

Gambogic Acid Induces Apoptosis and Regulates Expressions of Bax and Bcl-2 Protein in Human Gastric Carcinoma MGC-803 Cells

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The selective induction of apoptosis of gambogic acid (GA) on MGC-803 cells and its probable molecular mechanism were studied. GA greatly inhibited (24, 48, 72 h) the growth of MGC-803 cells (by MTT); the IC₅₀ value was 0.96 μ g/ml at 48 h. Meanwhile, no influence was observed on body weight, number of WBC (white blood cells) in blood or karyote in marrow of rats after GA was injected intravenously. We conclude that GA does not affect normal cells, but that it can induce apoptosis in tumor cells selectively and there were marked morphological changes. A great quantity of apoptotic cells and increasing G₂/M phase cells were observed by flow cytometry, and a significant percentage of early apoptotic cells were observed by Annexin-V/PI double staining assay. The increase of bax gene and the decrease of bcl-2 gene expressions were detected by immunohistochemistry. Activation of bax and suppression of bcl-2 may contribute to the apoptosis mechanism.

Key words gambogic acid; MGC-803; apoptosis; inhibition rate; bax; bcl-2

Gamboge is a dry resin secreted from *garcinia hanburryi*, and GA (C₃₈H₄₄O₈, mol.wt 628)¹⁾ is the main active compound of Gamboge. It is reported in traditional Chinese medicine that GA is cold, acidic, acerbic, and poisonous. GA also has effects of detoxification, hemostasis, and as a parasiticide. Early investigations on GA from the 1960s were mainly on its separation and evaluation of its structure. In the 1980s, the structure, anti-tumor activity *in vitro*, absorption, distribution and excretion of GA in mouse were studied,²⁾ but the crude ethanolic extraction was used, and there were many other compounds in the extraction, including organic acids, terpene and alkaloids.³⁾ Later a Cooperative Gamboge Anti-tumor Investigation (CGAI) separated and identified the chemical structure of GA, studied antitumor activity of its crude extract in ethanol *in vitro* and *in vivo*, as well as its absorption, distribution and excretion in mice.^{4,5)} The antitumor activity and toxicity of the crude extract was also researched clinically.^{2,6)} In the 1990s, Kong L. D.⁷⁾ of Nanjing Chinese Medicine University investigated its activity and components by various separations. In 1996, some Japanese scholars reported they had extracted 11 compounds from Gamboge. Recently some foreign scientists modified carboxylic to acidamide on GA to discover other active compounds. They applied for an American patent, which is the only one to date on GA.

Recently, researchers of China Pharmaceutical University applied a novel method to extract GA from Gamboge and the contents of GA in the extract were no less than 95% (the yield of extracts was about 1.5%).⁸⁾ Results from our present

study *in vitro* showed that GA had significant inhibitory effect on cultured human hepatoma SMMC-7721 cells and SGC-7901 cells, and could induce apoptosis in these tumor cells. Observations from our studies *in vivo* also indicated that GA exhibited inhibition on Heps, S₁₈₀, EAC and EC in a Kunming strain of mice, and on Lewis lung carcinoma in C57BL/6 mice. This paper was undertaken to evaluate this inhibition and apoptosis induction of MGC-803 cells, and the probable molecular mechanism.

MATERIALS AND METHODS

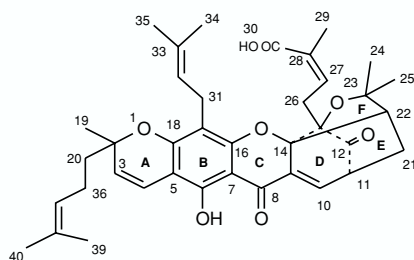
Medicine GA, supplied by the School of Pharmacy in China Pharmaceutical University (Norms: 20 mg/bottle). The sample was dissolved in RPMI-1640 medium (GIBCO, U.S.A.).

Animals SD rats (weighing 120—130 g), SPF, were made up of equal number of each sex. They were from the animal center of China Pharmaceutical University, maintained on a standard pellet diet with free access to water, and housed in an air-conditioned room at 24 \pm 2 °C with lighting from 8:00 to 20:00.

Tumor Cells MGC-803, supplied by the Cell Bank of Shanghai Institute of Cell Biology, were maintained in RPMI-1640 medium supplemented with 10% heated-inactivated calf serum (Sijiqing, Hangzhou, China), benzylpenicillin 100 KIU/l, and streptomycin 100 mg/l, pH 7.4 in an incubator (HIRASAWA, Japan) with a humidified atmosphere of 95% air + 5% CO₂ at 37 °C.

Reagents Calf serum (product of Sijiqing Biology Engineering Company, Hangzhou province) after a water bath at 56 °C, inactivated for 30 min and kept at -20 °C; penicillin and streptomycin, supplied by Lukang Pharmaceutical Company Ltd., Shandong, batch no. 20000628; Culture medium, RPMI-1640 culture powder, product of GIBCO Company (U.S.A.), dissolved in tri-stilling water, plus 10% calf serum and 100 U/ml of penicillin and streptomycin, respectively, and held at 4 °C. MTT (product of Fluka) was dissolved in 0.01 M PBS.

Changes of Body Weight, Number of WBC in Blood



Molecular structure of GA

and Karyotes in Marrow after Injection of GA into the Veins of Healthy Rats Forty rats were divided into 4 groups, 10 per group, 5 were male and 5 were female. The NS group was negative control and the GA group: 3 doses (6, 3, 1.5 mg/kg) of medication on alternate days, 4 times in all. The day following the 4th medication, blood was drawn from the eye orbit, and WBC counted. To cut one femur, all of the myelocytes were washed away with 10 ml 3% acetic acid solution and WBC counted.

Cell Growth Inhibition By colorimetric MTT-assay, the logarithmic cells were dispersed with 0.02% EDTA to prepare a 3×10^4 /ml cell suspension, and partitioned into wells of 40-well plates at 100 μ l/well for 4 h culture in a 5% CO₂ incubator under 37 °C. The cell culture wells were then exposed to different concentrations GA (100 μ l/well). After 24, 48 and 72 h culture, 5 mg/ml MTT solution (20 μ l/well) was added, cultured for 4 h, the supernatant was discarded and DMSO were added (100 μ l/well) in. The suspension was placed on micro-vibrator for 5 min and the absorbance (A) was measured at λ 570 nm by an enzyme immunoassay instrument (DJ-3200, Huadong Electron Tube Company). Cell inhibitory ratio was calculated by the following formula:

$$\text{inhibitory ratio (\%)} = (1 - \text{average absorbance of treated group} / \text{average absorbance of control group}) \times 100\%$$

IC₅₀ was calculated by SAS statistical software.

Cell Morphological Assessment Exponentially grown cell were dispersed with 0.02% EDTA to prepare a 1×10^6 /ml cell suspension. They were exposed to GA 1 μ g/ml for 48 h, collected, fixed in 3% glutaraldehyde, washed with 0.01 M PBS buffer, dehydrated in alcohol, embedded in EPOR812, stained with acetic acid uranium and citromalic acid and observed by electron microscope and inverted-microscope (Olympus, Japan).

DNA Content and Cell Cycle Analyzed by Flow Cytometry The logarithmic cells were dispersed with 0.02% EDTA to prepare the 1×10^6 /ml cell suspension. We added a treated group with GA 1 μ g/ml and used no medicine in the control group. Cells were collected after 24, 48 and 72 h culture, washed with 0.01 M PBS buffer, fixed in 70% alcohol, treated with 1% RNase A and stained with propidium iodide (PI) (GIBCO) before DNA content was determined. DNA content and cell cycle were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, U.S.A.), using the software MODFIT and CELLQUEST.

Assessment of Apoptosis by Annexin-V/PI Double-Staining Assay 1×10^6 cells were washed and resuspended with PBS. Apoptotic cells were identified by double supravital staining with recombinant FITC (fluorescein isoth-

iocyanate)-conjugated Annexin-V and PI, using the Annexin V-FITC Apoptosis Detection kit (Becton Dickinson, U.S.A.) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after supravital staining. Data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using CellQuest software.

Expressions of Bcl-2 Protein and Bax Protein 1×10^6 /ml cells were incubated with GA 1 μ g/ml for 48 h, washed with cold PBS buffer, collected, smeared, fixed with acetone at 4 °C, and incubated with anti-Bax protein mouse monoclonal antibody and anti-Bcl-2 protein mouse monoclonal antibody, respectively, for 60 min at room temperature or overnight at 4 °C. These cells then incubated with biotinylated goat antimouse IgG for 60 min at 37 °C, and treated with avidin-biotin complex for 60 min at room temperature. After being detected by DAB, they were stained again with lignins, mounted, and examined by electron microscope. Positive judgment: plasma and membrane were marked with a brown or coffee colour, \pm , +, ++, +++ represented 1—10%, 11—30%, 31—50%, >51% positive cells in 200 cells under a microscope.

RESULTS

Changes in Body Weight, Numbers of WBC in Blood and Karyotes in Marrow after Injecting GA Intravenously into Normal Rats As shown in Table 1, compared with negative control, GA (6, 3, 1.5 mg/kg) had no influence on any of these factors.

Cell Growth-Inhibitory Effect A dose-dependent inhibition of GA on the MGC-803 cell proliferation was found

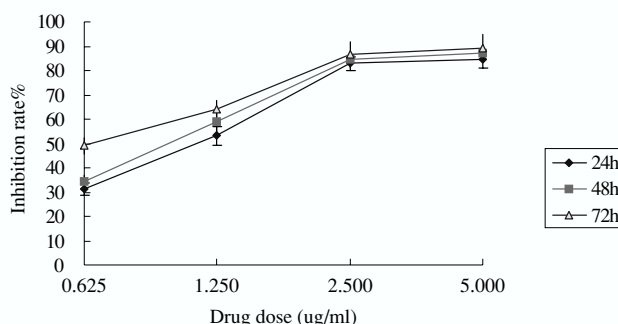


Fig. 1. Inhibitory Effect of GA on Proliferation in MGC-803 Cells

Incubating with GA for 48 h, MGC-803 cell proliferation was inhibited in a dose-dependent manner. Inhibitory ratio reached 89.4% at 72 h, 87.3% at 48 h and 84.4% at 24 h by 5 μ g/ml GA. At 48 h, the inhibitory ratio was 34.5, 59.1, 84.5, and 87.3% by 0.625, 1.25, 2.5, and 5 μ g/ml GA respectively. Data shown are as means \pm S.D. of five experiments.

Table 1. The Body Weight and Number of WBC in Blood and Karyotes in Marrow after Injecting GA Intravenously into Normal Rats

Group	Dosage (mg/kg)	Weight (g)		WBC (10^9 /l)	Karyote ($\times 10^4$)
		Pre-medication	Post-medication		
Control		125.4 \pm 5.38	149.6 \pm 10.13	8.52 \pm 1.58	8528 \pm 2540.17
GA	6	121.3 \pm 12.66	145.3 \pm 11.96	6.84 \pm 1.51	7390 \pm 1830.08
	3	121.0 \pm 9.67	145.0 \pm 12.31	7.28 \pm 1.43	7906 \pm 2129.04
	1.5	124.5 \pm 9.90	147.8 \pm 13.31	7.34 \pm 1.17	6930 \pm 2906.72

After rats were injected with GA (6, 3, 1.5 mg/kg) 4 times, we found no influence on body weight, WBC number in blood or karyotes in marrow compared with negative control. Results = $\bar{X} \pm$ S.D. ($n=10$). $p < 0.01$, vs. control.

for 24, 48 and 72 h. IC_{50} at 48 h was $0.96 \mu\text{g/ml}$ (Fig. 1).

Cell Morphological Assessment Under an inverted-microscope, after cultured with GA $1 \mu\text{g/ml}$ for 48 h, many MGC-803 cells turned round in shape and necrosed; the untreated cells grew well and the skeleton was clear (Fig. 2).

Under electron microscope, after being cultured with GA $1 \mu\text{g/ml}$ for 48 h, “dotted” chromatins were found; in a large quantity of tumor cells these condensed chromatin divided into “Apoptosis bodies” (Fig. 3).

DNA Content and Cell Cycle Analyzed by Flow Cytometry After exposure of MGC-803 cells to GA ($1 \mu\text{g/ml}$) for 24, 48, and 72 h, the apoptosis rate was 38.56, 73.70, and 71.77%, respectively, statistically different from the control, which was 23.27, 20.49, and 18.97%, respectively (Table 2).

The proportion of G_2/M phase cells increased after being treated with GA ($1 \mu\text{g/ml}$) (Fig. 4).

Apoptosis Assessment by Annexin-V and PI Double-Staining Assay For each sample, viable cells, necrotic cells and early apoptotic cells were obtained separately by Annexin-V and PI double-staining assay. The early apoptotic cells were divided from the necrotic cells (including late apoptotic cells) and the viable cells. Those labeled with Annexin-V⁺/PI⁻ cells were early apoptotic cells. The percentage increased from 0.98% (control) to 7.83% (24 h), 11.95% (48 h) and 21.81% (72 h) in a time-dependent manner (Figs. 5, 6).

Expressions of Bcl-2 Protein and Bax Protein Fixed Quality: After incubating with GA ($1 \mu\text{g/ml}$) for 48 h, more

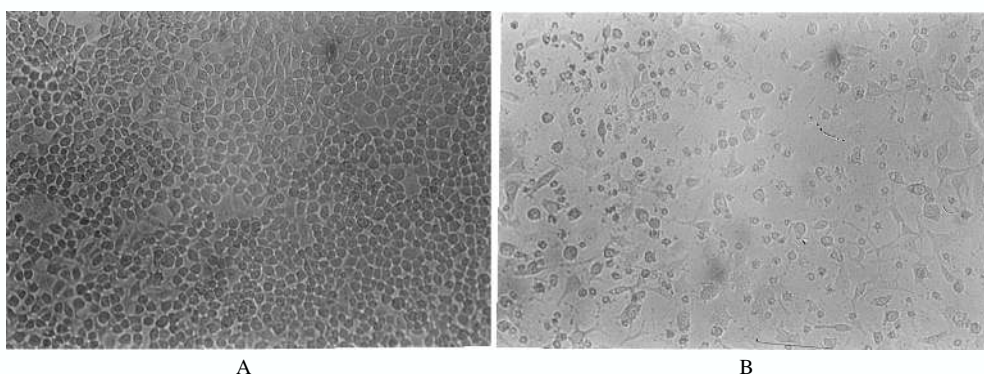


Fig. 2. Cell Morphological Changes under Inverted-Microscope (×100)

(A) Control; (B) MGC-803 cell treated with GA ($1 \mu\text{g/ml}$) for 48 h.

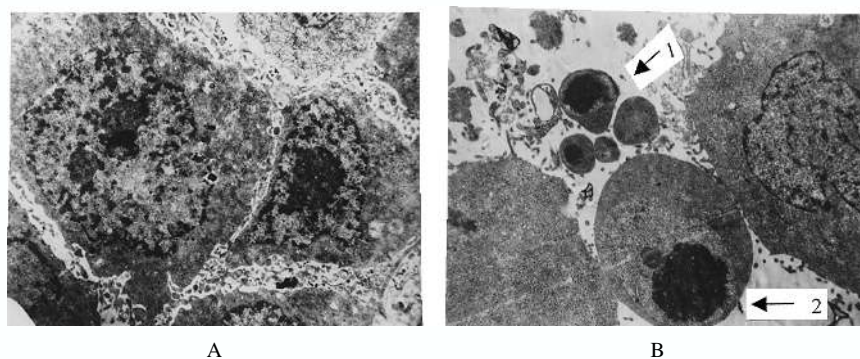


Fig. 3. Cell Morphological Changes under Electronmicroscope (×7500)

(A) Control; (B) MGC-803 cell treated with GA ($1 \mu\text{g/ml}$) for 48 h. Cells were changed morphologically after $1 \mu\text{g/ml}$ GA for 48 h was added. Under inverted-microscope, many cells were floating and separating from each other. Under electronmicroscope, some apoptotic bodies were found, some chromatins condensed, and the villus on cell membrane disappeared. Arrows 1 and 2 indicate apoptotic bodies and chromatin condensation, respectively.

Table 2. DNA Content and Cell Cycle with GA ($1 \mu\text{g/ml}$)

Group	Cell cycle			Apoptosis (%)
	G_0/G_1 (%)	S (%)	G_2/M (%)	
24 (control)	41.23 ± 4.52	32.03 ± 2.51	25.21 ± 2.97	23.27 ± 3.03
24 (treated)	43.58 ± 3.55	27.13 ± 4.14	$33.85 \pm 2.93^*$	$38.56 \pm 3.94^{**}$
48 (control)	57.45 ± 4.98	26.11 ± 3.52	17.85 ± 3.44	20.49 ± 3.01
48 (treated)	55.06 ± 5.08	25.89 ± 4.36	$21.44 \pm 4.58^*$	$73.70 \pm 6.12^{**}$
72 (control)	45.33 ± 6.55	39.32 ± 4.85	15.35 ± 2.15	18.97 ± 2.54
72 (treated)	45.06 ± 7.24	38.49 ± 3.12	16.45 ± 3.85	$71.77 \pm 7.56^{**}$

Apoptosis increased after MGC-803 cells were incubated with GA ($1 \mu\text{g/ml}$) for 24, 48, and 72 h. The apoptosis of the treated cells was 38.56% at 24 h, 73.70% at 48 h, and 71.77% at 72 h, compared with the control, which was significantly different in the *t*-test. Meanwhile, we found that GA also affected the amount of cells in different phases, but there was no statistical difference. Results = $\bar{X} \pm S.D.$ ($n=5$) $p < 0.01$, $0.01 < p < 0.05$, vs. control.

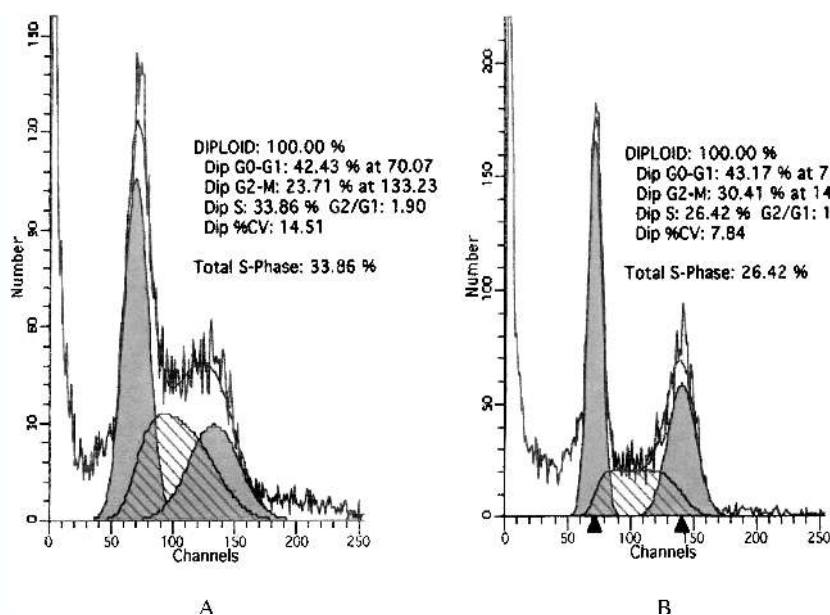


Fig. 4. Cell Cycle Treated with GA (1 μ g/ml) for 24 h

(A) Control; (B) treated with GA (1 μ g/ml). After incubation with GA (1 μ g/ml) for 24 h, the proportions of cells were 26.42% in S phase and 30.41% in G₂/M phase, while those of the control cells were 33.86% and 23.71%, respectively. Note that there is a comparatively distinct difference in G₂/M phase.

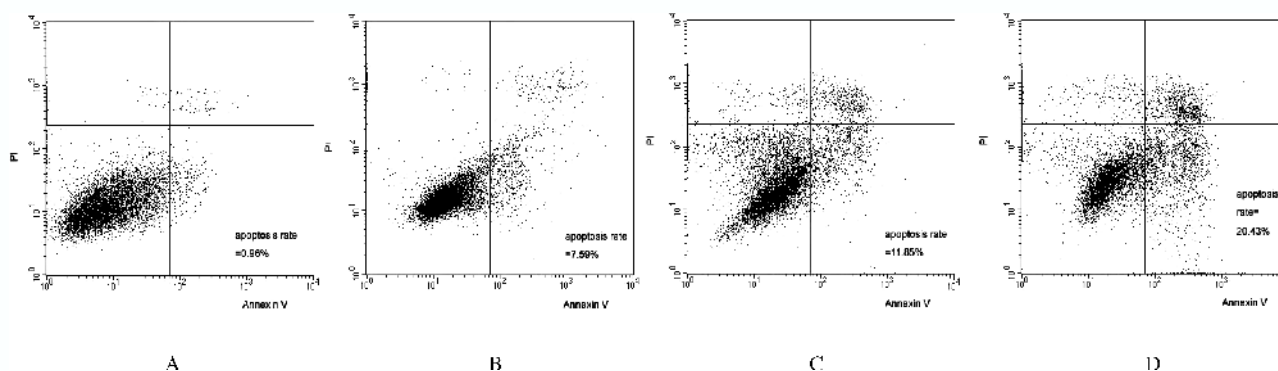


Fig. 5. Fluorescence-Activated Cell Sorter Analysis for Annexin-V and PI Staining of MGC-803 Cells Incubated with GA (1 μ g/ml)

(A) Control; (B) 24 h; (C) 48 h; (D) 72 h. UR (upright): necrotic cells and late apoptotic cells labeled with PI and Annexin V-FITC. LL (lower left): fully viable cells. LR (lower right): early apoptotic cells labeled with Annexin V-FITC but not with PI. After exposure to GA, the cells of LR had increased from 0.96% to 7.59% (24 h), 11.85% (48 h), 20.43% (72 h) in a time-dependent manner.

coffee color in plasma and cell membrane of Bax protein was found (Fig. 7).

Half-Fixed Quantity: In untreated cells, expression of Bcl-2 was (++), Bax was (\pm); after incubating with GA (1 μ g/ml) for 48 h, Bcl-2 became \pm , and Bax became +++ (Table 3).

DISCUSSION

The results of our finding demonstrated GA can inhibit the proliferation of human gastric carcinoma MGC-803 cells *in vitro* in a dose-dependent manner. The inhibition rate reached 89.45% when the cells were exposed to GA 5 μ g/ml for 72 h. The value of IC₅₀ at 48 h was 0.96 μ g/ml. At the same time, our experiments showed that GA did not affect body weight, WBC number in blood or karyotes in marrow in rats. We reached the conclusion that GA can't induce cell death in normal unimmortalized cells, but it can kill tumor cells selectively. MGC-803 cells incubated with GA underwent

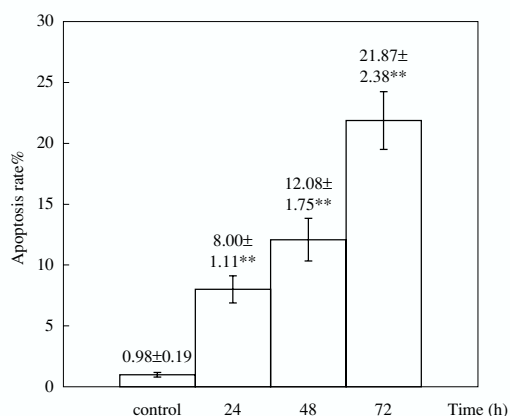


Fig. 6. Apoptosis Rate of MGC-803 Cells Induced by GA (1 μ g/ml) in Annexin V/PI Assay

The percentage of early apoptotic cells (Annexin V⁺/PI⁻) after exposure to GA (1 μ g/ml) was significantly different from the untreated group. The apoptotic cells were increased from 0.98% (control) to 7.83% (24 h), 11.95% (48 h) and 21.81% (72 h). The results = $\bar{X} \pm S.D.$ ($n=3$). represent $p < 0.01$ vs. control.

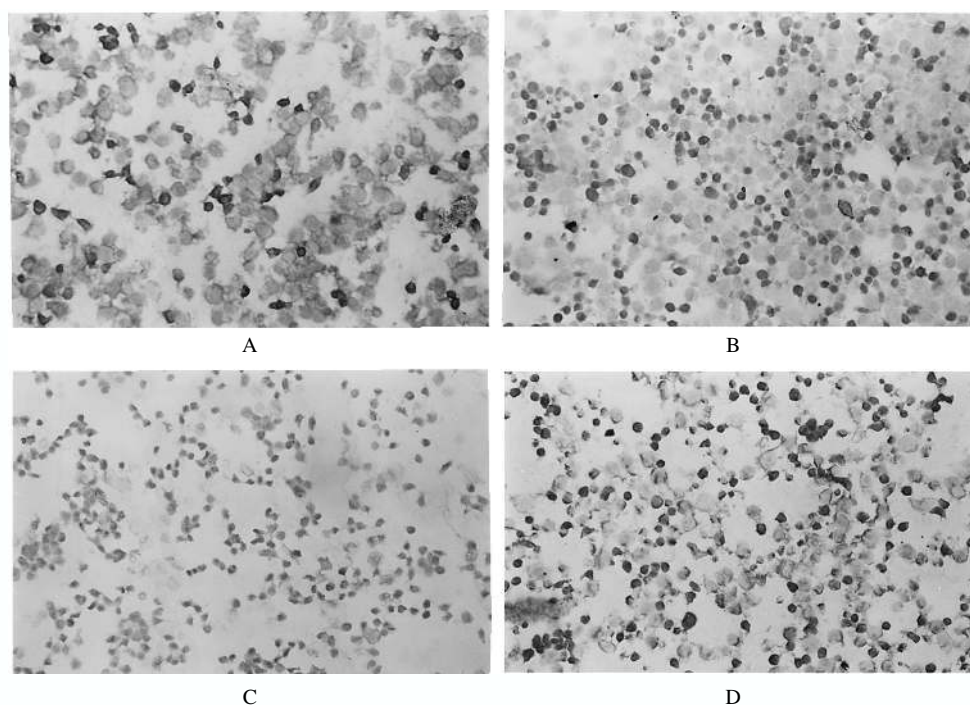


Fig. 7. Expressions of Bcl-2 and Bax Protein Incubated with GA ($1 \mu\text{g/ml}$) for 48 h

(A) Bcl-2 (control); (B) Bcl-2 (treated); (C) Bax (control); (D) Bax (treated).

Table 3. Effect of $1 \mu\text{g/ml}$ GA on Expressions of Bcl-2 and Bax in MGC-803 Cell ($n=3$)

Group	Bcl-2			Bax		
	1	2	3	1	2	3
Control	++	+	++	±	±	±
Treated	±	±	±	+++	+++	+++

Cultured with GA ($1 \mu\text{g/ml}$), less plasma and cell membrane of proteins (expressions of the Bcl-2) were dyed on coffee color, and more of proteins (expressions of the Bax), compared with the control. GA reduced the expressions of Bcl-2 to 1–10%, and increased the expressions of Bax to over 51%.

apoptosis with typically apoptotic characteristics including ultrastructural changes of cytoplasm condensation, nucleus disruption and apoptotic body formation. The results of flow cytometry confirmed the occurrence of apoptosis and the increased proportion of G_2/M phase cells in MGC-803 treated with GA. Phosphatidylserine (PS), normally present in the inner cytoplasmic leaflet of the plasma membrane of healthy cells, is translocated and exposed on the outer leaflet at the beginning of apoptosis.⁹⁾ Annexin-V has a high affinity for PS and binds to cells with exposed PS. When conjugated to FITC fluorochrome Annexin-V retains its high affinity for PS and, therefore, serves as a sensitive probe that can be used for flow cytometric detection of cell death (apoptotic or necrotic) characterized by the loss of membrane asymmetry. Use of Annexin-V in combination with PI allows the detection of early apoptotic and necrotic cells distinct from viable cells. Our results proved GA did induce MGC-803 cell apoptosis in a time-dependent manner. The apoptotic rate reached 21.87% after incubation for 72 h. A decrease of Bcl-2 protein and increase of Bax protein expressions were detected. All of these showed that GA could inhibit tumor cell growth contributing to the induction of cell apoptosis. The molecular

mechanism might be related to the reduction of expression of apoptosis-regulated gene bcl-2, and the improvement of the expression of apoptosis-regulated gene bax.

Apoptosis is an important metabolic step in regulating the number of cells and their growth. If it is blocked, the metabolism will become disordered and tumors will develop and grow.¹⁰⁾ Whether or not tumor cell apoptosis is induced has become a chief factor in an anti-tumor medicine. Apoptosis is a complex procedure involving many pathways. Recent evidence suggests there are two kinds of genes which control apoptosis: the active genes WTP53, E1A, ced3 and ced4, Fas, and the suppressive genes mutant p53, bcl-2, c-fos and c-myc.^{11,12)} Bax and bcl-2 both belong to the bcl-2 family.¹³⁾ Overexpression of Bax has been shown to accelerate cell death,^{14,15)} while overexpression of antiapoptotic proteins such as Bcl-2 represses the death function of Bax.¹⁶⁾ Thus, the ratio of Bcl-2/Bax might be one a critical factor of a cell's threshold for undergoing apoptosis.¹⁷⁾ We deduce that Bax leads to the release of cytochrome c which is restrained by binding to Bcl-2. The activation of cytochrome c is suppressed, and subsequently apoptosis occurs.^{18,19)} The high apoptosis rate of MGC-803 treated with GA was shown in our study, and lowering the ratio of Bcl-2/Bax might be one of its molecular mechanisms. Besides bax and bcl-2, whether or not GA affects another genes, how it might affect them, and which other mechanisms contribute to changes in the cell cycle, should be explored in our future research.

At present, the majority of anti-tumor medicines have serious side effects accompanying their therapeutic effects. So traditional Chinese medicine with high activity and low toxicity should be developed. GA is different from other anti-tumor drugs, for it can induce tumor cell death selectively, which offers a unique prospect in the development of new anti-tumor medicine, and also inspires new anti-tumor treat-

ment. GA is promising and so should have a significant influence.

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