

RESEARCH ARTICLE

Anti-Angiogenic Activity of Gecko Aqueous Extracts and its Macromolecular Components in CAM and HUVE-12 Cells

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Abstract

Gecko is a kind of traditional Chinese medicine with remarkable antineoplastic activity. However, undefined mechanisms and ambiguity regarding active ingredients limit new drug development from gecko. This study was conducted to assess anti-angiogenic properties of the aqueous extracts of fresh gecko (AG) or macromolecular components separated from AG (M-AG). An enzyme-linked immunosorbent assay (ELISA) approach was applied to detect the vascular endothelial growth factor (VEGF) secretion of the tumor cells treated with AG or M-AG. The effect of AG or M-AG on vascular endothelial cell proliferation and migratory ability was analyzed by tetrazolium dye colorimetric method, transwell and wound-healing assays. Chick embryo chorioallantoic membrane (CAM) assays were used to ensure the anti-angiogenic activity of M-AG *in vivo*. The results showed that AG or M-AG inhibited the VEGF secretion of tumor cells, the relative inhibition rates of AG and M-AG being 27.2% and 53.2% respectively at a concentration of 20 $\mu\text{L}/\text{mL}$. AG and M-AG inhibited the vascular endothelial (VE) cell proliferation with IC50 values of $11.5 \pm 0.5 \mu\text{L}/\text{mL}$ and $12.9 \pm 0.4 \mu\text{L}/\text{mL}$ respectively. The VE cell migration potential was inhibited significantly ($p < 0.01$) by the AG ($\geq 24 \mu\text{L}/\text{mL}$) or M-AG ($\geq 12 \mu\text{L}/\text{mL}$) treatment. *In vivo*, neovascularization of CAM treated with M-AG was inhibited significantly ($p < 0.05$) at a concentration of 0.4 $\mu\text{L}/\text{mL}$. This study provided evidence that anti-angiogenesis is one of the anti-tumor mechanisms of AG and M-AG, with the latter as a promising active component.

Keywords: Gecko - anti-angiogenesis - aqueous extracts - macromolecular component - vascular endothelial growth

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Introduction

Cancer is still a malignant disease which is difficult to cure and with higher mortality rate. Although radiotherapy, surgery and chemotherapy, as regular therapies in clinic, could relieve the symptoms of patients to some extent, the toxic side effects of these treatments shouldn't be ignored (Sinclair, 1998). Traditional Chinese medicine (TCM), a treasure trove evolved over thousands of years, was more widely used to treat malignant tumors in Chinese clinic due to its multi-target therapy and multilevel regulation (Sun, 2014; Liu et al., 2015).

Gecko, also known as shou gong, the most species-rich group of lizards, has been used in TCM for hundreds of years. As "Compendium of Materia Medica" recorded, gecko could cure "Blood plot into a ruffian, Pandora wind scrofula". Modern pharmacology studies have shown that gecko and prescriptions containing it are effective to treat malignant tumors, tuberculosis, osteomyelitis and syring. Recently, gecko has been increasingly concerned by the medical world due to its strong anti-tumor activity, especially for digestive system tumors such as esophageal cancer, gastric cancer, and liver cancer (Jiang et al., 2014). Jin Long capsule, fresh gecko as the main ingredients, was

a successful case and the sales had been in the top 10 list of antineoplastic Chinese patent medicines for several years in TCM hospital (Yang et al., 2003; Zeng, 2012). Although the antitumor activity of gecko is explicit, the investigation of mechanism and active substance is still superficial (Jiang et al., 2014).

Our laboratory has investigated the antitumor effect and active substance of gecko for many years, especially for liver cancer. We confirmed that AG could inhibit the growth of tumor in H22 (mouse hepatocellular carcinoma cell line H22) hepatocarcinoma-bearing mice (Hou et al., 2008). In order to illuminate the antitumor components, AG was separated into small molecular components (S-AG), macromolecular components (M-AG) and the valley fractions (V-AG) by Sephadex G-25, M-AG has been proved to have highly antineoplastic activity by previous studies in our group (data not given), it meant that M-AG was a promising active component. *In vitro*, AG or M-AG was found to have an inhibitory effect on the proliferation of BEL-7402 (Human hepatocarcinoma cell line). In addition, they could decrease the vascular endothelial growth factor (VEGF) secretion of BEL-7402. This suggested that AG and M-AG may have an effect on angiogenesis. Angiogenesis, a required progress for

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tumor growth (Folkman, 1972), is regarded as a promising therapeutic strategy for oncotherapy (Polverini, 2002; Li et al., 2014). Thus, this study is set to investigate the effect of AG or M-AG on angiogenesis.

Materials and Methods

Materials

Gecko (*Gekko swinhonis* Guenther) was collected from Duoshi Town, Jiyang, Jinan City, Shandong Province, China. Gecko was identified by Huzhan Zheng, professor of Beijing University of Chinese Medicine. All the specimens were deposited at the -80°C refrigerator in Department of Biological pharmaceuticals, School of Chinese Materia Medica, Beijing University of Chinese Medicine.

Dulbecco's Modified Eagle Medium (DMEM) and RPMI-1640 medium were purchased from Gibco (Grand Island, NY, USA). Foetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Trypsin, DMSO (dimethyl sulfoxide), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) and microporous membrane (0.22 μm) were obtained from Beijing BioDee BioTech Corporation Ltd. (Beijing, China). Transwell (8- μm pore size) was purchased from Corning Costar (Cambridge, MA, USA). The enzyme-linked immunosorbent assay (ELISA) kit for human VEGF was purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Eggs were obtained from the Institute of Laboratory Animals, China Agricultural University.

The preparation of AG and M-AG

Three hundred grams of gecko died of frost were put into a mortar, containing 1200 mL of sterile water, with continuous grinding for one hour in ice. Crude tissue homogenates were repeatedly frozen to -80°C and thawed in room temperature for five times. The crude tissue homogenates were filtered by filter paper, using a Buchner funnel and water suction, then were centrifuged for 10 min at 12,000 rpm. The supernatants were then filtered through a microporous membrane (0.22 μm) and collected as the AG. In order to obtain M-AG, AG (no more than 10% of column volume) was loaded onto a Sephadex G-25 column (Pharmacia, Cambridge, MA, USA) previously equilibrated with high purity water. The column was eluted with high purity water at 1.0 mL/min. The fractions were monitored at A280, the elution monitored as first peak was collected as M-AG. AG and M-AG was freeze-dried by a vacuum freeze drier (FD-1A-50, Beijing, China) and the freeze-dried powders were dissolved in high pure water respectively as the stock solution (1 mL stock solution was obtained from 2.5 g of fresh gecko), which was used in the subsequent experiment.

The analysis of AG and M-AG

AG was monitored by Sephadex G-25 column at A280 as mentioned above. To maintain the stable quality of the M-AG for each preparation, HPLC was used to check the components of the M-AG. The chromatographic column TSK-Gel G4000PW_{XL} (7.8 \times 300 mm, HOSOH, Japan) was

chosen. Pure water was used to be the mobile phase and the flow rate was 0.8 mL/min. The detection wavelength was 280 nm, and the column temperature was 25°C .

Cell lines and cell culture

Human umbilical vein endothelial cells 12 (HUVE-12) and human hepatoma cell line BEL-7402 were purchased from Shanghai cell Biology Institute of Chinese Academy of Sciences (Shanghai, China). HUVE-12 cells were cultured in DMEM medium supplemented with 10% FBS and BEL-7402 cells were cultured in RPMI 1640 medium with 10% FBS. All cells were grown in a humidified atmosphere, containing 5% CO_2 at 37°C .

VEGF Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA kit specific for human VEGF (Sangon, China) was used to quantify VEGF secretion of BEL-7402. BEL-7402 cell was plated in 6-well plates at a density of 2×10^5 cells /well. Cells were cultivated with serum-free RPMI 1640 medium containing AG or M-AG ranging from 4 $\mu\text{L}/\text{mL}$ to 20 $\mu\text{L}/\text{mL}$ for 24 h, then the medium was collected and centrifuged at 2000 rpm for 10 min at 4°C . The next actions were performed as the ELISA Kit Instruction Manual described.

Cell proliferation assay

The proliferation of the HUVE-12 cells was detected by the tetrazolium dye colorimetric assay. HUVE-12 cells growing in the log phase were treated with trypsin and suspended as single-cell suspension. Then, the cells dispersed evenly in DMEM medium were seeded in a 96-well plate with a density of 4000 cells/well and treated with various concentrations of AG, M-AG or high purity water as a negative control with six replicates of each treatment. After cultivating for 72 h in a humidified atmosphere, containing 5% CO_2 at 37°C , 50 μg of MTT was added to each well for addition of 4 h incubation. After discarding the supernatant, the formazane product was dissolved in 150 μL of DMSO. Then, the optical density was measured at 570 nm using a pan-wavelength microplate reader (Model: EPOCH, Serial: #130315A, BioTek, USA). The relative inhibition of cell proliferation was calculated as follows: relative inhibition $\% = (1 - A_{\text{treated}}/A_{\text{control}}) \times 100\%$ (Mosmann, 1983).

In vitro wound-healing assay

To determine the effects of AG or M-AG on the migration of HUVE-12 cells, an *in vitro* wound-healing repair assay was performed. HUVE-12 cells with a density of 4×10^5 cells/well were seeded into 6-well culture dishes and grown in DMEM medium containing 10% FBS to a nearly confluent cell monolayer. At the confluence, the cell monolayers were wounded using a 20 μL micropipette tip. The floating cells were washed away by phosphate buffer saline. Then the cell monolayers with wound were incubated in 1% FBS DMEM medium containing various concentrations of AG, M-AG or high purity water as a negative control for 24 h. The situation of wound healing was photographed at a 200 \times magnification using a microscope (Nikon, Japan).

Transwell migration assay

Briefly, 650 μ L of DMEM medium with 20% FBS were placed in the bottom chamber of the transwell. Top chambers were seeded with 4×10^4 HUVE-12 cells in 100 mL of serum-free medium, where the transwell membrane was coated with 1% gelatin in order to provide a better adherence. The cells were incubated with various concentrations of AG, M-AG or high purity water (negative control). The migration assay proceeded in a cell culture incubator (37°C, 5% CO₂) for 6 h. Then, the 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 90% methanol for 10 min and washed for three times with sterile water. Further, the HUVE-12 cells were stained with 1% crystal violet. The cells that were trapped in or attached to the reverse side of the membrane were photographed by a microscope (Nikon, Japan) at a 100 \times magnification, and was quantified by manual counting in four microscopic fields per sample at a 400 \times magnification. The number of pierced cells represented average of the pierced cells in the four microscopic fields per sample.

Chick embryo chorioallantoic membrane assay

Anti-angiogenic activity of M-AG was measured by CAM assay as described with minor modification (Brooks et al., 1999). One hundred fertilized eggs which were incubated at 37.5 \pm 0.5°C with 65-70% relative humidity were divided into five groups, treated with 0, 0.4, 0.8, 1.6 or 4 μ L/mL M-AG in sequence. On day 7, a 2.5 cm² window was cut on the broad side of the egg, which could candle the egg to assure existence of embryonic blood vessels, the shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away. Ten microliters of M-AG or high purity water as a negative control was applied on gelatin sponges with 2 mm in diameter and then placed onto the 7-day-old CAM surface, after which a permeable sticky tape was immediately appended to the window. Three days later, methanol was used to immobilize blood vessels and the vessels were photographed. Anti-angiogenic effect of M-AG on CAM was quantified by counting the number of visible microvessels (diameter <0.05 mm) within the defined area of the gelatin sponges.

Statistical analysis

Student's t-test and one-way analysis of variance (ANOVA) were used to determine the statistical significance of differences between the values for the various experimental and control groups. All data are expressed as the mean \pm S.D. a P-value < 0.05 was considered statistically significant.

Results

The analysis of AG and M-AG

Three fraction pools (1 (peak 1, macromolecular components; M-AG), 2 (peak 2, small molecular components; S-AG) and 3 (valley, valley fractions; V-AG)) were collected by passing AG through a Sephadex G-25 gel filtration column which was monitored at A280

(Figure 1A). The yields of M-AG, S-AG and V-AG were 5.86%, 2.14% and 1.50%, respectively. Duo to the separation range of Sephadex G-25 is 1-5 kD, we inferred that the molecular weight of M-AG was more than 5 kD, and the molecular weight of S-AG was less than 5 kD. M-AG appeared tawny and clear. HPLC method was used to test the components of M-AG. Three main peaks (1, 2 and 3) and several small peaks were observed on the HPLC graph (Figure 1B), and the three main peaks were used to evaluate the sample quality.

Down-regulated secretion of VEGF

The concentration of VEGF, secreted over the 24 h period by BEL-7402 cells treated with 4 to 20 μ L/mL AG or M-AG, were illustrated as shown in Figure 2. AG or M-AG reduced VEGF secretion in BEL-7402 cells in a dose-dependent manner. M-AG had a stronger effect on inhibiting the secretion of VEGF than AG. At the same concentration of 20 μ L/mL, the relative inhibition rate of AG for 24 h was 27.2%, obviously less than that of M-AG (53.2%). This suggested that M-AG had more intensive suppression on the secretion of VEGF in BEL-7402 cells.

Anti-proliferative effects on HUVE-12 cells

HUVE-12 (Wang et al., 2010; Lou et al., 2012) which was widely applied in the study of angiogenesis, was chosen to investigate the potential effects of AG or M-AG on vascular endothelial cell viability. HUVE-12 cells were exposed to 6-20 μ L/mL of AG or M-AG for 24-72 h. The dose-dependent inhibitory effects of AG or M-AG were observed at 72 h (Figure 3). The IC₅₀ of AG or M-AG

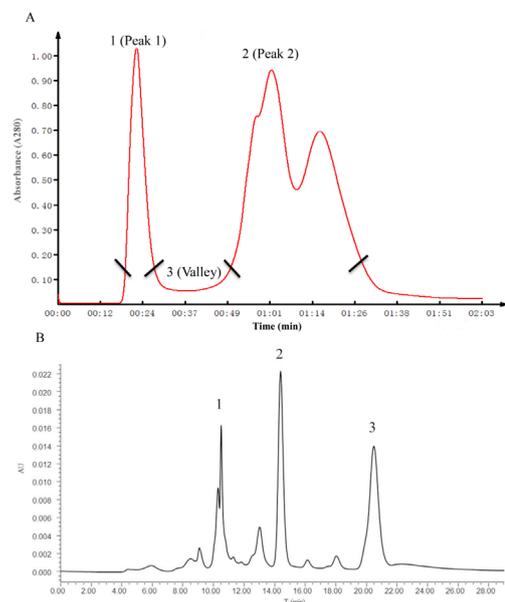


Figure 1. The chemical profiles of AG and M-AG. (A) The chemical profiles of AG. AG was analysed through a Sephadex G-25 gel filtration column. The column was eluted with high purity water at 1.0 mL/min and the fractions were monitored at A280. Three fraction pools (1 (peak 1, M-AG), 2 (peak 2, S-AG) and 3 (valley, valley fractions; V-AG)) were obtained. (B) Chromatogram of the HPLC (TSK-Gel G4000PW_{XL}) analysis of M-AG. M-AG was analyzed by HPLC. Pure water was chosen to be the mobile phase. The flow rate was 0.8 mL/min and the detection wavelength was 280 nm. AU: absorbance unit; 1: Peak 1; 2: Peak 2; 3: Peak 3

was $11.5 \pm 0.5 \mu\text{L/mL}$ and $12.9 \pm 0.4 \mu\text{L/mL}$ respectively, the maximum inhibitory rate of AG or M-AG was 80.12% and 65.81% respectively (Figure 3B).

Inhibitive effects on the migration of HUVE-12 cells

The wound-healing assay is a classical and commonly subjecting method for studying cell migration (Yarrow et al., 2004). The results of microscopic observation showed that AG or M-AG decreased the migration of HUVE-12 cells in a concentration-time-dependent manner. Almost no cell migration was observed when the cells treated with $40 \mu\text{L/mL}$ of AG or $28 \mu\text{L/mL}$ of M-AG (Figure 4A). In

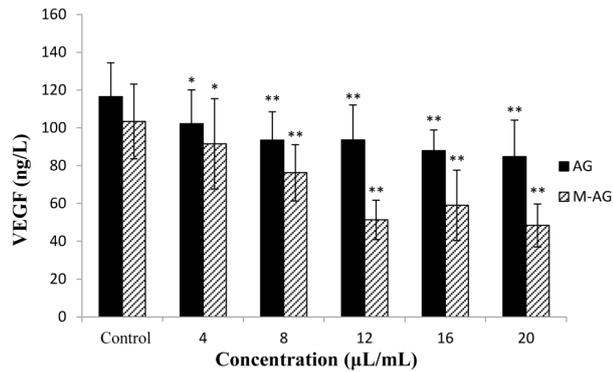


Figure 2. Effects of AG or M-AG on VEGF Secretion of BEL-7402 Cells. The BEL-7402 cells were treated with different concentrations of AG or M-AG for 24 h, then the supernatant was measured as the ELISA Kit Instruction Manual described. Data were presented as mean ± S.D. The single asterisk (*) indicates a significant difference from the control ($p < 0.05$); the double asterisk (**) indicates a very significant difference from the control ($p < 0.01$)

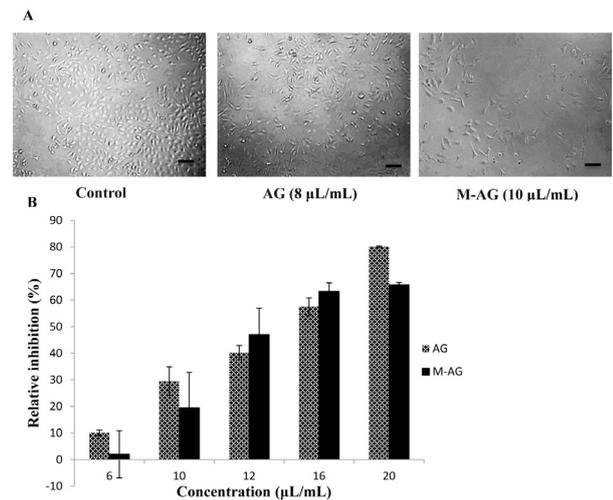


Figure 3. Effects of AG or M-AG on the Morphology and Proliferation of HUVE-12 Cells. HUVE-12 cells were seeded in 96-well plates with a density of 4000 cells/well and treated with 0, 6, 10, 12, 16, 20 $\mu\text{L/mL}$ of AG or M-AG for 72 h with six replicates of each treatment, negative control group was treated with high purity water. (A) Morphology of HUVE-12 cells treated with various concentrations of AG or M-AG. The figures were photographed at a $200\times$ magnification. Scale bar = 100 μm . (B) Relative inhibitory rates of AG or M-AG on HUVE-12 cells proliferation. The light density was measured by using a Pan-wavelength microplate reader at 570 nm ($n=4$)

order to quantify the migration degree of HUVE-12 cells, transwell assay was used to determine the inhibitory effect of AG or M-AG migratory activity (Wang et al., 2014). According to the result, the migration potential was inhibited significantly ($p < 0.01$) when HUVE-12 cells treated with AG ($24 \mu\text{L/mL}$) or M-AG ($12 \mu\text{L/mL}$) for 6 hours (Figure 4B, C).

Inhibitive effect of M-AG on vessels formation of CAM

To ensure the inhibitory effect of M-AG on angiogenesis *in vivo*, CAM assay was performed. CAM is a successful model for studying angiogenesis which has been applied in research on morpho-functional aspects of the angiogenesis due to its abundant vascularization and easy accessibility (Auerbach et al., 2003; Chen et al., 2013). The blood vessel was observed under the microscope, the CAMs on the edge of gelatin sponges with high purity water did not show obvious changes in vascular density, and a normal branching pattern of blood vessels was presented. Vasculatisation in chick embryo was significantly inhibited by M-AG at $0.4 \mu\text{L/mL}$. The displayed images in Figure 5A show that the vasoganglion at the edge of the gelatin sponge got fuzzy and damaged, as the extension of time and concentration, the damaged area increased gradually and the vascular branch got fractured

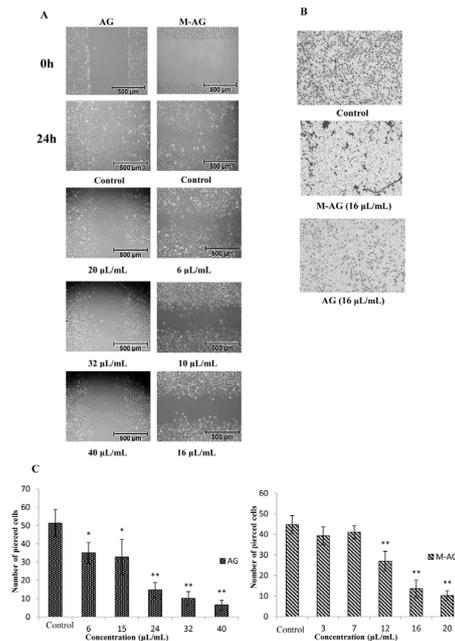


Figure 4. Effects of AG or M-AG on Migration of HUVE-12 Cells. (A) Pictures of cell migration in wound-healing repair assay. HUVE-12 cells were scratched and treated with various concentrations of AG or M-AG for 24 h. Migration was observed under a phase-contrast microscope at a $200\times$ magnification. (B) Photos of HUVE-12 cells on the bottom surface of the membrane in transwell assay. Cells were photographed by a microscope at a $100\times$ magnification (C) Average of the pierced HUVE-12 cells in the four microscopic fields. Data were determined by counting cells in four microscopic fields per sample at a $400\times$ magnification. Results were presented as mean ± S.D. ($n = 3$). The single asterisk (*) indicates a significant difference from the control ($p < 0.05$); the double asterisk (**) indicates a very significant difference from the control ($p < 0.01$)

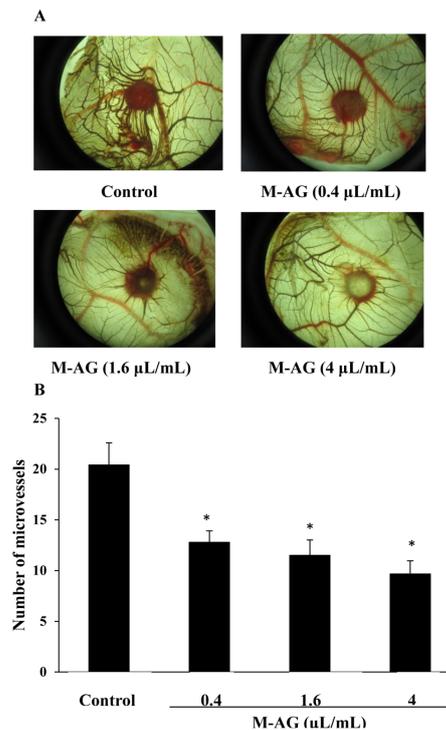


Figure 5. Effects of M-AG on New Vessels Formation in CAMs.

Chicken embryos were incubated at 37°C with 65% relative humidity for seven days, then the gelatin sponges adsorbed with various concentrations of M-AG or high purity water were placed onto the CAMs surface and incubated for 72 h. Vasculogenesis was quantified by counting the number of visible new vessels (diameter < 0.05 mm) within the defined area of the gelatin sponges. (A) Photos of the CAMs with different treatment. (B) The number of newly formed vessels on the edge of gelatin sponges. Results were presented as mean ± S.D. (n = 3). The single asterisk (*) indicates a significant difference from the control ($p < 0.05$); the double asterisk (**) indicates a very significant difference from the control ($p < 0.01$)

and haemolytic. The quantitative results shown in Figure 5B indicated that M-AG had an inhibitory effect on angiogenesis of CAMs in a dose-dependent manner. The inhibition ratio of M-AG on the formation of new blood vessels was $37.4 \pm 5.6\%$ at a concentration of $0.4 \mu\text{L/mL}$.

Discussion

Ever since the mechanism of tumor angiogenesis was revealed by Folkman's research (Folkman, 1972; Folkman et al., 1987), it is generally accepted today that tumor growth is angiogenesis-dependent, and the anti-angiogenic therapy is also regarded as a promising therapeutic strategy for oncotherapy (Polverini, 2002; Naqane, 2014). Tumors secrete various growth factors (e.g. VEGF) which activate receptors on endothelial cells present in pre-existing blood vessels. The activated endothelial cells release proteases to allow endothelial cells to escape from the original (parent) vessel walls. The endothelial cells then proliferate into the surrounding matrix and form solid sprouts connecting neighboring vessels. As sprouts extend toward the source of the angiogenic stimulus, endothelial cells migrate in tandem. These sprouts then form loops to become a full-fledged vessel lumen as cells migrate to the

site of angiogenesis. In this study, we showed that both AG and M-AG could reduce VEGF secretion of BEL-7402 cells. At a concentration of $20 \mu\text{L/mL}$, the relative inhibition rate of AG and M-AG effecting on the VEGF secretion was 27.2% and 53.2% respectively, suggesting that M-AG had a more intensive inhibition on the secretion of VEGF than AG. AG or M-AG inhibited the HUVE-12 cells proliferation in a dose-dependent way, the IC₅₀ of AG or M-AG was $11.5 \pm 0.5 \mu\text{L/mL}$ and $12.9 \pm 0.4 \mu\text{L/mL}$ respectively. The transwell and wound-healing assay demonstrated that both AG and M-AG significantly ($p < 0.01$) inhibited the HUVE-12 cells migration when treated with AG ($24 \mu\text{L/mL}$) and M-AG ($12 \mu\text{L/mL}$) for 6 hours. All the data proved that VEGF secretion, endothelial cells proliferation and migration, which were key steps for tumor angiogenesis, were blocked partly by AG or M-AG. What's more, M-AG, a macromolecular component with a more than 5 kD molecular weight separated from AG, was proved to have a strong anti-tumor activity (63% inhibition rate) by previous studies (data not given) through H22 hepatocarcinoma-bearing mice *in vivo*. In this research M-AG displayed stronger inhibitory effects on angiogenesis, and this activity was further identified by CAM assays. Therefore, we inferred that the major active components from gecko might be macromolecules and anti-angiogenic might be one of the anti-tumor mechanisms of gecko.

Because of the good therapeutic effects in cancer clinical, the active ingredients of gecko were searched by several labs. Gekko sulfated polysaccharide was separated from water extraction of gecko with alcohol precipitation, which showed a differentiation-inducing activity in hepatic cancer cell line SMMC-7721, however, it didn't show anti-tumor activity in mice (Xin et al., 2011). The *in vivo* anti-tumor effect of the imidazole alkaloids extracted from gecko was reported when it was encapsulated in liposomes (Kang et al., 2009). But there was no report to show if the imidazole alkaloids itself was orally effective while the gecko powder did. The anti-tumor crude peptide separated from gecko was reported but its components still need to be verified (Wu et al., 2013). Therefore, the anti-tumor single active ingredient from the gecko was still need to find. M-AG is a promising resource to find anti-angiogenic component from gecko. With the deepening of research on tumor dormancy, there has been growing attention on the tumor microenvironment, anti-angiogenesis drugs is an instance. However, the development of anti-angiogenic drugs moves slowly, only few were commonly used in clinic. The most common of them was Bevacizumab, a recombinant humanized monoclonal antibody that produces angiogenesis inhibition by inhibiting vascular endothelial growth factor A (VEGF-A) (Los et al., 2007). It is worth noting that Bevacizumab has been proved to have issues of toxicity and drug resistance by the FDA, such as hypertensive crisis, nephritic syndrome and congestive heart-failure. The promising evolutionary trend was to develop multi-target and broad-spectrum angiogenesis inhibitors (Limaverde-Sousa et al., 2014). Separating active substance from TCM is a high-efficiency and secures way for research and development of new drugs due to the multi-targeted and the thousands of years of

application of TCM. Therefore, M-AG was a promising anti-angiogenic resource for new drug development.

The mechanism of the antitumor activity of gecko was deemed to be various although the studies were not deep enough (Jiang et al., 2014). Several studies indicated that the extracts of gecko could induce the tumor cell apoptosis *in vitro* (Yang et al., 2007; Song et al., 2011; Jeong et al., 2012). Other data proved that gecko could inhibit the tumor growth by adjusting the immune system (Wu et al., 2008; Liu et al., 2008). Besides, differentiation-inducing activity of gecko was also confirmed by our laboratory (Wang et al., 2014). All of the data indicated that various active substances in gecko had therapeutic action on oncology in different ways. So, making certain what the active substances are and what the mechanism they depend on is still a daunting task.

Our research confirmed that anti-angiogenesis is one of the anti-tumor mechanisms of gecko, M-AG is a promising anti-tumor substance depending on inhibiting anti-angiogenesis and is well worth being researched and separated deeply.

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