

## Original Article

# Anti-tumor effects of Gecko ethanol extract on human cervical cancer SiHa cells

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**Abstract:** Gecko has been applied as an anti-tumor agent in traditional Chinese medicine for hundreds of years. Gecko ethanol extract (GEE) which was extracted from the powder of whole Gecko, has shown the obvious antitumor activity. It is still unclear the effect of GEE on human cervical cancer cells. In this study we investigated the anti-tumor effect of GEE on human cervical cancer SiHa cells and its potential molecular mechanism. The growth inhibitory effect of GEE on SiHa cells were assessed by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay and colony forming assay in vitro. The results showed that GEE significantly inhibited proliferation of SiHa cells in a dose- and time- dependent manners. The typical morphological changes of apoptosis were observed in SiHa cells, such as chromatin condensation, nuclear fragmentation and reduction of cell volume. The effects of GEE on proteins expression of SiHa cells were detected by Western blot assay. The protein expression levels of Bcl-2 and VEGF were down-regulated, whereas Bax, cytochrome c (Cyt c), caspase-9 and caspase-3 protein were up-regulated after treatment of GEE in vitro. In a word, these results suggested that GEE play an antineoplastic role on SiHa cells, which may be concerned with the effect of anti-proliferation, anti-angiogenesis and apoptosis-induction.

**Keywords:** Gecko ethanol extract, human cervical cancer, anti-tumor, apoptosis

## Introduction

Cervical cancer is one of the most menace to women health, and the incidence and mortality of the cancer keep increasing all over the world [1-3]. Current treatment for cervical cancer includes surgery, radiation therapy, chemotherapy, and concurrent chemoradiation therapy [4]. However, it is important to note that lots of conventional chemotherapeutic drug cause serious cytotoxic, numerous side effects sometimes, and multiple drug resistance [5]. Therefore, identification of novel antitumor agents from natural products with better effectiveness is an alternative choice for management of cervical cancer. Traditional Chinese medicine is more widely used to treat malignant tumors in Chinese clinic due to its high activity and low toxicity [6, 7]. Therefore, many research groups are actively investigating the role of different traditional Chinese medicine in anticancer pharmacological effect [8].

Gekko swinhonis Guenther, commonly known as Gecko, has been applied in traditional

Chinese medicine for hundreds of years [9, 10]. As "Compendium of Materia Medica" recorded, Gecko could cure "Blood plot into a ruffian, Pandora wind scrofula". Recently, Gecko were reported to show its strong antineoplastic activity on different cancer, such as liver cancer, colorectal cancer, bladder cancer and esophageal cancer [11-14]. Jin Long capsule, fresh gecko as the main ingredients, was used to treat malignant tumors for several years. Recent studies have shown that Jin Long capsule could improve the clinical effect, boost living quality and reduce the adverse reaction of chemotherapy [15, 16]. Although the anti-tumor activity of Gecko is explicitly confirmed, the investigation of its mechanism is still superficial.

Gecko ethanol extract (GEE) has antineoplastic activity in previous experiment. The results of our previous studies revealed that GEE can inhibit the proliferation and induce apoptosis of human laryngeal carcinoma Hep2 cells [17]. Further researches were proved that the antitu-

mor effect of GEE on HepG2 cells in vitro and in a mouse xenograft model of ascites H22 tumors [18, 19]. Although the antitumor effect of Gecko has been reported, there is still limited information about its effect in human cervical cancer. Therefore, we emphatically studied that the anti-cancer activity of GEE on human cervical cancer and its underlying mechanism of action in this paper.

## Materials and methods

### *Preparation of Gecko ethanol extract (GEE)*

The protocol used to obtain GEE from *Gecko* was based on the previously method [17-19]. Whole-dried *Gecko japonicus* were purchased from Anhui Bozhou Yonggang Co. Ltd (Bozhou, China). In brief, dry powder of *Gecko* (400 g) was dissolved in 400 mL double distilled water, and then the mixture was put into a lapping machine to grind into homogenate four hours continuously. Following centrifugation at 5000 rpm for 5 min, the precipitation was collected and extracted by soaking in 400 mL 55% ethanol solution for 4 h at 4°C. The supernatant solution was further concentrated using a rotary evaporator to remove the ethanol, and finally lyophilized in a freeze-dryer to collect golden extration powder GEE. Then the lyophilized of GEE were deposited at the -80°C refrigerator, which was used in the subsequent experiments.

### *Cell line and cultures*

The human cervical cancer cell line SiHa was kindly provided by the First Affiliated Hospital of Henan University of Science and Technology. Dulbecco's Modified Eagle Medium (DMEM) medium was obtained from Gibco (Grand Island, NY, USA), and fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd. (Hangzhou, China). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, and incubated at 37°C in a humiditive atmosphere with 5% CO<sub>2</sub>. Cells in the exponential growth phase were collected for the subsequent experiments.

### *MTT assay*

The inhibition of cell growth was measured by MTT assay [20]. SiHa cells in logarithmic growth

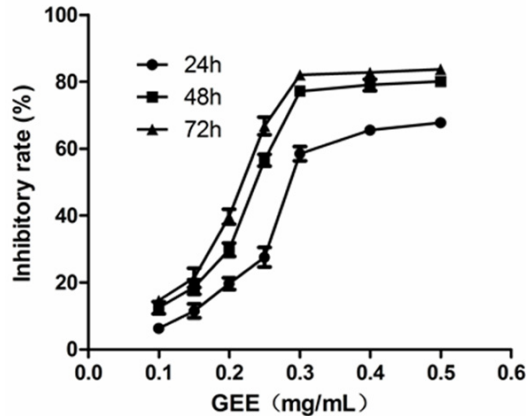
were dispensed into 96-well plates at a density of 2.5×10<sup>4</sup> cells/mL with 200 µL and incubated 24 h to allow the cells to attach. The cells were exposed to different doses of GEE (0.1, 0.15, 0.2, 0.25, 0.3, 0.4 and 0.5 mg/mL) for 24 h, 48 h, and 72 h, respectively, while cells cultured without GEE served as a control group and cells treated with 0.01 mg/mL 5-Fu served as a positive control group. After incubation for specified time at 37°C in a humidified incubator, 20 µL MTT (5 mg/mL) was added to each well and additional incubated for 4 h. After 4 h, medium was removed and replaced by 200 µL dimethyl sulfoxide (DMSO) in each well to solubilize the formazan product, and the plate was placed on a plate shaker for 10 min at room temperature. Finally, the absorbance (A) was recorded on an ELX800 Universal Microplate Reader (Bio-Tek Instruments) at the wavelength of 490 nm. The inhibition rate (IR) was calculated using the following formula:  $IR\% = [1 - \frac{A_{drug}}{A_{control}}] \times 100\%$ .

### *Plate clone formation assay*

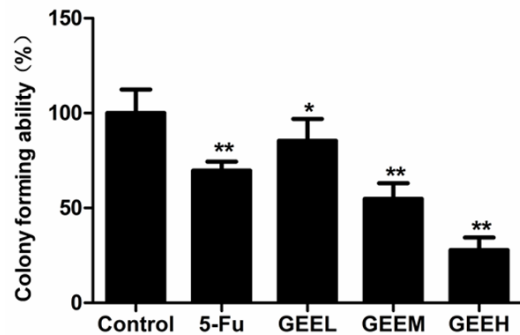
The multiplication capacity of SiHa cells were observed by plate clone formation assay. SiHa cells in the logarithmic growth phase were suspended and transferred into six-well plate at 5000 cells per well. After 24 h, cells were cultured with GEE (0, 0.04, 0.06 and 0.1 mg/mL) and 0.01 mg/mL 5-Fu, respectively. Then the plates were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 10 d, until the cell clones could be observed directly. At the end of the incubation period, the cells were stained with crystal violet for 20 min. The number of colonies was counted with an inverted microscope. Each experiment was repeated three times. The colony forming ability (CFA) was calculated using the following formula:  $CFA\% = \frac{\text{Colony counts in experiment}}{\text{Colony counts in control group}} \times 100\%$ .

### *Cell morphological observation*

SiHa cells were seeded in 96-well plates with a density of 5000 cells/well and treated with 0, 0.1, 0.2, 0.3 mg/mL of GEE, positive control group was treated with 0.01 mg/mL 5-Fu. Following treatment for 48 h, the morphological changes of SiHa cells were observed under an inverted microscope at 100× magnification and photographed. The experiment was repeated three times.



**Figure 1.** The anti-proliferative effect of GEE on SiHa cells. The growth inhibitory effect of GEE on SiHa cells were assessed by MTT assay in vitro. Values are mean  $\pm$  SD (n = 5).



**Figure 2.** The effects of GEE on the clone ability of SiHa cells. The clone ability of SiHa cells was assessed by the plate clone formation assay. Control, control group; 5-Fu, cells treated with 0.01 mg/mL 5-Fluorouracil served as a positive control group; GEEL, low dose of atorvastatin (0.1 mg/mL); GEEM, medium dose of GEE (0.2 mg/mL); GEEH, high dose of GEE (0.3 mg/mL). Values are mean  $\pm$  SD (n = 5). Compared with control group, \* $P < 0.05$ , \*\* $P < 0.01$ .

## Hoechst 33258 staining

Hoechst 33258 staining was used to observe morphologic changes of cell nuclei in vitro. SiHa cells, which were in exponential growth, were seeded in a six-well plate at a dose of  $2.5 \times 10^4$ /well for 24 h. Afterward, cells were exposed to GEE (0, 0.1, 0.2 and 0.3 mg/mL) and 0.01 mg/mL 5-Fu for an additional 48 h. Following treatment with GEE, cells were washed twice with PBS and fixed in 4% paraformaldehyde for 15 min at 4°C. After washing twice with PBS, cells were Hoechst 33258 staining solution for 10 min at room temperature in the dark.

Afterwards, cells were washed twice again before observed under the fluorescence microscope. All experiments were performed in triplicate.

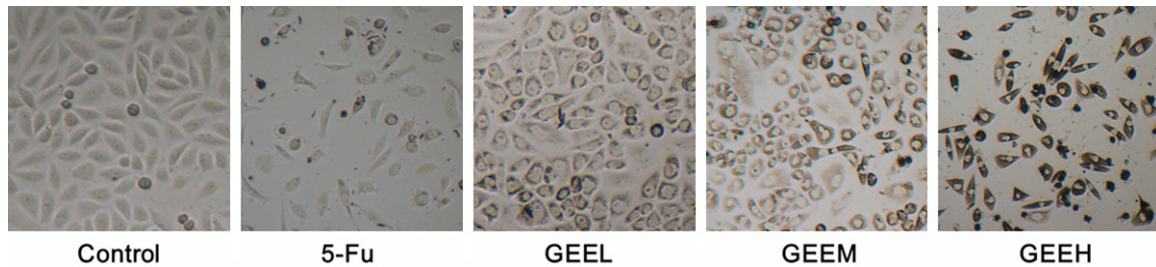
## Annexin V-FITC/PI (propidium iodide) double staining

Apoptosis was determined by Annexin V-FITC staining and PI labeling. To quantify apoptosis, prepared cells were washed twice with cold PBS and then resuspended in 500  $\mu$ l binding buffer at a concentration of  $1 \times 10^6$  cells/ml. Five microliters annexin-V-FITC and 5  $\mu$ l PI were then added to these cells, which the were kept in the dark at RT (25°C) for 10 min. Data acquisition and analysis were performed in a FACScalibur flow cytometer (Becton Dickinson) and calculated by CellQuest software (BD Biosciences, Franklin Lakes, NJ).

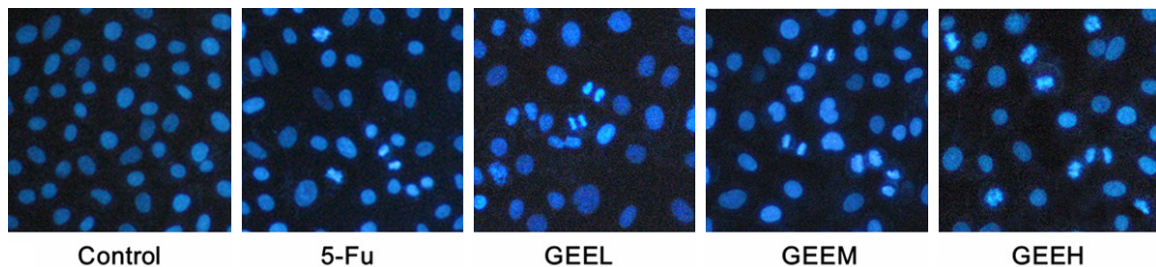
## Western blotting

SiHa cells were seeded into four 25 cm<sup>2</sup> culture flasks at a density of  $5 \times 10^5$  cells/mL and incubated for 24 h. Varied doses of GEE (0, 0.1, 0.2 and 0.3 mg/mL) and 0.01 mg/mL 5-Fu were added to each well and the cells were incubated for 48 h. Afterward, the cells were centrifuged and washed twice with pre-cooled PBS, and lysed in RIPA lysis buffer with protease inhibitors for 30 min on ice. Following centrifugation at 14,000 rpm for 20 min, the supernatants were removed and total protein dose was measured using the BCA protein assay kit. BCA protein quantification kit was purchased from Solarbio Science and Technology Co. Ltd (Beijing, China), the antibodies used for Western blotting were purchased from Proteintech Group, Inc (Wuhan, China). Equal amounts of protein were separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes at 200 mA for 4 h. The membranes were blocked in 5% skim milk at room temperature for 1 h and then incubated overnight with primary antibody at 4°C. Subsequently, the membrane was further incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and then washed with PBST 3 times. Finally, the protein bands were visualized using the DAB chromogenic reagent, and the intensity ratios of the bands compared with control bands.

## Anti-tumor effects of GEE on SiHa cells



**Figure 3.** The effects of GEE on the morphologic change of SiHa cells (100×). Following treatment for 48 h, the morphological changes observed in SiHa cells via inverted microscope. Abbreviations as above. Representative pictures of the morphologic change in SiHa cells.



**Figure 4.** The effects of GEE on the nuclear morphologic change of SiHa cells (400×). The nuclei morphological changes observed in SiHa cells via Hoechst 33258 staining assay. Abbreviations as above. Representative pictures of the morphologic change in SiHa cells.

### Statistical analysis

The results were expressed as the mean  $\pm$  standard deviation (SD). Statistical analysis was performed using SPSS 16.0 software. One-way ANOVA was applied to analyze the data by SPSS 16.0 system to determine differences between groups. *P*-values less than 0.05 ( $P < 0.05$ ) was considered statistically significant.

### Results

#### Effects of GEE on the proliferation of SiHa cells

SiHa cells were treated with increasing doses of GEE for 24, 48, 72 h, respectively, and cell viability was assessed by the MTT assay. The MTT assay data indicated that exposure to GEE at 0.1~0.5 mg/mL significantly inhibited the viability and proliferation of SiHa cells, and these effects occurred in a dose- and time-dependent manner (**Figure 1**). Additionally, our data demonstrated that incubation with 5-Fu (0.01 mg/mL) for 24, 48, 72 h resulted in a marked decrease in cell viability, and the inhibition ratio were 33.43%, 43.47% and 44.77%, respectively. After treatment of GEE for 24, 48,

72 h, the 50% inhibitory dose ( $IC_{50}$ ) values were 0.318 mg/mL, 0.239 mg/mL, 0.219 mg/mL, respectively.

#### Effects of GEE on the colony forming ability of SiHa cells

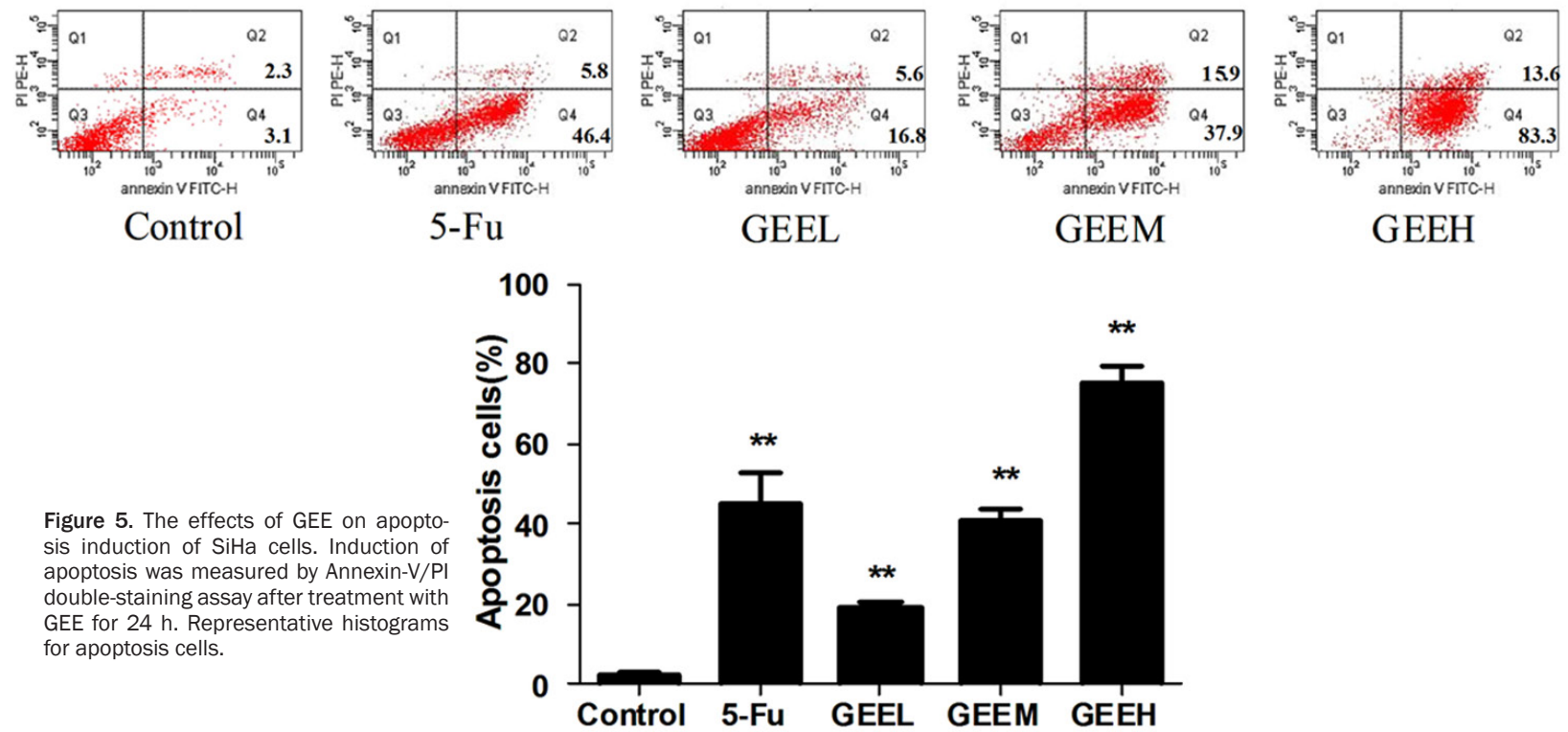
To evaluate the effect of GEE on the clone ability of SiHa cells, plate clone formation assay was employed. As shown in **Figure 2**, the colony forming ability of GEE cells was decreased compared with the control group. The colony formation assay further confirmed that GEE may inhibit the proliferation of SiHa cells.

#### Effects of GEE on the morphologic change of SiHa cells

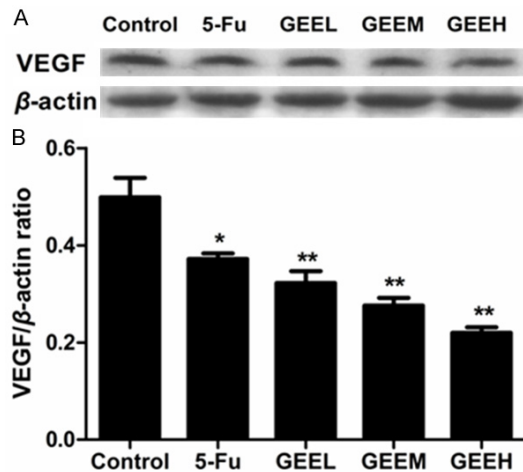
Following treatment of GEE for 48 h, the morphological changes observed in SiHa cells via inverted microscope. As shown in **Figure 3**, cells of the control group were adherent, spindle-shaped, and tightly packed. Compared with the control group, cells treated with varying doses of GEE (0.1, 0.2, 0.3 mg/mL) were markedly shrunken, cell adhesion reduced, and the cell membrane were partially broken. These changes were more severe or more evident with increasing doses of GEE.



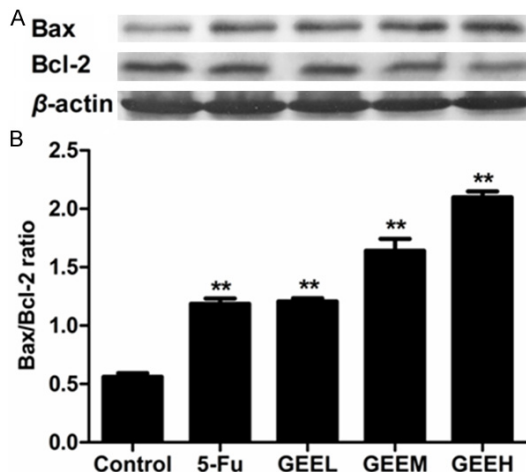
# Anti-tumor effects of GEE on SiHa cells



**Figure 5.** The effects of GEE on apoptosis induction of SiHa cells. Induction of apoptosis was measured by Annexin-V/PI double-staining assay after treatment with GEE for 24 h. Representative histograms for apoptosis cells.



**Figure 6.** The effect of GEE on the expression of VEGF in SiHa cells. The expression of VEGF in SiHa cells was examined via Western blot analysis. A. Representative Western blot band photographs of VEGF. B. Ratio of the protein expression of VEGF to  $\beta$ -actin. Abbreviations as above. Changes were significantly different compared with control group (\* $P < 0.05$ , \*\* $P < 0.01$ ).  $\beta$ -actin was used as the internal loading control.



**Figure 7.** The effect of GEE on the expression of Bcl-2, Bax and  $\beta$ -actin in SiHa cells. Western blot analysis was employed to detect the protein expression of Bcl-2, Bax and  $\beta$ -actin. A. Representative Western blot band photographs. B. Ratio of the protein expression of Bax to Bcl-2 in SiHa cells. Abbreviations as above. Changes were significantly different compared with control group (\* $P < 0.05$ , \*\* $P < 0.01$ ).  $\beta$ -actin was used as the internal loading control.

#### Effects of GEE on the apoptosis of SiHa cells

The nuclei morphological changes were observed in SiHa cells via Hoechst 33258 staining

assay are shown in **Figure 4**. As indicated in **Figure 4**, the nuclei of SiHa cells in control group were similarly sized, regularly shaped, and evenly stained. However, cells treated with GEE showed conspicuous morphological changes, such as chromatin condensation, nuclear fragmentation and reduction of cell volume. Especially in 0.3 mg/mL GEE group, cells revealed serious nuclear condensation, fragmentation, and apoptotic bodies, all of which are characteristics of apoptosis.

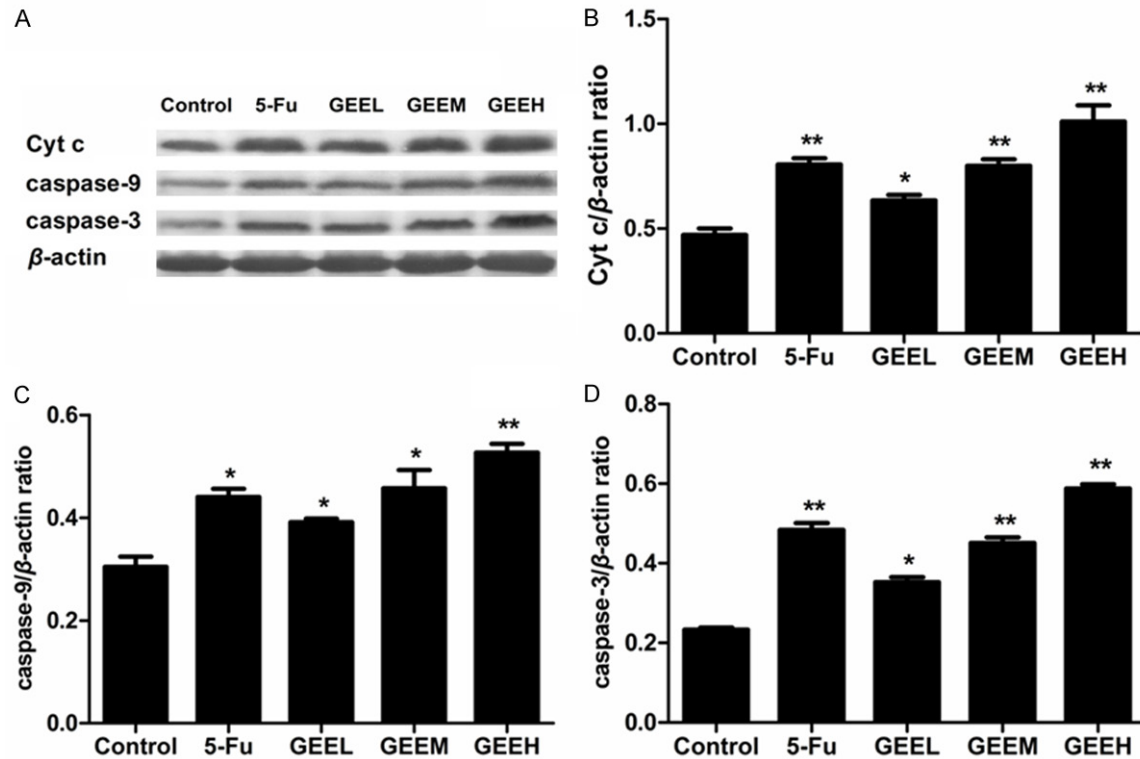
GEE-induced apoptosis in SiHa cells was further determined using an Annexin V-FITC/PI staining assay. As shown in **Figure 5**, compared to the control group, GEE dramatically triggered apoptosis in SiHa cells in a dose-dependent manner. The percentage of apoptotic cells in the control group was 3.6%. After treatment with GEE for 24 h, the percentages of apoptotic cells were 16.8%, 37.9% and 83.3%, respectively.

#### Effects of GEE on the expression of VEGF in SiHa cells

Obviously, angiogenesis have been suggested a prerequisite progress for tumor growth, meanwhile VEGF play an important role in angiogenesis. Therefore, Western blot analysis was employed to detect the protein expression of VEGF. As shown in **Figure 6**, compared to the control group, VEGF protein expression decreased in the GEE-treated groups. This indicated that there was decrease in the VEGF/ $\beta$ -actin ratio.

#### Effects of GEE on the expression of apoptotic-related proteins

Western blot analysis was applied to observe the expression of apoptotic-related proteins and investigate the mechanism responsible for the apoptosis induced by GEE in SiHa cells. As shown in **Figure 7**, western blotting analysis demonstrated that Bcl-2 protein expression was down-regulated while Bax protein expression was increased. Compared with control group, both of them are statistically significant ( $P < 0.05$ ). As shown in **Figure 8**, Cyt c, caspase-3 and caspase-9 were significantly enhanced in GEE groups. These data together suggested that intrinsic mitochondrial pathway may be involved in GEE-induced apoptosis.



**Figure 8.** The effect of GEE on the expression of apoptosis-related proteins in SiHa cells. Western blot analysis was employed to detect the protein expression of Cyt c, caspase-9 and  $\beta$ -actin. A. Representative Western blot band photographs. B. Ratio of the protein expression of Cyt c to  $\beta$ -actin in SiHa cells. C. Ratio of the protein expression of caspase-9 to  $\beta$ -actin in SiHa cells. D. Ratio of the protein expression of caspase-3 to  $\beta$ -actin in SiHa cells. Abbreviations as above. Changes were significantly different compared with control group (\* $P < 0.05$ , \*\* $P < 0.01$ ).  $\beta$ -actin was used as the internal loading control.

## Discussion

In recent years, many traditional Chinese medicines have been gradually discovered to be potential sources of antitumor drugs for its role in killing tumor cells more actively and less toxically [8]. Because of the good therapeutic effects in cancer, the active ingredients of *Gecko* were searched by several labs [7, 9, 14]. A number of studies have demonstrated that *Gecko* could inhibit proliferation and also could induce apoptosis in liver cancer, colorectal cancer, bladder cancer and esophageal cancer [11-14]. In addition, *Gecko* has also been indicated as a suppressant in angiogenesis and cell motility of liver cancer [7, 9, 21, 22]. All these findings provide a certain rationale for therapeutic properties of *Gecko* on carcinoma. However, the potential molecular mechanisms are still elusive and require further validation. Therefore, we aimed to elucidate the inhibitory proliferation effect of GEE on human cervical cancer SiHa cells in this study. In the present study, we demonstrated that GEE could signifi-

cantly inhibit the proliferation of SiHa cells in a dose- and time- dependent manners via MTT assay and plate clone formation assay. Moreover, Hoechst staining revealed that GEE could induce the apoptosis of SiHa cells in vitro.

Angiogenesis, the growth of new blood vessels from the pre-existing ones, is essential for the development and progression of malignant tumors. It supplies nutrients and oxygen for cell proliferation, penetrating the whole growth of tumor [23, 24]. VEGF is regarded as the important regulatory protein of the angiogenic process. It can promote proliferation of endothelial cells, angiogenesis and increase the permeability of blood vessels [25, 26]. Several studies reveal that blocking of VEGF function could inhibit angiogenesis, and then suppress tumor growth and metastasis [27, 28]. In the current study, the expression of VEGF was decreased following the treatment with GEE. This finding indicates that GEE may have an effect on angiogenesis. Moreover, suppressed expression of VEGF can induce release of Cyt c and activation

of caspase-3, which ultimately results in apoptosis [29].

Apoptosis is essential for maintaining the physiologic balance between cell death and cell growth. When disrupted, an imbalance between life and death of cells can lead to tumor initiation, progression and metastasis [30, 31]. Currently, inducing apoptosis plays a major role in cancer treatment, serving as the main effector function of anti-cancer therapies [32]. Consequently, we aimed to elucidate that whether apoptosis is involved in the antineoplastic effect of GEE on human cervical cancer SiHa cells in this study. Mainly two apoptotic pathways are known as the extrinsic (death receptor-mediated) and the intrinsic (mitochondrial-mediated) pathway [33, 34]. In the intrinsic pathway, mitochondria play a pivotal role in mediating apoptosis [35]. The mitochondrial pathway is regulated by the Bcl-2 family proteins which includes the anti-apoptotic proteins (Bcl-2, etc.) and the proapoptotic proteins (Bax and Bak) [31, 36, 37]. Our data showed that after GEE treatment, the expression of Bcl-2 in SiHa cells was significantly decreased in a dose-dependent manner. On the contrary, the activity of Bax was decreased when the amounts of GEE were increased. Accumulation of proapoptotic proteins on the mitochondrial outer membrane results in the increase of mitochondrial membrane permeability, and causing the release of Cytochrome C (Cyt c) into the cytoplasm [38, 39]. Release of Cyt c from the intermembrane spaces of the mitochondria into the cytosol is a key event in apoptosis [40]. In this study, we demonstrated that the expression of Cyt c was down-regulated. Cyt c binds to the cytosolic protein Apaf-1 to facilitate the formation of apoptosomes, which can then recruit and activate the inactive procaspase-9 [41]. Only the procaspase-9 bound to the apoptosome is able to efficiently cleave and activate downstream executioner caspases such as caspase-3, and trigger a cascade of events leading to apoptosis [42-44]. To clarify whether the mitochondrial pathway is involved in GEE-induced apoptosis, we examined the activation of some apoptosis-associated proteins by Western blotting. As shown, both caspase-9 and caspase-3 were up-regulated in cells following GEE treatment. These results suggested that the GEE-induced apoptosis might be through the intrinsic mitochondrial

pathway. In brief, these results suggested that GEE induced the apoptosis of human cervical cancer SiHa cells through promoting the release of Cyt c, then activation of caspase family, which may be concerned with the endogenous mitochondrial pathway.

In summary, these results demonstrated that GEE play a antineoplastic role on SiHa cells, which may be concerned with the effect of anti-proliferation, anti-angiogenesis and apoptosis-induction. These effects may owe to the increase of Cyt c, caspase-3, caspase-9 and the decrease of VEGF, Bcl-2 proteins. Although GEE could inhibit the proliferation of SiHa cell, the antitumor mechanism is quite complicated, which is need further investigations.

## Disclosure of conflict of interest

None.

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