors was measured with a caliper every 3-4 days and was calculated using the formula: (length × width)^2/2. On day 22, mice were anaesthesized, blood was collected by cardiac puncture and the plasma was stored at -80°C until plasma enzyme analysis. Tumors were excised from mice after cervical dislocation. The tumor weights were recorded. The changes on hemato-biochemical markers were assessed by measuring the activities of liver specific (AST, ALT), bone-related (AP) and heart-related (CK) enzymes in the plasma using commercially available kits (Stanbio Laboratory, TX, USA).

In order to evaluate the effect of APW on tumor metastasis, in another set of experiments, nude mice were inoculated intraperitoneally with EC-109 cells (5×10^6 cells in 200 μL PBS) on day 0 and the tumors were allowed to grow. On day 1, APW (320 or 1600 mg/kg) was administered orally daily for 3 weeks. On day 22, mice were sacrificed and the tumor nodules in peritoneal cavity were collected, counted and weighed.

Isolation of isoandrographolide from AP aqueous extract

The dried herb of AP (2.4 kg) was powdered and extracted twice under reflux with distilled water for 1 h. The combined solution was concentrated under reduced pressure at 60°C, and then partitioned twice with ethyl acetate to yield dark brown residue. The dark brown residue (38.8 g) was applied to a column of silica gel (4 cm × 55 cm), and eluted with a gradient mixture of chloroform/ethyl acetate/methanol (10:0:0, 8:2:0, 5:5:0, 3:7:0, 0:10:0, 0:49:1, 0:19:1, 0:9:1, 0:0:10 v/v/v) to yield five fractions (A to E). Bioassay-guided fractionation was accomplished by scratch wound migration assay in EC-109 cells and monitored by TLC (Silica Gel 60 F254, Merck, Germany), and spots were visualized by spraying the plates with 10% H_2SO_4-ethanol reagent followed by heating. Further separation of fraction C (6.88 g) was performed by silica gel column chromatography eluted with ethyl acetate/hexane (6:4 to 10:0 v/v) to obtain three subfractions (C-A to C-C). Pure isoandrographolide (80.8 mg, **Figure 6E** was obtained from subfraction C-B (580 mg) by silica gel column chromatography using chloroform/ethyl acetate (10:0 to 6:4 v/v) as eluents. The structure elucidation was based on the ^1H and ^{13}C NMR spectral analysis and mass spectrometry as described in previous studies [35, 36]. In brief, ^1H, ^{13}C, and 2D NMR spectra were measured in C_5D_5N solution and recorded on a Bruker AV-400 and DRX-500 spectrometer. Chemical shifts were reported in units of δ(ppm) and coupling constants (J) were expressed in Hz (Figures in Supplementary Materials). HRESIMS was recorded on Aligent 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS.

Isolated pure compound, isoandrographolide, was dissolved in dimethyl sulfoxide (DMSO) at 100 mM and stored at -20°C and reconstituted in appropriate media prior to the experiments.

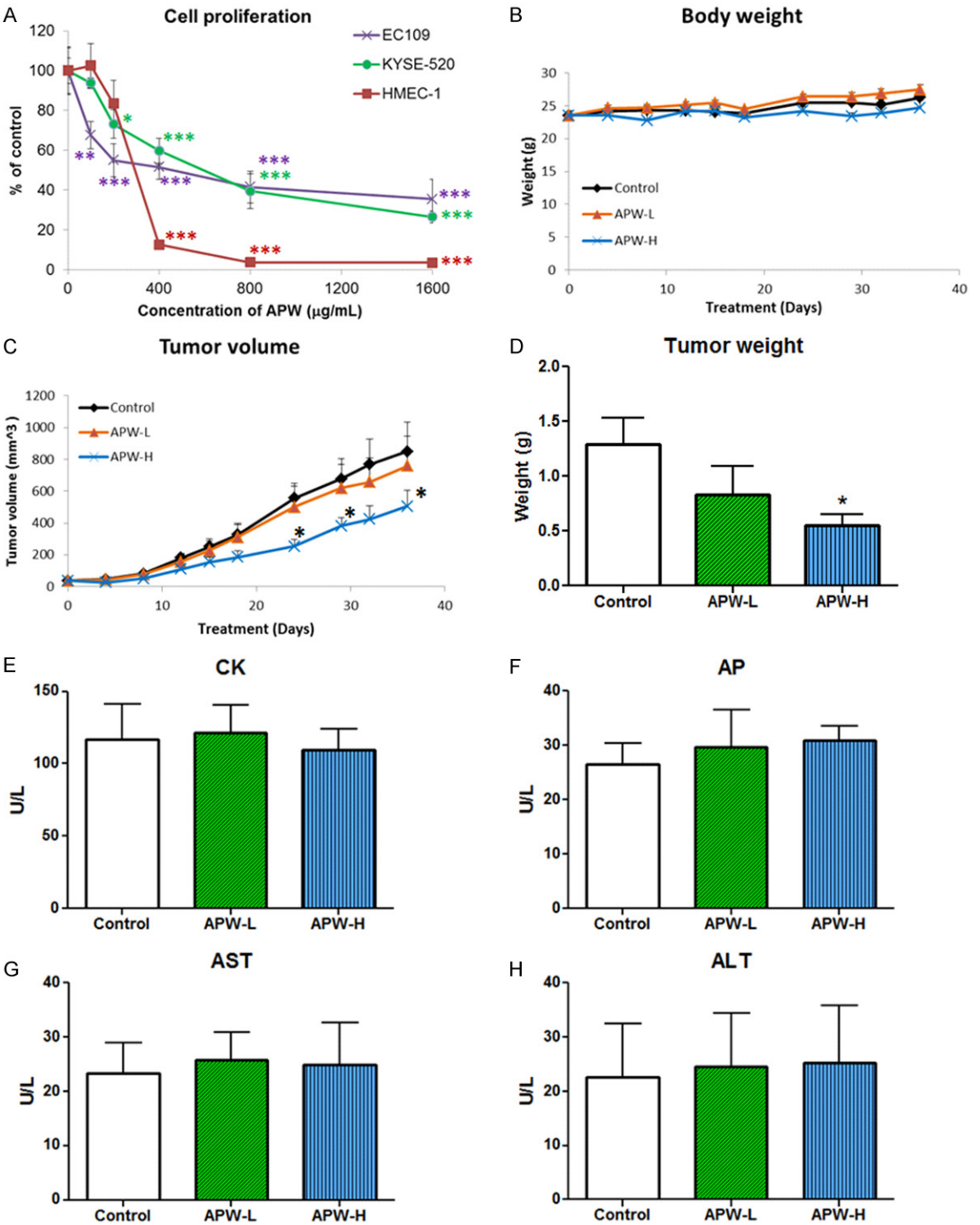


Figure 2. Effects of APW on cell proliferation of esophageal cancer cells and endothelial cells and tumor growth in esophageal xenograft-bearing mice. (A) Cells were treated with increasing concentrations of APW for 48 h, cell proliferation was determined by the [methyl-^{3}H] thymidine incorporation assay. Results are expressed as the mean % ratio of count per minute in treated and vehicle-treated control cells (mean + SD of 3 independent experiments with 5 wells each). Differences between the treated and untreated control groups were determined by one-way ANOVA followed by *post-hoc* Tukey's multiple comparison test. $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to the control group. (B-H) EC-109 cells were inoculated in the flank of nude mice. The xenograft-bearing mice were treated with APW-L (320 mg/kg) or APW-H (1600 mg/kg) for 3 weeks. (B) Body weight, (C) tumor volume and (D) final tumor weight were recorded. (E-H) The plasma enzymes (CK, AP, AST and ALT) activities in the plasma collected from APW-treated and untreated mice were measured. All results are mean + SEM of 7-8 mice in each group. Differences between the treated and untreated control groups were compared using one-way ANOVA followed by *post-hoc* Tukey's multiple comparison test. $^{*}P < 0.05$ as compared to the control group.

Table 1. IC_{50} values of APW in tested human esophageal cancer cells and endothelial cells

Assays	IC_{50} (mg/mL)		
	Human esophageal squamous carcinoma cells		Human microvascular endothelial cells
	EC-109	KYSE-520	HMEC-1
Thymidine incorporation	409.3	578.1	275.4
MTT	1479.1	1689.3	647.1

The vehicle control cultures received the vehicle solvent (0.5%, v/v, DMSO).

Statistical analysis

Data were expressed as mean + SD for *in vitro* studies or mean + SEM for *in vivo* studies. Statistical analyses and significance were analyzed by one-way ANOVA with Tukey's *post-hoc* test using GraphPad PRISM software version 5.0 (GraphPad Software, CA, USA). In all comparisons, $P < 0.05$ was considered statistically significant.

Results

Phytochemical analysis of Andrographis paniculata aqueous extract (APW)

The chemical markers of AP, andrographolide and dehydroandrographolide, were shown at R_f 0.32 and 0.60, respectively, in TLC (**Figure 1B**). The contents of andrographolide and dehydroandrographolide in APW were 1.0% and 0.21% (w/w), respectively, which were quantified by UPLC. Seven bioactive compounds previous reported [37], andrographolide, andropanoside, dehydroandrographolide, deoxyandrographolide, neoandrographolide, 5-hydroxy-7,8-dimethoxyflavone and 5-hydroxy-7,8-dimethoxyflavanone could be detected in the APW and their chemical profiles were shown in **Figure 1C**.

Effects of APW on proliferation of esophageal cancer cells and endothelial cells

Andrographis paniculata aqueous extract (APW) significantly inhibited the proliferation of esophageal cancer cells and endothelial cells (**Figure 2A**) which was assessed by thymidine incorporation assay after 48 h incubation. The IC_{50} values ranged from 275.4 to 578.1 μg/mL in the tested cell lines (**Table 1**). Meanwhile, APW at 125-1000 μg/mL did not show obvious cytotoxicity in esophageal cancer cells EC-109 & KYSE-520 and the IC_{50} values of APW in these cells by MTT assay were larger than 1400 μg/mL. For the human microvascular endothelial cell (HMEC-1), APW at relatively low concentration (i.e. 275.4 μg/mL) inhibited the proliferation, which may result from high cytotoxicity of APW in this cell type.

APW reduced the tumor size in human esophageal xenograft-bearing mice

As EC-109 cells were more sensitive to APW treatment than KYSE-520 cells *in vitro*, esophageal xenografts were established using EC-109 cells in nude mice to evaluate the *in vivo* effect of APW. The xenograft-bearing mice were treated with APW-L (320 mg/kg) or APW-H (1600 mg/kg), which were the 1X or 5X human equivalent doses recommended in Chinese Pharmacopeia. It was observed that no significant body weight change was found in mice in APW-treated groups (**Figure 2B**) during the treatment period. Tumor volumes and weights were significantly decreased in the group treated with APW-H (1600 mg/kg) by 40.6% and 56.9%, respectively, when compared with untreated control group (**Figure 2C** and **2D**). Nevertheless, there was no significant change on the activities of heart-related (CK), bone-related (AP) and liver specific (AST, ALT) plasma enzymes (**Figure 2E-H**) after 3-weeks of APW treatments.

Inhibitory effects of APW on migration of cancer cells and endothelial cells

Apart from cancer cell proliferation, cell migration and invasion are also essential components in cancer progression. APW were shown to inhibit the proliferation of esophageal cancer cells and endothelial cells without strong cytotoxic effect, as well as to reduce tumor size *in vivo*. The role of APW in migration of cancer cells and endothelial cells was further explored. In scratch wound assay, the non-cytotoxic dose ranges of APW in different cell types were applied to the cell monolayers and incubated for 16 or 24 h. As shown in **Figure 3A-D**, APW significantly decreased the closed wound area of EC-109, KYSE-520 and HMEC-1 cells, implying the inhibitory effects on cell motility induced by APW. In **Figure 3E** and **3F**, APW was shown to

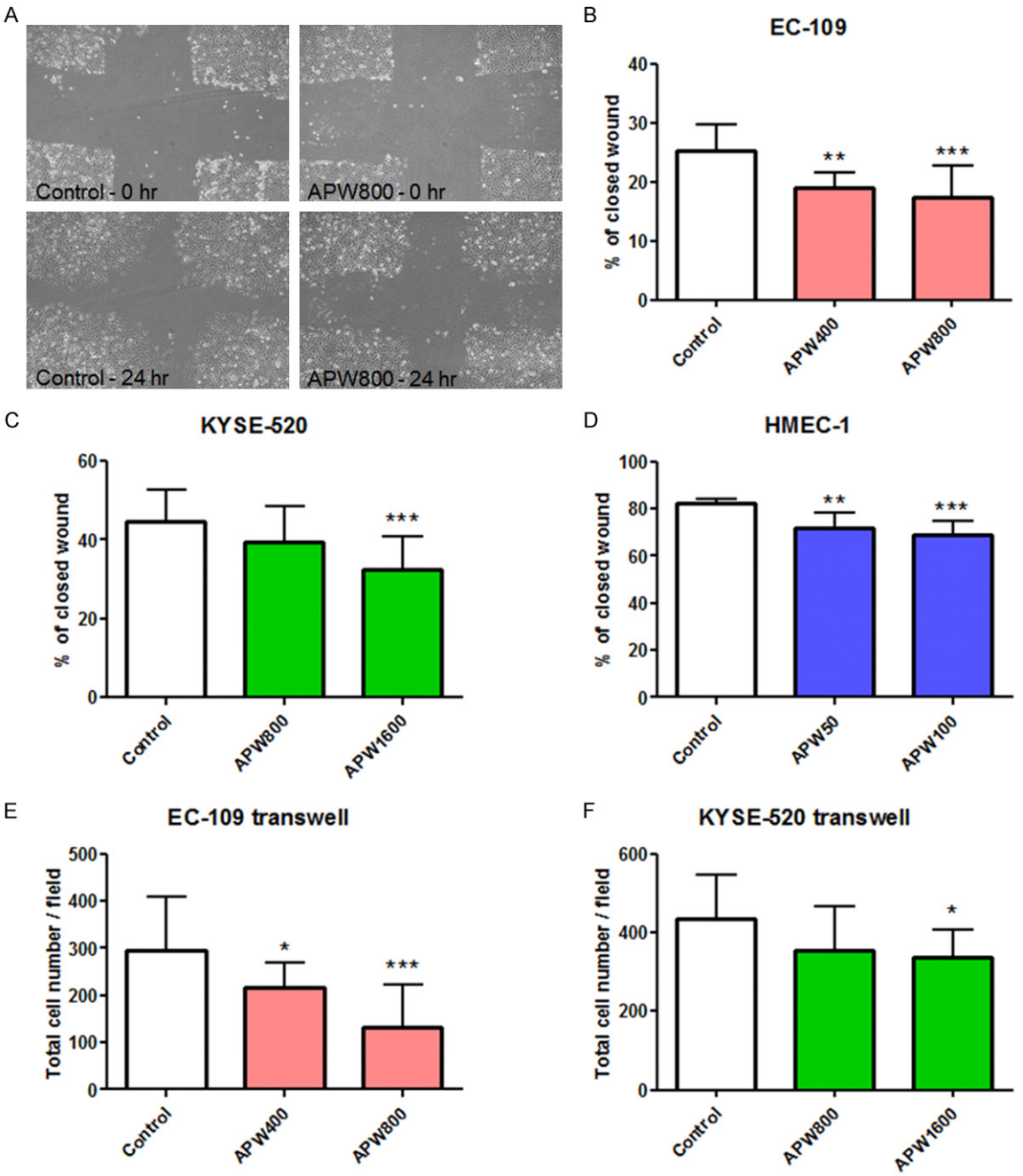
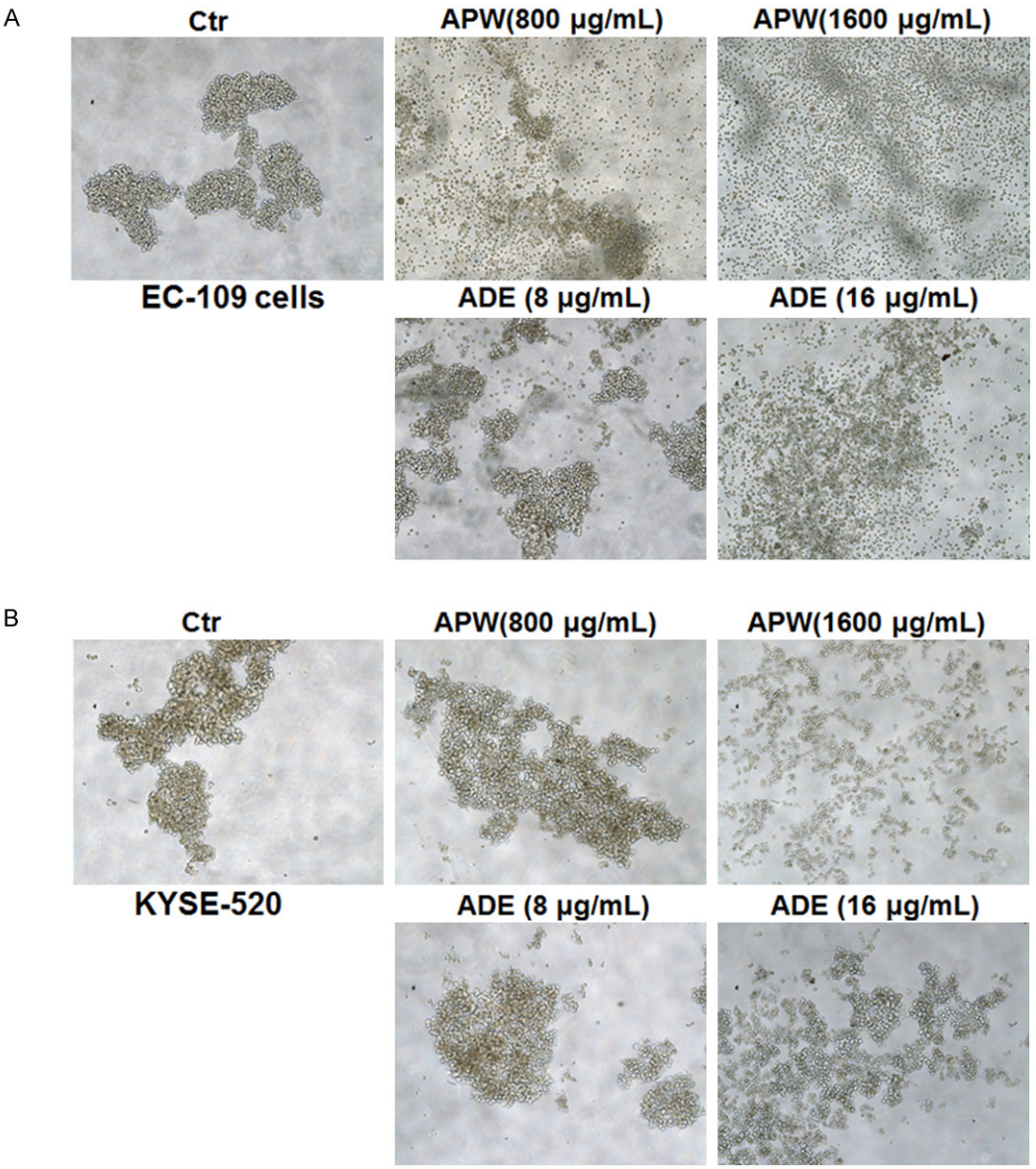


Figure 3. Effects of APW on migration of esophageal cancer cells and endothelial cells. (A) Representative photomicrographs showing the EC-109 cells migrated across the scratch wound in the presence or absence of APW (800 μg/mL) after 24 h of incubation. Quantifications of wound-induced cell motility in (B), EC-109, (C) KYSE-520 and (D) HMEC-1 cells were shown. The concentrations of APW in each type of cell were added as indicated on the X-axis. Quantifications of cell migration of (E) EC-109, (F) KYSE-520 in modified Boyden chambers were shown. Results are expressed as the mean percentage of control (mean + SD of 3 independent experiments). Differences among treatment groups were determined by one-way ANOVA followed by *post-hoc* Tukey's multiple comparison test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ as compared to the control group.

inhibit the cell invasion in esophageal cancer cells with the increased concentrations. In the presence of 800 and 1600 μg/mL APW, cell invasion of EC-109 and KYSE-520 cells significantly reduced by 55.2% and 22.1%, respectively.


A
Ctr
APW(800 μg/mL)
APW(1600 μg/mL)
EC-109 cells
ADE (8 μg/mL)
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B
Ctr
APW(800 μg/mL)
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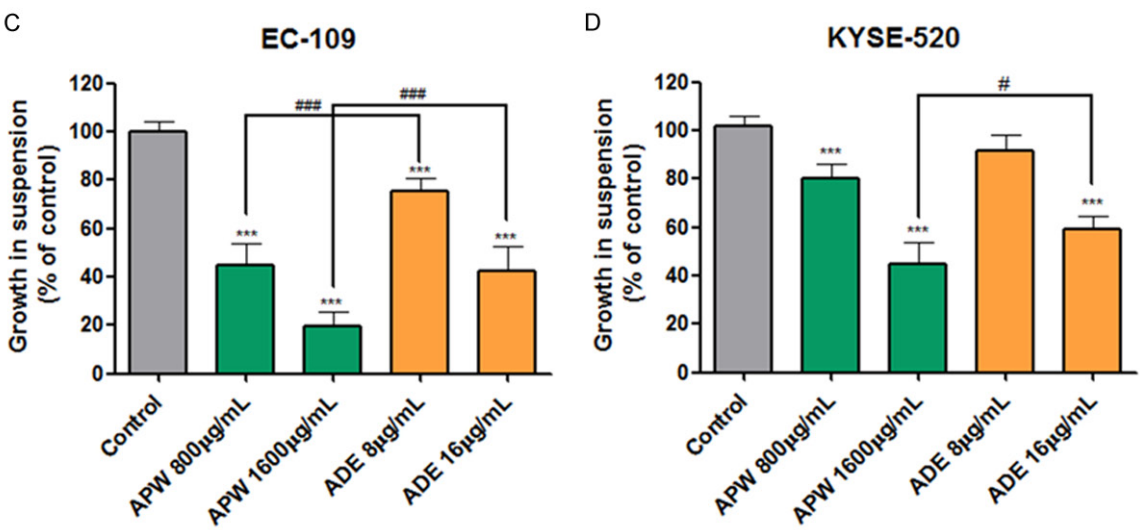
KYSE-520
ADE (8 μg/mL)
ADE (16 μg/mL)


C
EC-109
Growth in suspension (% of control)
120
100
80
60
40
20
0
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Control
APW 800μg/mL
APW 1600μg/mL
ADE 8μg/mL
ADE 16μg/mL
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KYSE-520
Growth in suspension (% of control)
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Control
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ADE 8μg/mL
ADE 16μg/mL

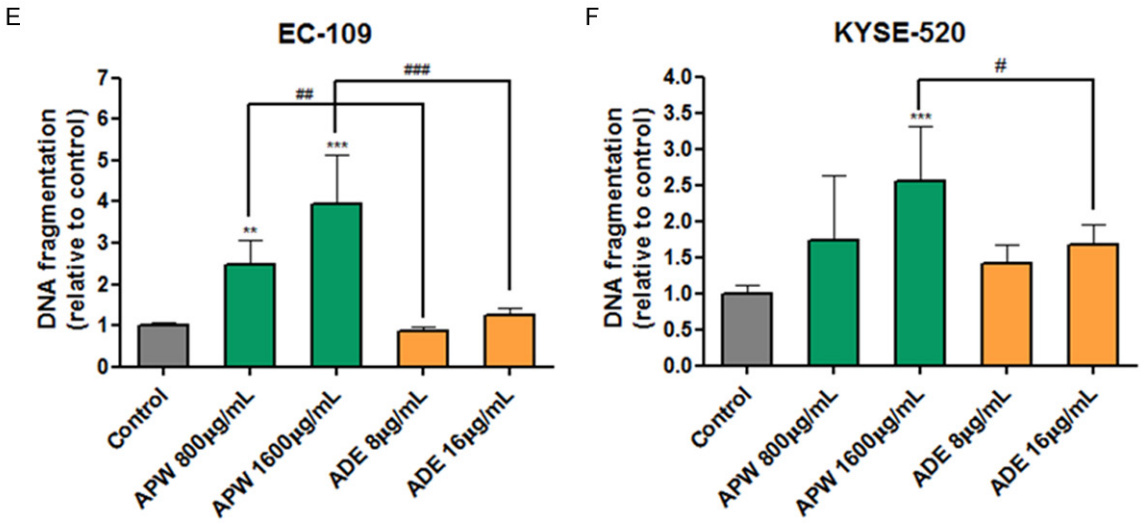

Figure 4. APW and andrographolide (ADE) were able to sensitize anoikis (detachment-induced cell death) in EC-109 or KYSE-520 cells. EC-109 cells and KYSE-520 cells were resistant to detachment-induced cell death (i.e. anoikis-resistant) when grown in suspension (or detached state in ultra-low attachment cell culture plates; Corning Life Sciences, USA). The photos shown are representative photos after APW or ADE treatment for 48 h. APW or ADE reduced the ability of (A), EC-109 cells or (B), KYSE-520 cells seeded at a density of 2.5×10^4 cells/well (EC-109) or 2.0×10^4 cells/well (KYSE-520) in the presence of 10% FBS for 48 h in ultra-low attachment cell culture plates. After 48 h, the cells grew into large multicellular spheroids. (C, D) MTT assay is used to measure survival and growth in suspension culture. The percentage growth in suspension was calculated as OD drug-treated/OD vehicle-treated × 100%. (Means + SD of three independent experiments with two wells each). (E, F) APW or ADE increased the apoptosis activity of EC-109 and KYSE-520 cells/spheroids in detached state as quantified of histone-complexed DNA fragments by the Cell Death ELISA assay. Data were expressed as means + SD from three independent experiments with two wells each. Differences among all groups were determined by a one-way ANOVA followed by *post-hoc* Tukey's multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to vehicle control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared 1600 μg/mL APW treatment with 16 μg/mL ADE treatment (or comparing 800 μg/mL APW with 8 μg/mL ADE).

APW reversed the anoikis resistance of esophageal cancer cells

To further investigate the potential effect of APW on the metastasis of esophageal cancer cells, anoikis, which is defined as detachment-induced apoptosis or cell death [31], was assessed in APW-treated esophageal cancer cells. Both esophageal cancer cell lines EC-109 and KYSE-520 showed the ability to proliferate (from very low density) in suspension to large multicellular 3D spheroids in detached state (**Figure 4A** and **4B**). From the results of the MTT assay for 3D culture (**Figure 4C** and **4D**), the proliferation of EC-109 and KYSE-520 in suspension was reduced by APW or andrographolide (ADE) treatments in a dose-dependent manner. When exposed to 800 and 1600 μg/mL APW for 48 h, the proliferation of EC-109 cells was significantly reduced to 44.9% and 19.3% of control, respectively. Meanwhile, ADE treatments exerted relatively weaker inhibitory effects as ADE treatments reduced the cell proliferation to 75.3% (8 μg/mL ADE) and 42.3% (16 μg/mL ADE) of control. The similar inhibitory effects were also observed in KYSE-520 cells. After treatment with 800 and 1600 μg/ml of APW, the proliferation of KYSE-520 cells was significantly reduced to 80.2% and 45.0% of control respectively. On the other hand, exposure to ADE significantly reduced the proliferation to 91.8% (8 μg/mL ADE) and 59.2% (16 μg/mL ADE) of control, respectively. To further investigate whether the reduced proliferation in detached state was associated with apoptosis, quantification of DNA fragmentation of apoptotic cells in suspension culture was analyzed by the Cell Death Detection ELISA. As shown in **Figure 4E** and **4F**, our data revealed a concentration-dependent induction of cellular apoptosis by APW or ADE. Notably, APW induced greater extent of DNA fragmentation than ADE did after 48 h of exposure in both cell lines. The differences between APW and ADE treatments were statistically significant ($P < 0.001$).

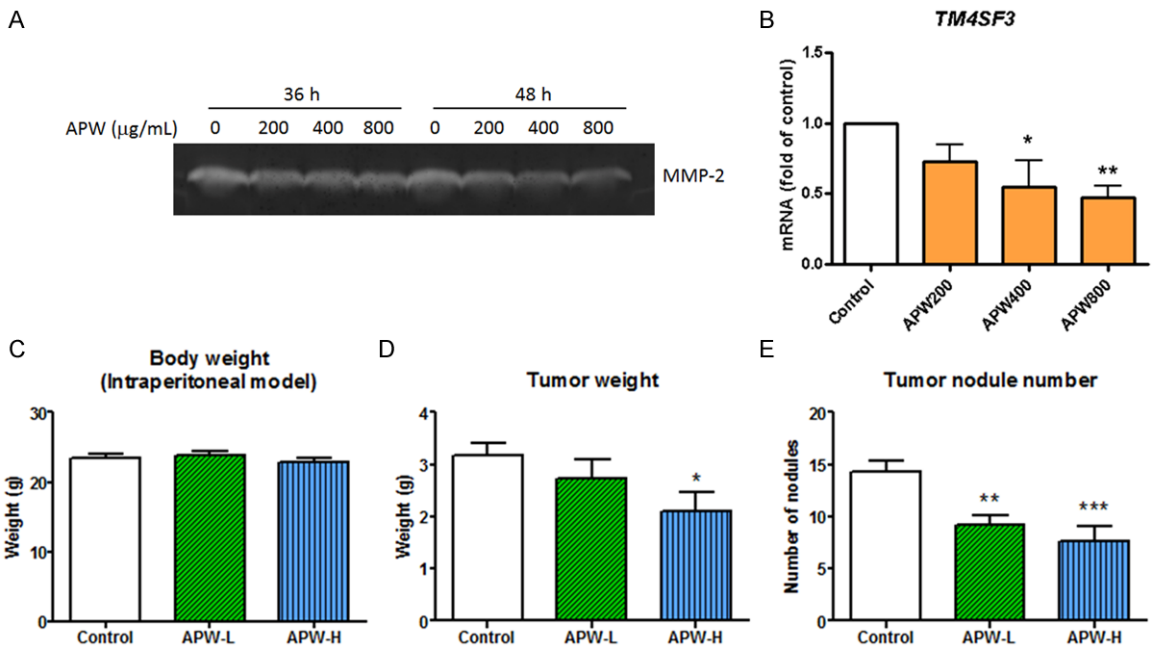

Figure 5. Effects of APW on the activity of MMP2 and expression of *TM4SF3* gene in EC-109 cells and on the tumor growth in intraperitoneal xenograft-bearing mice. (A) Cells were treated with APW (200-800 μg/mL) for 36 or 48 h and the MMP-2 activities were determined by zymography and representative zymograms were shown. (B) Cells were treated with APW (200-800 μg/mL) for 24 h and then the cells were collected for RNA extraction. Quantitative RT-PCR analyses of *TM4SF3* gene mRNA were shown. Data were normalized to corresponding *GAPDH* expressions in control cells. mRNA expressions results are expressed as fold of control (mean fold of control + SD from 3 independent experiments). EC-109 cells were inoculated in the peritoneal cavity of nude mice. The xenograft-bearing mice were treated with APW-L (320 mg/kg) or APW-H (1600 mg/kg) for 3 weeks. (C) Body weight, (D) tumor weight and (E) tumor nodule number were recorded. All results are mean + SEM of 7 mice in each group. Differences among treatment groups were determined by one-way ANOVA followed by *post-hoc* Tukey's multiple comparison test. $*P < 0.05$, $***P < 0.001$ as compared to the control group.

Effects of APW on the activity of matrix metalloproteinase-2 and the expression of metastasis-related gene in EC-109 cells

As the inhibitory activities of APW on the migration of EC-109 cells were stronger than that of KYSE-520 cells, further investigation on the effects of APW in EC-109 cells was performed *in vitro* and *in vivo*. Gelatin zymography was carried out to evaluate the effect of APW on the activity of matrix metalloproteinase (MMP)-2, which played an important role in extracellular matrix degradation [38]. MMP-2 in EC-109 cell culture supernatant was detected in the gel at molecular weight of 72 kDa. The enzyme activity of MMP-2 was suppressed by APW in a concentration- and time-dependent manner (**Figure 5A**). Furthermore, the effect of AP extract on the expression of metastasis-related gene *TM4SF3* in EC-109 cells was also examined using real-time PCR. Results showed that the mRNA expression of *TM4SF3* was significantly decreased in a concentration-dependent manner in AP extract-treated cells (**Figure 5B**).

APW inhibited tumor growth and metastasis in intraperitoneal esophageal xenograft-bearing mice

To investigate the activity of APW on esophageal tumor growth and metastasis, intraperitoneal EC-109 xenograft-bearing mouse model [39] was employed. It was observed that no significant body weight loss was found in mice in APW-treated groups (**Figure 5C**) during the treatment period. However, intraperitoneal tumor weights and tumor nodule numbers were significantly decreased in APW-H-treated group by 34.1% and 46.8% when compared with untreated control group, respectively (**Figure 5D** and **5E**). The results suggested that the APW was effective in decreasing tumor growth and spreading in peritoneal cavity, implying the anti-metastatic activities of APW in xenograft-bearing mice.

Isoandrographolide in APW inhibited cell motility of esophageal cancer cells

Based on the aforementioned *in vitro* and *in vivo* results, we carried out bioassay-guided fractionation of the APW in order to search for the active component(s) responsible for the inhibitory effects on esophageal cancer cell migration and metastasis. A series of column chromatography fractionation was accomplished by scratch wound migration assay in EC-109 cells. A subfraction was shown to be effective in decreasing the closed wound area in EC-109 cells. The structure of the major compound isolated from subfraction C-B was identified as isoandrographolide (**Figure 6E**), using ^{1}H, ^{13}C, HRESIMS and 2D-NMR methods.

Isoandrographolide: $C_{20}H_{30}O_5$. HRESIMS m/z 351.2172 $[M+H]^+$ (calcd. for $C_{20}H_{31}O_5$ $[M+H]^+$, 351.2166). ^{1}H NMR (C_5D_5N, 500 MHz): 0.71 (3H, s, 20-CH3), 1.17 (2H, m, 5-CH and 1-CH), 1.33 (1H, m, 6-CH), 1.49 (3H, s, 18-CH3), 1.77 (3H, m, 1-CH, 6-CH and 9-CH), 1.87 (1H, s, 7-CH), 1.95 (2H, m, 2-CH_2), 2.31 (1H, m, 7-CH), 3.06 (2H, m, 11-CH), 3.61 (2H, m, 3-CH, 19-CH), 4.39 (1H, dd, J = 9.5, 3.0 Hz, 15-CH), 4.44 (1H, d, J = 11.0 Hz, 19-CH), 4.52 (1H, dd, J = 9.5, 6.0 Hz, 15-CH), 4.71 (1H, br s, 17-CH), 4.88 (1H, br s, 17-CH), 5.05 (1H, br, 14-CH), 6.71 (1H, t, J = 7 Hz, 12-CH). ^{13}C NMR (C_5D_5N, 100 MHz): 169.98 (C-16), 148.41 (C-12), 148.22 (C-8), 129.87 (C-13), 108.20 (C-17), 79.93 (C-3), 74.36 (C-15), 69.17 (C-14), 64.22 (C-19), 56.74 (C-9), 55.37 (C-5), 43.27 (C-4), 39.38 (C-10), 38.33 (C-7), 37.27 (C-1), 29.03 (C-2), 24.48 (C-6), 23.79 (C-11), 23.69 (C-18), 15.34 (C-20).

The ^{1}H and ^{13}C NMR spectroscopic data (listed in Supporting Information) were compared and found to be consistent with previously reported values for isoandrographolide [35, 36]. The percentage yield of isoandrographolide from APW was 0.021% (w/w).

The inhibitory activities of isoandrographolide on motility of EC-109 and HMEC-1 cells were shown in **Figure 6F** and **6G**, in which the percentages of closed wound area were significantly decreased after isoandrograpolide (5 μg/mL) treatment. Besides, when the motility of EC-109 cells was induced by epidermal growth factor (EGF at 50 ng/mL), isoandrographolide could significantly decrease the closed wound area. The results suggested the novel anti-migratory activities of this compound.

Discussion

Esophageal cancer is usually aggressive and invasive in nature. Despite the recent advances in surgery, radiotherapy, chemotherapy and targeted-therapy, limited improvement in treating metastatic esophageal has been achieved. Although new triplet chemotherapy have shown favourable efficacies in advanced esophagogastric cancer patients, the survival benefit is limited and the risks of serious adverse effects, such as grade 3-4 thrombocytopenia, infection, and mucositis, are increased [6]. Hence, searching for alternative treatments for metastatic esophageal cancer is still urgently needed. Natural products, including herbal medicines, could be a good source of anti-metastatic agents.

Andrographis paniculata (AP), is a herb widely used in both Chinese and Indian traditional medicines. The major constituent, andrographolide, has been shown to induce apoptosis in esophageal cancer cells [21] and exhibit anti-invasive activity in colon cancer cells [28] and breast cancer cells [40]. Nonetheless, the anti-tumor and anti-metastasis abilities of the aqueous extract of AP (APW), the common preparation for traditional Chinese medicine users, have not been explored. Our results showed that APW significantly inhibited the proliferation of human esophageal cancer cells and microvascular endothelial cells without cytotoxicity. In addition, APW (< 1600 μg/mL) did not cause cytotoxicity in human primary normal esophageal epithelial cells (data not shown). On the other hand, the anti-tumor activity of APW was firstly demonstrated here in EC-109 esophageal subcutaneous xenograft-bearing mice. Even though the effective anti-tumor dose of APW was 5-times of human equivalent dose, there was no significant change of body weight and heart- or liver-specific plasma enzyme activities, implying the non-toxic properties of APW at this dose. There were only a few studies reported the *in vivo* activities of AP extract. Sheeja and Kuttan showed that AP ethanolic extract inhibited the tumor growth in thymoma cells-carrying mice [26] and augmented cell-mediated immune response in metastatic melanoma tumor-bearing mice [29]. The chemo-

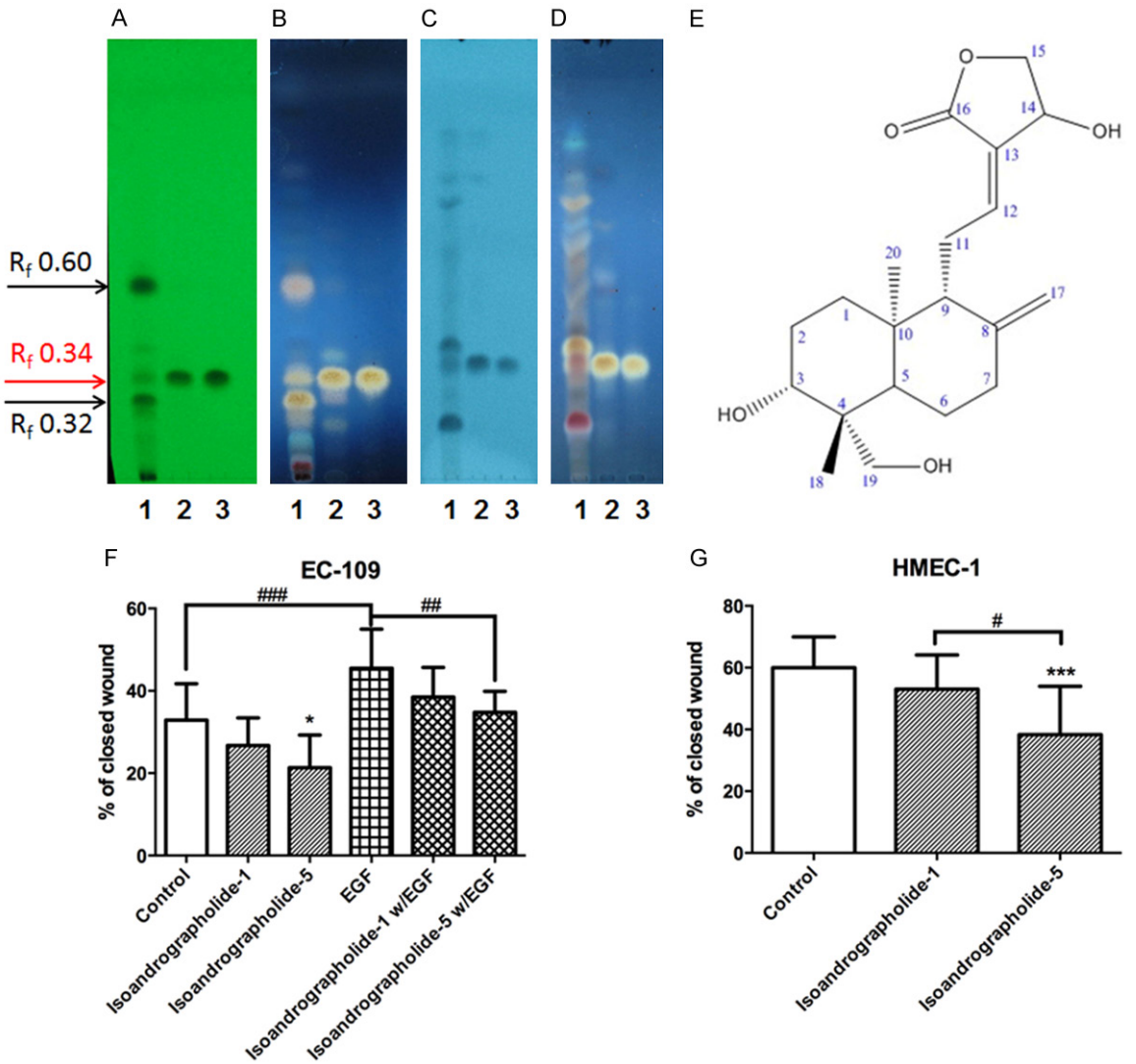

Figure 6. Isolation of isoandrographolide and its effects on motility of EC-109 and HMEC-1 cells. (A-D) The TLC chromatograms of AP aqueous extract subfractions and isoandrographolide, using (A and B) Silica gel 60 F_{254} or (C and D) RP-18 (20 × 10 cm, Merck, Germany). TLC plates were observed under (A and C) UV 254 nm or (B and D) UV 365 nm after spraying with 10% H_2SO_4-ethanol reagent followed by heating. The mobile phase was composed of (A and B) chloroform:ethyl acetate:methanol = (4:3:0.4); (C and D) Methanol:water = (3:2). Lane 1: Subfraction C; Lane 2: Subfraction C-B; Lane 3: Isoandrographolide. (E) Chemical structure of isoandrographolide. Quantifications of wound-induced cell motility in (F), EC-109 and (G), HMEC-1 cells were shown. Cells were incubated with isoandrographolide (1 or 5 μg/mL) for 16 h or 24 h and the area of the scratch wound was assessed. In another set of experiment, epidermal growth factor (EGF, 50 ng/mL) was added in the EC-109 cells with isoandrographolide (1 or 5 μg/mL). Results are expressed as the mean percentage of control (mean + SD of 3 independent experiments). Differences among treatment groups were determined by one-way ANOVA followed by *post-hoc* Tukey's multiple comparison test. $^{*}P < 0.05$, $^{***}P < 0.001$ as compared to the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared among treatment groups.

protective effects of AP ethanolic extract on colorectal cancer were shown in the azoxymethane (AOM)-induced aberrant crypt foci (ACF) rat model [41]. However, none of the studies has evaluated the activity of AP aqueous extract in esophageal cancer animal model.

In view of the anti-tumor effects of APW shown in the xenograft-bearing mice, we further hypothesized that APW may exert inhibitory effect on metastasis in esophageal cancer cells and in metastatic mouse models. Hence, the motility and invasion of esophageal cancer cells

after APW treatment were evaluated by scratch wound and transwell invasion assays. Our results showed that the non-cytotoxic concentrations of APW significantly inhibited the motility and invasion of esophageal cancer EC-109 and KYSE-520 cells. To further investigate the anti-metastatic effect of APW and ADE, the anoikis-sensitizing activities of APW and ADE in esophageal cancer cells were evaluated. Anoikis is the process of losing adhesion that would induce apoptosis in cancer cells. However, the ability to resist anoikis may allow for the survival of cancer cells to travel through the lymphatic and circulatory systems, and eventually facilitating secondary tumor formation in distant organs [42]. The agents that reduce anoikis resistance can inhibit metastasis of cancer cells [43]. To our knowledge, the present results demonstrated for the first time that APW and ADE could reverse the anoikis resistance of esophageal cancer cells. Besides, the inhibitory activities of APW and ADE were compared. The content of ADE in APW was around 1% and thus when comparing the activities of APW at 1600 μg/mL with ADE at 16 μg/mL (or APW at 800 μg/mL with ADE at 8 μg/mL), APW showed stronger inhibitory effects than ADE, implying that other components in APW may involve in the anoikis sensitization in esophageal cancer cells.

Furthermore, our data suggested that the activity of matrix metalloproteinase 2 (MMP-2) was suppressed by APW in esophageal cancer EC-109 cells. Matrix metalloproteinases are well known to play important roles in the process of tumor invasion and metastasis of different types of cancer, including esophageal cancer [44, 45]. Previous studies showed that andrographolide inhibited MMP2 activity in colon cancer cells [28]. The inhibitory effect of APW on MMPs activities and expressions in esophageal cancer cells will be further studied. Our data also demonstrated that APW could inhibit human endothelial cell proliferation and wound-induced motility, suggesting its potential anti-angiogenic properties. New vessels formation (angiogenesis) involves multistep process such as cell proliferation, migration and remodeling. Suppression at any step may result in new blood vessel formation inhibition. The concept of anti-angiogenesis is now an important component in cancer treatment and it is believed that blocking angiogenesis could be a strategy to arrest tumor growth and metastasis [30].

In addition, the expression of gene *TM4SF3* in EC-109 cells was assessed after APW treatment and our results showed that APW significantly decreased the expression of *TM4SF3* in a concentration-dependent manner. *TM4SF3* a member of tetraspanin family, has been reported as a metastasis associated gene in many types of tumors [46]. Zhou *et al*. suggested that the overexpression of *TM4SF3* in esophageal cancer conferred advantage to the invasion and metastasis in this disease [34]. Our findings extend the understanding of mechanisms for the abilities of APW to inhibit esophageal cancer metastasis.

Based on the above-mentioned activities of APW, i.e. anti-migration, anti-angiogenesis, anoikis sensitization, inhibition on MMP-2 activity and metastatic gene expression, the anti-metastatic efficacies of APW were further confirmed in intraperitoneal esophageal xenograft-bearing mouse model. The reduced tumor weights and tumor nodule numbers after APW treatment provided the evidences of multi-function of APW, a mixture of active components including andrographolide. Although plentiful studies demonstrated that andrographolide was responsible for the anti-cancer activities on hepatoma and nasopharyngeal carcinoma *in vitro* and *in vivo* [47, 48], and it inhibited the breast cancer with bone metastasis [40], the role of this compound on the metastasis of esophageal cancer has not been fully understood. Moreover, previous studies reported that the oral bioavailability of andrographolide is poor and modifications of formulations (such as nanoparticle, microspheres, micelles...etc.) are in progress [49, 50]. Our present data revealed that the oral administration of aqueous extract of AP, which contains 1% andrographolide only, significantly exert anti-metastatic activities in esophageal xenograft-bearing mice. It is possible that components other than andrographolide may possess anti-metastatic effects. Alternatively, the other components in APW may facilitate the absorption of andrographolide.

Another active component in APW that is responsible for the anti-metastatic effects has been identified for the first time here using bioassay-guided fractionation. Isoandrographolide was found to potently inhibit the motility of EC-109 cells as its effective concentration was around 5 μg/mL, while andrographolide

at 50 μg/mL exerted similar inhibition (data not shown). Besides, isoandrographolide was reported to effectively reduce the epidermal growth factor-induced motility [51]. The underlying mechanism of this compound on the epithelial-mesenchymal transition will be further investigated.

Overall, our novel findings suggested that the aqueous extract of *Andrographis paniculata* (APW) possesses anti-tumor and anti-metastatic effects in esophageal xenograft-bearing mouse models. As the multi-function biological activities and the chemical composition of the mixture of active components in APW have been characterized, the adjuvant effects of APW with chemotherapeutics could be further elucidated in metastatic esophageal cancer, both in pre-clinical and clinical setting. As mentioned above, chemotherapy for metastatic cancer is effective with many side effects, herbal extract such as APW may play multi-targeted role in the combined treatment [52], so as to provide the beneficial outcome for the patients.

In conclusion, the AP aqueous extract exerted inhibitory activities on the migration of esophageal cancer cells and endothelial cells. It also inhibited the growth of esophageal subcutaneous xenografts as well as the metastasis of intraperitoneal xenografts in mice models, which were reported for the first time. Our findings suggested the potential anti-metastatic effects of AP aqueous extract, which may further enhance the understanding of the therapeutic mechanisms of this herb in metastatic esophageal cancer.

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Disclosure of conflict of interest

None.

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