

# Gambogic acid suppresses colon cancer cell activity *in vitro*

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**Abstract.** The aim of the present study was to elucidate the underlying mechanism of antitumor activity of gambogic acid (GA) in colon cancer. Human colon cancer SW620 cells were divided into five treatment groups, including no-treatment control (NC), low dose GA (10  $\mu$ g/ml), medium dose GA (50  $\mu$ g/ml), high dose GA (100  $\mu$ g/ml) and 5-fluorouracil (10  $\mu$ g/ml). Differences in cell proliferation, apoptosis and cell cycle, invasion, and migration were measured between groups using MTT, flow cytometry, transwell and wound-healing assays, respectively. Western blotting was used to analyze relative protein expression levels of phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), P21, and matrix metalloproteinase (MMP)-2 and -9 between groups. Compared with the NC group, GA (low, middle and high) inhibited SW620 cell proliferation, invasion and migration (all  $P < 0.05$ ). Furthermore, there were significant differences in proliferation, invasion and migration between groups administered with different doses of GA (all  $P < 0.05$ ). Compared with the NC group, the expression levels of PI3K, AKT, phosphorylated-AKT, P21 and MMP-2 and -9 were significantly altered in a dose dependent manner following treatment with GA (all  $P < 0.05$ ). The results of the current study indicated that GA suppressed proliferation and dispersion of human colon cancer cells in a dose-dependent manner, possibly through a PI3K/AKT/P21/MMP-2/9-dependent pathway.

## Introduction

Colon cancer is a common malignancy of the digestive tract with an increasing incidence each year (1). At present, 5-fluorouracil (5-Fu)-based combination chemotherapy is considered the gold standard treatment; however, it is associated

with significant side effects and poor tolerability (2). Despite the improved prognosis associated with the use of targeted drugs, the mortality rate remains high among patients with colon cancer, and there is an evident clinical requirement for new treatment methods (3). Gambogic acid (GA), a bioactive compound extracted from certain *Garcinia* species, exhibits a variety of antitumor activities against several types of cancer (4-6). The limited information available suggests that GA may inhibit colon cancer by suppressing cellular activities, including proliferation, apoptosis, invasion and migration (7).

The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway is an important signaling pathway in cell development and growth (8-10). Previous studies revealed that suppressing the PI3K/AKT signaling pathway may inhibit cancer cell proliferation and increase cancer cell apoptosis by regulating P21 expression (11,12). Other studies confirmed that inhibition of the PI3K/AKT signaling pathway may suppress cancer cell invasion and migration, possibly by regulating matrix metalloproteinase (MMP)-2 and -9 expression (13,14). The current study evaluated the antitumor effect of GA and the underlying mechanism in a human colon cancer cell line.

## Materials and methods

**Cells and reagents.** Human colon cancer cell line SW620 was purchased from American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS), RPMI-1640 medium and pancreatin were purchased from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA). GA (purity,  $\geq 95\%$ ) was purchased from Chengdu Pulis Biotech Co., Ltd. (Chengdu, China). MTT, dimethylsulfoxide (DMSO) and 5-Fu were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), while the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis and cell cycle assay kits [Cell cycle and apoptosis (PI) kit] were purchased from BD Biosciences (San Jose, CA, USA). Antibodies against PI3K (1:500; cat. no. ab32089), AKT (1:500; cat. no. ab8805), phosphorylated (p)-AKT (1:500; cat. no. ab8933), P21 (1:500; cat. no. ab109520), MMP-2 (1:500; cat. no. ab37150), MMP-9 (1:500; cat. no. ab73734) and GAPDH (1:1,000; cat. no. ab181602) were purchased from Abcam (Cambridge, MA, USA). Furthermore, a DM3000 microscope (Leica Microsystems GmbH, Wetzlar, Germany) and LightCycler® 480 instrument (Roche Molecular Diagnostics, Pleasanton, CA, USA) were used in the present study.

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**Cell culture and experimental design.** SW620 cells were cultured in RPMI-1640 medium with 10% FBS in an incubator at 37°C and 5% CO<sub>2</sub> and passaged upon reaching 90% confluence. The culture was divided into five groups based on subculturing conditions: i) No-treatment control (NC) group cultured in RPMI-1640 medium with 10% FBS; ii) NC group medium + 10 µg/ml GA (low group); iii) NC group medium + 50 µg/ml GA (medium group); iv) NC group medium + 100 µg/ml GA (high group); and v) NC group medium + 10 µg/ml 5-Fu (5-Fu group). Cells were cultured in their respective experimental group media as described below for each specific assay. Cells were cultured until the logarithmic growth stage and digested with trypsin prior to seeding into culture plates for specific experiments.

**MTT assay.** A 200-µl cell suspension (4x10<sup>3</sup> cells/ml) was cultured in 6-well-plates for 5 h to allow time for cell adherence, after which the medium was replaced with the group-specific experimental culture media, as described above. Each group was cultured in triplicate. After 48 h at room temperature, 20 µl MTT (5 g/ml) was added to each well and the plates were cultured for 30 h at room temperature. DMSO was added to each well to stop the reaction, and the OD value was measured at 490 nm on the plate reader to calculate cell proliferation.

**Apoptosis assay.** SW620 cells (1x10<sup>6</sup> cells/ml) were seeded in a 6-well plate (2 ml/well) and cultured for 24 h in group-specific media at room temperature. Media were removed and cells collected by resuspending in 300 µl binding buffer, to which 5 µl Annexin V-FITC was added according to the manufacturer's protocol. Suspensions were incubated at room temperature in the dark for 15 min, after which 5 µl PI was added. Apoptosis was detected by flow cytometry (FACSARIA; BD Biosciences; wavelength, 488 nm) supplemented with 200 µl 1X binding buffer (diluted in double distilled water) at room temperature after 1 h. All tests were performed in triplicate. Data were analyzed using Diva software (version 8.0.1; FACSARIA; BD Biosciences).

**Cell cycle assay.** SW620 cells in the logarithmic growth phase were seeded in a 7.5-cm culture flask and cultured for 24 h, then digested by pancreatin and pelleted by centrifugation (1,000 x g at 4°C for 5 min). Cell cycle status was detected using the aforementioned cell cycle kit according to the manufacturer's protocol. Briefly, the cells of each group were treated with their respective aforementioned conditioned media for 24 h at room temperature. Afterwards, 5 µl RNase (10 mg/ml) was added to the PBS-resuspended cell pellet, and the cells were cultured at room temperature for 1 h. PI (100 µg/ml) was subsequently added and the cells incubated at room temperature for 30 min. The cell cycle status was measured in triplicate by flow cytometry (FACSARIA; BD Biosciences) at a wavelength of 488 nm and data were analyzed using Diva software (version 8.0.1; FACSARIA; BD Biosciences).

**Transwell invasion assay.** Matrigel (1:3) was diluted with serum-free medium (RPMI-1640) and placed in the upper transwell chamber (pore size, 8 µm) at 37°C for 4 h, after which the transwell chambers were placed in 24-well plates. SW620 cells (1x10<sup>4</sup> cells/ml; 100 µl/well cell suspension) from

each individual experimental group were added to the upper chamber. Complete culture medium (RPMI-1640) containing 10% FBS was added to the lower chamber, and the cells were cultured for 24 h. Subsequently, noninvasive cells were removed with cotton swabs. The transwell chambers were removed from the wells, washed with phosphate-buffered saline (PBS) three times and the remaining cells in the lower chamber were fixed with 4% paraformaldehyde-PBS solution for 10 min at room temperature. The chambers were subsequently washed with PBS as aforementioned, inverted and dried. Cells were stained with 0.1% cresyl violet solution for 20 min at room temperature and washed with PBS three times. Cells were subsequently counted under an inverted optical microscope.

**Wound healing assay.** SW620 cells (5x10<sup>4</sup>/well) from each experimental group were seeded into a six-well plate and cultured for 24 h at room temperature. When the cells formed a single, tightly adherent layer, a scraper with a 2-mm wide tip was used to scratch the confluent cells. Images of each scratch were captured under a light microscope at 0 h. Plates were cultured and images were captured again under the light microscope at 48 h. Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used to measure the scratch width in the 0- and 48-h images and to calculate the cell migration rate from the difference.

**Western blotting.** Cells were incubated for 24 h at room temperature in the aforementioned conditioned media and collected using lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration was measured using a bicinchoninic acid Protein kit (Beyotime Institute of Biotechnology). Subsequently, cells of the different groups were washed three times with ice-cold PBS and lysed with buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Na-deoxycholate, 5 µg/ml aprotinin, 5 µg/ml leupeptin and 1 mM phenylmethylsulphonyl fluoride. Cell lysates were cleared via centrifugation at 12,000 x g at 4°C for 30 min and denatured by boiling in Laemmli buffer (Beyotime Institute of Biotechnology). Proteins were denatured by incubation in a boiling water bath for 5 min with 5X SDS gel buffer solution. A total of 15 µl/lane of each sample was electrophoresed on an 8% SDS-PAGE (110V; 4 h). The protein samples were transferred to polyvinylidene fluoride (PVDF) membranes using the semi-dry method. The PVDF membranes were blocked with 5% skim milk in PBS for 1.5 h at room temperature. The membranes were subsequently incubated with the aforementioned primary antibodies overnight at 4°C. Following this incubation, the membrane was washed three times in PBS and incubated in goat anti-rabbit second antibody (1:5,000; cat. no. ab205718; Abcam) solution at room temperature for 2 h. Anti-GAPDH antibody was used as an internal reference. Specific immune complexes were detected using the Western Blotting Plus Chemiluminescence Reagent (Thermo Fisher Scientific, Inc.). Band intensity was quantified via densitometry analysis using Image-Pro Plus version 4.5 software (Media Cybernetics, Inc.).

**Statistical analysis.** Data are presented as the mean ± standard deviation from three independent experiments. Quantitative results were analyzed via one-way ANOVA assay followed by

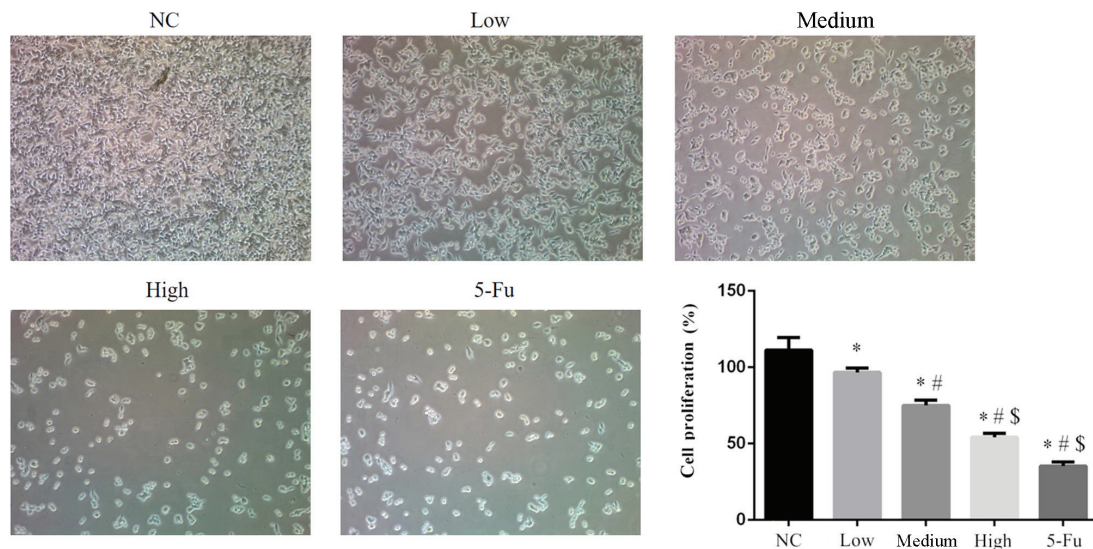


Figure 1. Cell proliferation rate among different groups (magnification, x100). \*P<0.05 vs. the NC group; #P<0.05 vs. the low group; \$P<0.05 vs. the medium group. NC, no-treatment control group; low, group treated with 10  $\mu$ g/ml GA; medium, group treated with 50  $\mu$ g/ml GA; high, group treated with 100  $\mu$ g/ml GA; 5-Fu, group treated with 10  $\mu$ g/ml 5-fluorouracil; GA, gambogic acid.

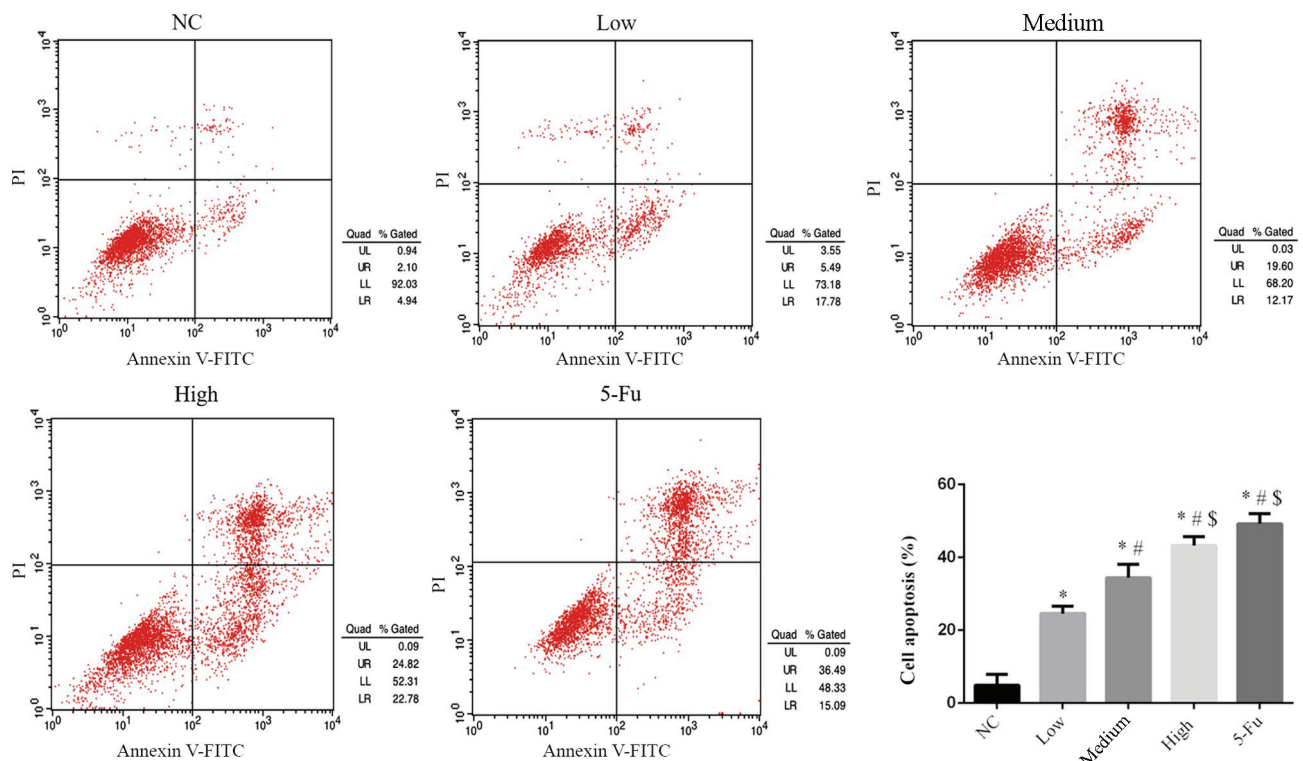


Figure 2. Cell apoptosis rate of different groups. \*P<0.05 vs. the NC group; #P<0.05 vs. the low group; \$P<0.05 vs. the medium group. NC, no-treatment control group; low, group treated with 10  $\mu$ g/ml GA; medium, group treated with 50  $\mu$ g/ml GA; high, group treated with 100  $\mu$ g/ml GA; 5-Fu, group treated with 10  $\mu$ g/ml 5-fluorouracil; GA, gambogic acid.

a Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**GA suppresses cell proliferation.** Cell proliferation rates of the GA-treated groups were significantly suppressed compared with the NC group (all P<0.05). The proliferation rate of 5-Fu

treated cells was significantly suppressed when compared with the NC, low and medium groups (each, P<0.05). Furthermore, significant differences in proliferation rates were identified between the GA-treated groups (low, medium and high; all P<0.05; Fig. 1).

**GA influences cell apoptosis rates.** Compared with the NC group, apoptosis rates in all GA-treated groups were



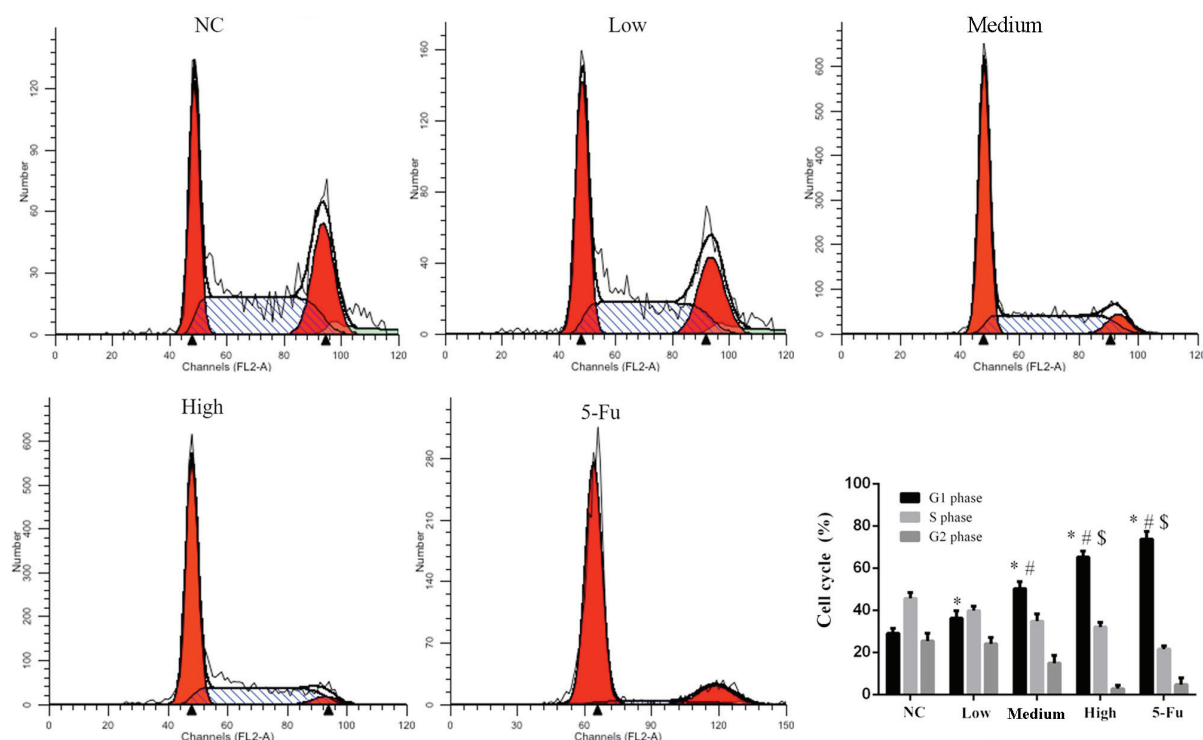


Figure 3. Cell cycle of different groups. \* $P < 0.05$  vs. the NC group; # $P < 0.05$  vs. the low group; \$ $P < 0.05$  vs. the medium group. NC, no-treatment control group; low, group treated with 10  $\mu\text{g/ml}$  GA; medium, group treated with 50  $\mu\text{g/ml}$  GA; high, group treated with 100  $\mu\text{g/ml}$  GA; 5-Fu, group treated with 10  $\mu\text{g/ml}$  5-fluorouracil; GA, gambogic acid.

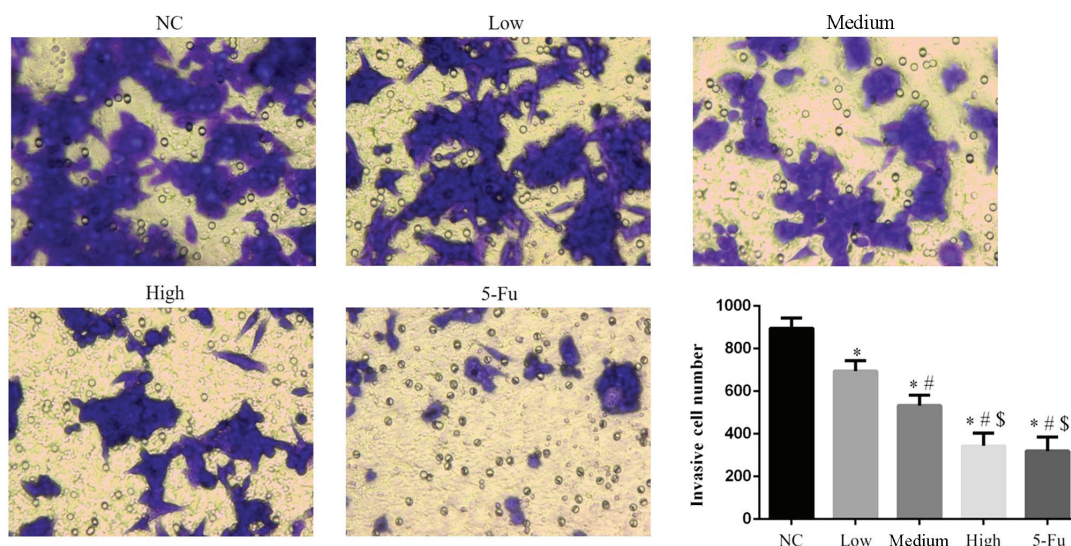


Figure 4. Invasive cell number determined in different groups using transwell assay (magnification, x200). \* $P < 0.05$  vs. the NC group; # $P < 0.05$  vs. the low group; \$ $P < 0.05$  vs. the medium group. NC, no-treatment control group; low, group treated with 10  $\mu\text{g/ml}$  GA; medium, group treated with 50  $\mu\text{g/ml}$  GA; high, group treated with 100  $\mu\text{g/ml}$  GA; 5-Fu, group treated with 10  $\mu\text{g/ml}$  5-fluorouracil; GA, gambogic acid.

significantly upregulated ( $P < 0.05$ ; Fig. 2). The rates of apoptosis increased in a dose-dependent manner. The apoptosis rate of the 5-Fu group was significantly upregulated compared with the NC, low and medium groups (each,  $P < 0.05$ ; Fig. 2).

**GA affects the cell cycle.** A significantly increased number of G1-phase cells was observed among GA treated groups compared with the NC group (all  $P < 0.05$ ; Fig. 3). The

number of 5-Fu treated cells in the G1 phase were significantly increased compared with the NC, low and medium groups (each,  $P < 0.05$ ; Fig. 3). The increase in the number of G1-phase cells was dose dependent and the differences between GA-treated groups were statistically significant ( $P < 0.05$ ).

**GA suppresses cell invasion.** Counts of invasive cells were significantly lower in all GA groups compared with the NC

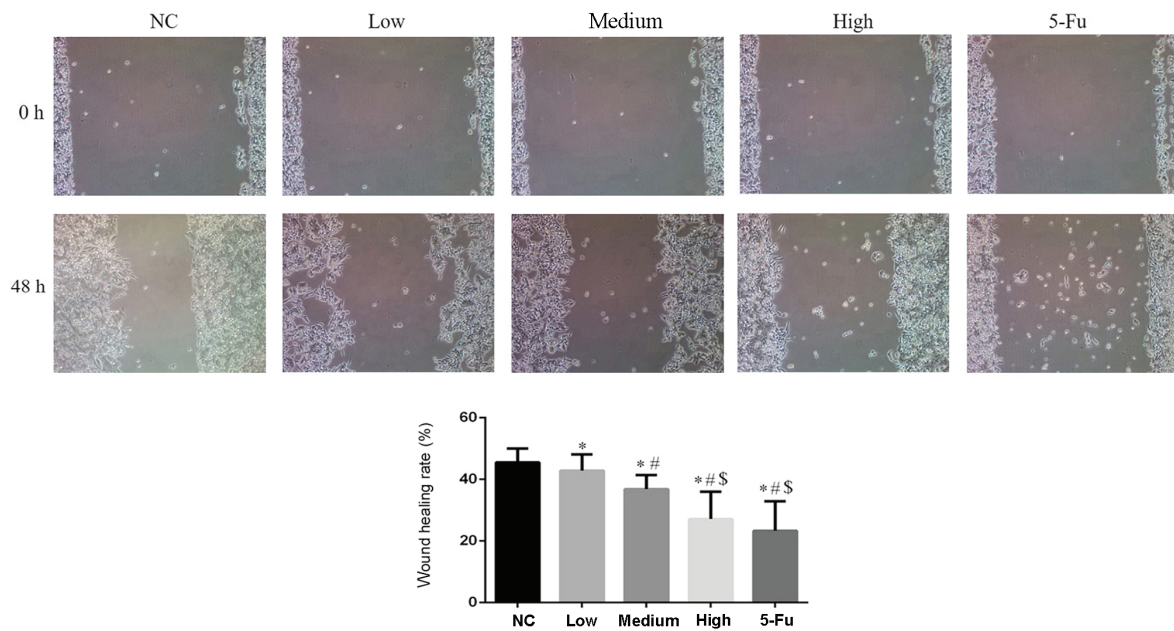


Figure 5. Wound healing rate of different groups. \*P<0.05 vs. the NC group; #P<0.05 vs. the low group; S P<0.05 vs. the medium group. NC, no-treatment control group; low, group treated with 10  $\mu$ g/ml GA; medium, group treated with 50  $\mu$ g/ml GA; high, group treated with 100  $\mu$ g/ml GA; 5-Fu, group treated with 10  $\mu$ g/ml 5-fluorouracil; GA, gambogic acid.

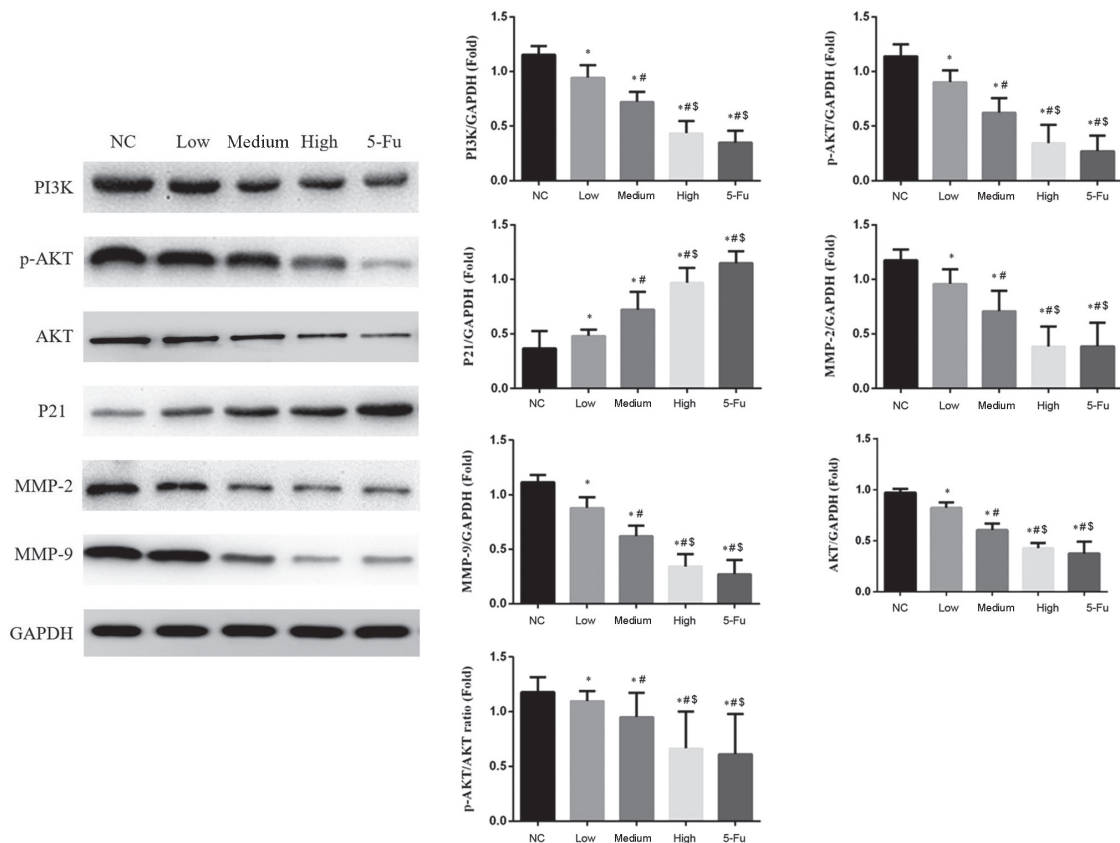


Figure 6. Relative protein expression levels in different groups. \*P<0.05 vs. the NC group; #P<0.05 vs. the low group; S P<0.05 vs. the medium group. NC, no-treatment control group; low, group treated with 10  $\mu$ g/ml GA; medium, group treated with 50  $\mu$ g/ml GA; high, group treated with 100  $\mu$ g/ml GA; 5-Fu, group treated with 10  $\mu$ g/ml 5-fluorouracil; GA, gambogic acid; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; p-, phosphorylated; MMP, matrix metalloprotease.

group (all P<0.05; Fig. 4). The number of invasive cells in the 5-Fu group were significantly reduced when compared with the

NC, low and medium groups (each, P<0.05). Significant differences were also observed between GA-treated groups (P<0.05).

**GA affects cell migration.** The wound healing rates of GA-treated groups were significantly inhibited compared with the NC group ( $P < 0.05$ ; Fig. 5). The wound healing rate of the 5-Fu group was significantly reduced compared with the NC, low and medium groups. This effect was dose-dependent.

**GA affects the PI3K/AKT pathway protein expression.** Compared with the NC group, protein expression levels of PI3K, AKT, p-AKT, MMP-2 and -9 significantly decreased, and the expression levels of P21 significantly increased following treatment with GA at low, medium and high doses ( $P < 0.05$ ; Fig. 6). Furthermore, the p-AKT/AKT ratio decreased in all GA-treated groups compared with the NC group and this effect was dose-dependent ( $P < 0.05$ ).

## Discussion

GA is the main active ingredient in gamboge. In Traditional Chinese Medicine, GA is used to treat ulcers, swelling and poisoning (15,16). Previous studies have revealed antitumor effects of GA, including induction of apoptosis, and inhibition of proliferation and pro-neoplastic protein expression (17-20). GA was determined to selectively kill cancer cells without affecting normal hematopoietic cells (21). Previous studies revealed that GA exhibited antitumor effects in colon cancer, however, the underlying mechanism of action remains unclear (22,23). In the present study, GA exhibited antitumor effects by inhibiting cell proliferation, invasion and migration and increasing apoptosis. Furthermore, GA arrested cells in the G1 phase of the cell cycle. At present, 5-Fu is the preferred drug for the clinical treatment of tumors. Therefore, 5-Fu was used as a positive control in the present study.

The PI3K/AKT signaling pathway is activated in the majority of tumors (24,25), which further enhances tumor-associated cell activity, including proliferation, invasion and migration (25). In the current study, GA inhibited the expression of PI3K, AKT and p-AKT in human colon cancer SW620 cells. Furthermore, the ratio of p-AKT to AKT decreased significantly following treatment with GA indicating that inhibition of the AKT-associated signaling may mediate the antitumor activity of GA. P21 is an important protein, which is downstream of AKT, that serves as a suppressor of tumor development (26) and can prevent cells from exiting the G1 phase (27-29). In the current study, following treatment with GA, the protein expression of P21 increased along with a dose-dependent increase in the number of cells in the G1 phase of the cell cycle.

Migration of cancer cells from the primary tumor and subsequent invasion through the tissue barrier is key to metastasis (30,31). Extracellular matrix and basement membranes are a natural barrier in the process of tumor infiltration and diffusion, and MMPs that degrade the matrix are important for promoting tumor invasion and metastasis (32,33). Previous studies indicated that MMP-2 and -9 increase cancer cell invasion and migration and are the downstream gene targets in the PI3K/AKT pathway (34,35). The present results suggest that GA suppressed SW620 cell invasion and migration, and regulated the MMP-2 and -9 protein expression via downregulation of the PI3K/AKT signaling pathway. GA appeared to suppress SW620 cancer cell pro-neoplastic and pro-metastatic

activities, including proliferation, invasion and migration, by altering the PI3K/AKT/P21 signaling and MMP-2 and -9 activity *in vitro*.

The current study was limited by the use of a single cell line and lack of *in vivo* experiments. In future studies, the effects of GA should be investigated in different colon cancer cell lines and *in vivo*.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

ZZ designed the current study, acquired/analyzed the data and drafted the manuscript. JM designed the current study, acquired the data and revised the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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