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Original Research Article

Studies on Anti-Hepatoma Effect of Gan-Ai-Xiao Decoction

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Abstract

Purpose: *To explore the anti-hepatoma effect of Gan-Ai-Xiao Decoction (GAXD), a folk remedy.*
Methods: *High performance liquid chromatography (HPLC) was used to identify the major chemical components of GAXD ethanol extract (EE). The cytotoxic effect of GAXD EE against HepG2 cells was measured by methyl thiazolyl tetrazolium (MTT) assay. Flow cytometry and Western blot were used to study the effect of GAXD EE on apoptosis and apoptotic proteins (Bcl-2, Bax and caspase-3) in HepG2 cells. Xenograft assay was used to evaluate the anti-hepatoma effect of GAXD EE in vivo.*
Results: *Four components were identified in GAXD EE by HPLC. The results of MTT and flow cytometry assays indicated that GAXD EE significantly reduced HepG2 cells viability ($p < 0.05$) and induced its apoptosis. The results of Western blot assay suggested that GAXD EE down-regulated the expression of anti-apoptotic protein (Bcl-2) and up-regulated the expression of pro-apoptotic proteins (Bax and caspase-3) in HepG2 cells. Furthermore, the results of xenograft assay showed that GAXD EE significantly inhibited the growth of HepG2 cells-induced tumor ($p < 0.05$) without any effect on the body weight of nude mice.*
Conclusion: *GAXD has anti-hepatoma activity, and the mechanism is associated with apoptosis.*
Keywords: *Gan-Ai-Xiao Decoction, Anti-hepatoma, Flow cytometry, Western blot, Xenograft, HepG2 cells, Apoptosis*

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INTRODUCTION

The liver plays an important role in metabolism and has many functions in human life, such as detoxification, glycogen storage and plasma protein synthesis. However, patients with liver cancer are facing afunction of the liver. Nowadays, the people's lifestyles such as obesity [1], smoking [2] and alcohol drinking [3] enhance the incidence of hepatoma. Hepatoma is the second leading cause of cancer mortality worldwide [4] and a number of therapies have been used to decrease the mortality of patients with hepatoma, such as surgical treatment [5], interventional therapy [6-8], biotherapy [9], radiotherapy [10,11], chemotherapy [12], adjunctive traditional Chinese medicine (TCM) therapy [13,14]. According to clinical observation, adjunctive TCM therapy has unique advantages, such as stabilizing patient condition, low toxicity and side effect, improving clinical symptoms, prolonging patients' survival time and low cost.

GAXD, a folk medicine, is composed of *Stigma Maydis*, Kummerowia Herbal, *Gleditsiae Spina*, *Astragali Radix*, Poria, *Glycyrrhizae Radix* (Table 1). GAXD has been used to cure hepatoma with a long history in Southwestern China, such as Tianshui, Baoji and Bazhong, but there were not any study reports about its anti-hepatoma effect.

In this work, we identified the major chemical components and studied anti-hepatoma effect of GAXD by HPLC, MTT, flow cytometry, western blot and xenograft assays.

EXPERIMENTAL

Plant materials

Stigma Maydis, Kummerowia Herbal, *Gleditsiae Spina, Astragali Radix*, Poria and *Glycyrrhizae Radix* were collected from Sichuan, Henan, Shanxi and Jiangxi in 2010. Their original plants were identified by Department of Pharmacy, People Hospital of Cangzhou city, Cangzhou Hebei. All air-dried plant materials were used to form GAXD. A series of voucher specimen (2010022-2010027/PHCZCHB) were deposited in Department of Pharmacy, People Hospital of Cangzhou city, Cangzhou Hebei for future reference.

Chemicals and reagents

HPLC grade methanol was purchased from Fisher (Fisher Scientific, USA). Analytical grade DMSO, phosphoric acid and ethanol were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Ultra-pure water was obtained from Milli-Q Advantage A10 (Millipore Crop., MA, USA). Isoorientin, quercetin, luteolin and apigenin were purchased from Shanghai shifeng biological technology Co. ltd (Shanghai, China) and the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Bcl-2, Bax and caspase-3 monoclonal antibodies were obtained from Abcam Co. (Hong Kong, China). Goat anti-rabbit/HRP was obtained from Shanghai Hao Yang Biotechnology Co. (Shanghai, China).

Animals

Nude mice (5 - 6 weeks old) were purchased from the SLRC Laboratory Animal Company (Shanghai, China). All animal treatments were strictly in accordance with the international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals [15]. Experiments were carried out with the approval of the Animal Experimentation Ethics Committee of People Hospital of Cangzhou city, Cangzhou Hebei.

Preparation of GAXD ethanol extract

All crude drugs of GAXD were powdered together according to the composition shown in Table 1. The powder was extracted 3 times (6 h × 3) with 75 % ethanol at room temperature. Evaporation of the 75 % ethanol under reduced pressure provided the GAXD ethanol extract (EE) (13.6 g/100 g), which was diluted with 0.5 % DMSO to obtain the required concentrations for the various tests.

Identification of major chemical components of GAXD EE

The GAXD EE was dissolved in methanol to get appropriate concentrations for HPLC analysis. The identification of chemical components of GAXD EE was performed with Agilent Technologies 1100 system (Agilent Crop., MA, USA). The chromatography was carried out on an Eclipse XDB-C18 column (4.6 mm × 250 mm, 5 µm). The mobile phase was composed of methanol (solvent A) and 0.2 % phosphoric acid (solvent B): 0 - 20 min (20 – 45 %, A), 20 - 30 min (45 – 70 %, A), 30 - 38 min (70 – 100 %, A). The column temperature was 30 °C. The injection volume was 15 µL. The flow rate was 1 mL/min. The detection wavelength was 355 nm.

Cell culture

Human hepatoma cell line (HepG2) was obtained from American Type Culture Collection (Manassas, Virginia, VA, USA). The cells were cultured in RPMI-1640 medium supplemented with antibiotics (100 µg/mL streptomycin and 100 U/mL penicillin) and 10 % fetal bovine serum. The cells were cultured with a humid atmosphere of 5 % CO_2 and 95 % air at 37 °C. Then the logarithmic-growth phase cells were used to evaluate the anti-hepatoma effect of GAXD EE.

Table 1: Composition of *Gan-Ai-Xiao Decoction* (GAXD)

Plant	Family	Original plant	Weight (g)
Stigma Maydis	Poaceae	*Zea mays* L.	30
Kummerowia Herbal	Leguminosae	*Kummerowia striata* (Thunb.) Schindl	30
Gleditsiae Spina	Leguminosae	*Gleditsia sinensis* Lam	15
Astragali Radix	Leguminosae	*Astragalus membranaceus* (Fisch.) Bunge.	15
Poria	Polyporaceae	*Wolfiporia cocos* (Schw.) Ryv.&Gibn	10
Glycyrrhizae Radix	Leguminosae	*Glycyrrhiza uralensis* Fisch	5

MTT assay

HepG2 cells (1 × 10^4/0.2 mL) were seeded on 96-well plates and incubated for 24 h. Then cells were separately treated with GAXD EE (20, 40 and 80 µg/mL) and 0.05 % DMSO (control) for 48 h. Subsequently, MTT assay was performed using a standard protocol and the absorbance of both control (Ac) and treated (At) samples was measured at 490 nm in a microplate reader (Multiskan FC, Thermo, MA, USA). Inhibition was calculated as in Eq 1 [16]:

Inhibition (%) = {(Ac – At)/Ac}100 …… (1)

Apoptosis assay by flow cytometry

HepG2 cells, separately treated with GAXD EE (20, 40 and 80 µg/mL) and 0.05 % DMSO (control) for 48 h, were harvested by centrifugation. Then the cells were washed with cold phosphate buffered saline (PBS) twice, stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions. Subsequently, the stained cells were analyzed by flow cytometry on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blot analysis

After treatment with GAXD EE (20, 40 and 80 µg/mL) and 0.05 % DMSO (control) for 48 h, HepG2 cells were harvested and their total proteins were extracted. The concentration of total proteins was determined by the Enhanced BCA Protein Assay kit (Beyotime, Haimen, Chian). About 40 µg total proteins were separated by SDS/PAGE and blotted on PVDF membrane. Then PVDF membranes with target protein were probed with anti-Bcl-2, Bax and caspase-3 rabbit polyclonal IgG and subsequently with goat anti-rabbit/HRP, and detected by chemiluminescence. Antibody directed against β-actin was used to measure protein loading.

Xenograft assay

Nude mice (5 - 6 weeks old) were randomly divided into two groups, control and treatment groups (n = 8). HepG2 cells (2 × 10^6 cells per nude mouse) were injected in the right flank of mice subcutaneously. When the HepG2-induced tumors grew to about 2 - 3 mm in diameter, the control and treatment groups were separately treated with 0.05 % DMSO and GAXD EE (100 mg/kg) for 15 days by gavage. The tumor sizes (width and length) and body weight of nude mice were measured in 5th, 10th and 15th days by a vernier caliper. The tumor volumes were calculated as in Eq 2 [17]. At last, all animals were sacrificed by injecting chloral hydrate.

Volume = (width^2 × length)/2 …… (2)

Statistical analysis

All data are presented as mean ± standard deviation (SD). The difference between groups was analyzed by two-tailed Student's t-test in SPSS 21.0. When *p*-value was less than 0.05, the differences were considered as statistically significant.

RESULTS

Major chemical components of GAXD EE

As shown in Figure 1, four components, isoorientin, quercetin, luteolin and apigenin, were identified in GAXD EE according to standard substance by HPLC.

Cytotoxic effects of GAXD EE on HepG2 cells

The results of MTT assay indicated that GAXD EE (20, 40 and 80 µg/mL) significantly ($p < 0.05$) reduced the HepG2 cells viability in a dose-dependent manner, compared with control. The results are shown in Figure 2.

Effects of GAXD EE on apoptosis of HepG2 cells

As depicted in Figure 3, the results of flow cytometry assay indicated that GAXD EE (20, 40 and 80 µg/mL) induced the apoptosis of HepG2 cells in a dose-dependent manner.

Effects of GAXD EE on apoptotic proteins of HepG2 cells

The results of western blot assay suggested that GAXD EE (20, 40 and 80 µg/mL) obviously down-regulated the expression of anti-apoptotic protein (Bcl-2) and up-regulated the expression of pro-apoptotic proteins (Bax and caspase-3) in a dose-dependent manner. The results are shown in Figure 4.

Anti-tumor effects of GAXD EE on HepG2 cells-induced tumor

Xenograft assay of nude mice was used to evaluate the anti-tumor effect of GAXD EE on HepG2 cells-induced tumor in vivo. Tumor volume and body weight of nude mice were used

to evaluate the anti-tumor effect. As depicted in Figure 5, GAXD EE significantly ($p < 0.05$) inhibited the growth of HepG2 cells-induced tumor without any effect on the body weight of the mice, compared with control.

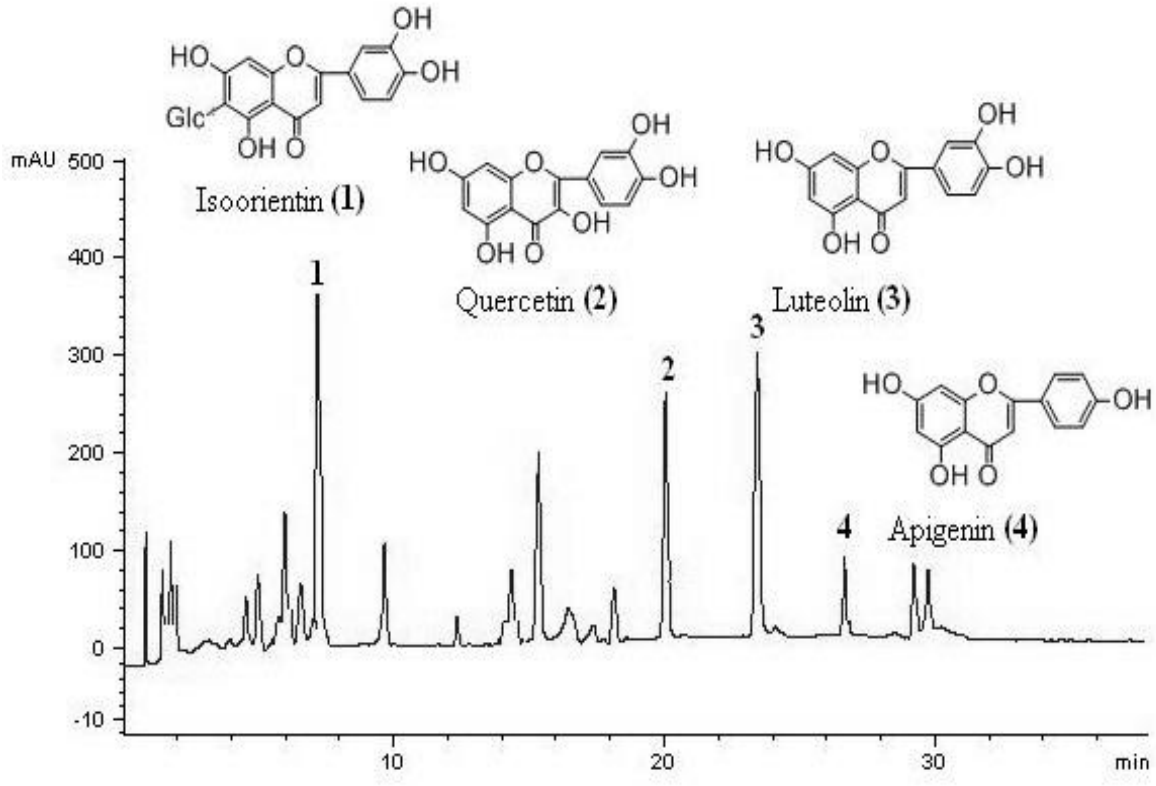

Figure 1: Chromatogram showing the major chemical components of GAXD ethanol extract

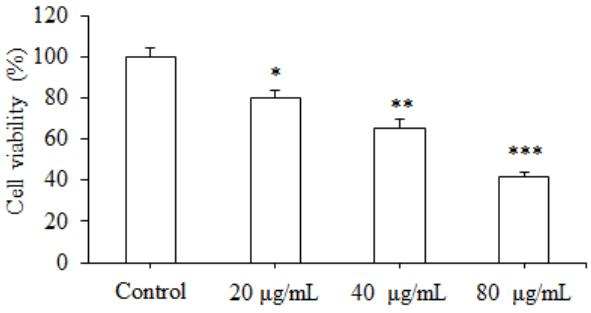

Figure 2: Cytotoxic effect of GAXD ethanol extract on HepG2 cells. HepG2 cells were treated with 0.05% DMSO (control) and GAXD ethanol extract (20, 40 and 80 μg/mL) for 48 h; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control

DISCUSSION

In this work, we research the major chemical components and anti-hepatoma effect of GAXD by HPLC, MTT, flow cytometry, Western blot and xenograft assays for the first time. The study on identification of major chemical components shows that what the active components are in GAXD.

MTT assay, a common method, is used to evaluate the cytotoxic effect of drug against cultured cells in vitro by determining cells viability [18,19]. Viable cells induce MTT to generate the purple-blue formazan precipitate, but dead cells lose this function. The absorbance of formazan dissolved in DMSO is used to analyze the cytotoxic effect of drugs on cells. The result of MTT assay indicated that GAXD EE had significant cytotoxic activity against HepG2 cells (Figure 2).

Flow cytometry assay [20,21] was used to study whether the cytotoxic activity of GAXD EE against HepG2 cells was associated with apoptosis. The cells in early apoptosis were stained with Annexin V-FITC and cells in late apoptosis were stained with Annexin V-FITC and PI. Subsequently, cells were analyzed by flow cytometry and different kinds of cells including dead cells, viable cells, cells in early apoptosis and cells in late apoptosis were counted respectively. According to the results of counting in flow cytometry, we can easily analyze whether drugs induce apoptosis of cells. The results of flow cytometry suggested that the cytotoxic activity of GAXD EE against HepG2 cells was associated with apoptosis (Figure 3).

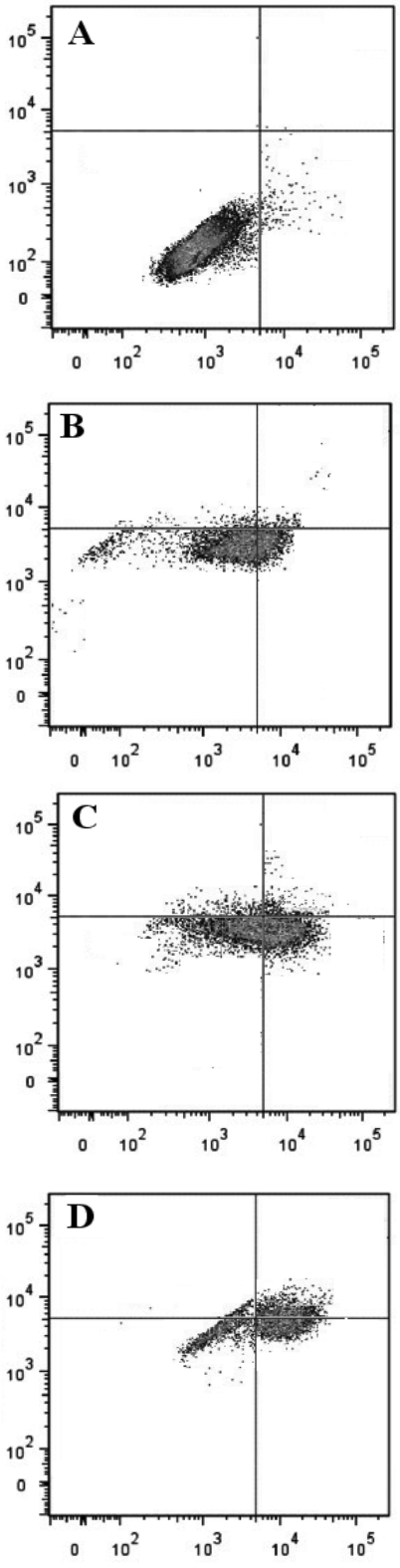

Figure 3: Effects of GAXD ethanol extract on apoptosis of HepG2 cells. HepG2 cells were treated with 0.05% DMSO (control) and GAXD ethanol extract (20, 40 and 80 μg/mL) for 48 h; A, B, C, D stood for 0.05% DMSO, 20, 40 and 80 μg/mL GAXD ethanol extract respectively

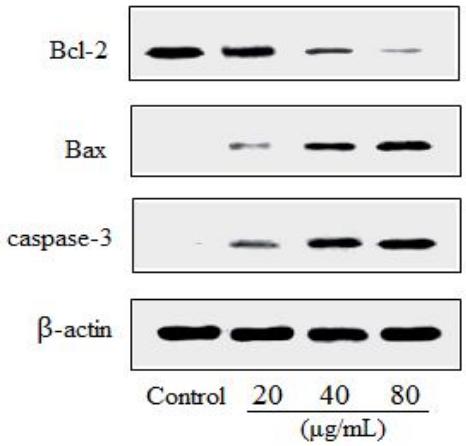

Figure 4: Effect of GAXD ethanol extract on apoptotic proteins (Bcl-2, Bax and caspase-3) of HepG2 cells. HepG2 cells were separately treated with 0.05% DMSO (control) and GAXD ethanol extract (20, 40 and 80 μg/mL) for 48 h

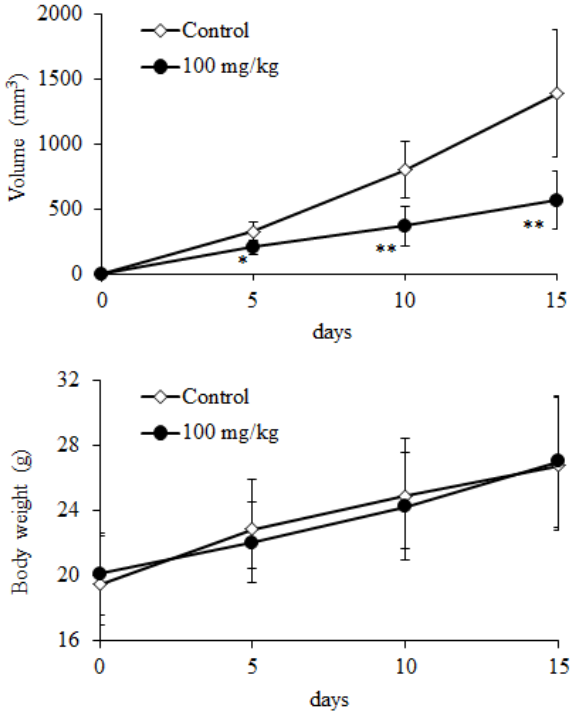

Figure 5: Anti-tumor effect of GAXD ethanol extract on HepG2 cells-induced tumor. Nude mice were treated with 0.05% DMSO (control) and GAXD ethanol extract (100 mg/kg) by gavage; * $p < 0.05$, ** $p < 0.01$, compared with control.

The results of MTT and flow cytometry assays indicated that GAXD indeed have anti-hepatoma activity, associated with apoptosis. Western blot assay was used to study the effects of GAXD on apoptotic proteins, which were immediately related to apoptosis of cells. The apoptotic proteins were a research hotspot in the aspect of anti-tumor mechanism [22,23]. Bcl-2 protein

inhibits apoptosis of tumor cells, and Bax and caspase-3 proteins induce apoptosis of tumor cells [24]. As depicted in Figure 4, GAXD EE down-regulated the expression of anti-apoptotic protein (Bcl-2), and increased the expression of pro-apoptotic proteins (Bax and caspase-3). Namely, the anti-hepatoma mechanisms of GAXD were associated with apoptosis.

Finally, the xenograft assay was used to verify the anti-tumor effect of GAXD in vivo. The results of xenograft assay indicated that GAXD EE significantly inhibited the growth of HepG2 cells-induced tumor without any effect on the body weight of nude mice.

CONCLUSION

GAXD shows anti-hepatoma activity in vitro and in vivo by inducing the apoptosis of HepG2 cells, associated with down-regulating the expression of anti-apoptotic protein (Bcl-2) and increasing the expression of pro-apoptotic proteins (Bax and caspase-3). Additionally, this work provides the evidences to support that GAXD is used to cure hepatoma. However, further studies to elucidate the anti-hepatoma mechanisms of GAXD are required.

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