*ORIGINAL ARTICLE*

# Toxicarioside A inhibits SGC-7901 proliferation, migration and invasion *via* NF-κB/bFGF signaling

Jun-Li Guo, Shao-Jiang Zheng, Yue-Nan Li, Wei Jie, Xin-Bao Hao, Tian-Fa Li, Li-Ping Xia, Wen-Li Mei, Feng-Ying Huang, Yue-Qiong Kong, Qi-Yi He, Kun Yang, Guang-Hong Tan, Hao-Fu Dai

Jun-Li Guo, Shao-Jiang Zheng, Yue-Nan Li, Xin-Bao Hao, Tian-Fa Li, Li-Ping Xia, Feng-Ying Huang, Yue-Qiong Kong, Qi-Yi He, Kun Yang, Guang-Hong Tan, Hainan Provincial Key Laboratory of Tropical Medicine, Cancer Research Institute of Affiliated Hospital, Hainan Medical College, Haikou 571199, Hainan Province, China
Wei Jie, Department of Pathology, Guangdong Medical College, Zhanjiang 524023, Guangdong Province, China
Wen-Li Mei, Hao-Fu Dai, Key Laboratory of Tropical Crop Biotechnology, Ministry of Agriculture, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, Hainan Province, China
Correspondence to: Hao-Fu Dai, Professor, Key Laboratory of Tropical Crop Biotechnology, Ministry of Agriculture, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, Hainan Province, China. hf_dai@yahoo.cn
Telephone: +86-898-66890292  Fax: +86-898-66890978

## Abstract

**AIM:** To investigate the inhibitory role of toxicarioside A on the gastric cancer cell line human gastric cancer cell line (SGC-7901) and determine the underlying molecular mechanism.

**METHODS:** After SGC-7901 cells were treated with toxicarioside A at various concentrations (0.5, 1.5, 4.5, 9.0 μg/mL) for 24 h or 48 h, cell viability was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay, and the motility and invasion of tumor cells were assessed by the Transwell chamber assay. Immunofluorescence staining, reverse transcription polymerase chain reaction and Western blotting were performed to detect the expression of basic fibroblast growth factor (bFGF) and fibroblast growth factor receptor-1 (FGFR1), and nuclear factor-kappa B (NF-κB) activation was examined by electrophoretic mobility shift assay.

**RESULTS:** The results showed that toxicarioside A was capable of reducing cell viability, inhibiting cell growth, and suppressing cell migration and invasion activities in a time- and dose-dependent manner in SGC-7901 cells. Further analysis revealed that not only the expression of bFGF and its high-affinity receptor FGFR1 but also the NF-κB-DNA binding activity were effectively blocked by toxicarioside A in a dose-dependent manner compared with the control group ($P < 0.05$ or $P < 0.01$). Interestingly, application of the NF-κB specific inhibitor, pyrrolidinedithiocarbamate (PDTC), to SGC-7901 cells significantly potentized the toxicarioside A-induced down-regulation of bFGF compared with the control group ($P < 0.05$).

**CONCLUSION:** These findings suggest that toxicarioside A has an anti-gastric cancer activity and this effect may be achieved partly through down-regulation of NF-κB and bFGF/FGFR1 signaling.

**Peer reviewer:** Hitoshi Tsuda, MD, PhD, Diagnostic Pathology Section, Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

## INTRODUCTION

*Antiaristoxicaria* (Pers.) *Lesch* (Moraceae) is a well known precious species widespread in the tropical rain forest of Southeast Asia. Its latex and seeds contain a complex mixture of cardenolide glycosides and is therefore toxic[1]. Representative toxicariosides A-L have recently been identified from the latex and seeds of *Antiaristoxicaria* in our laboratory and by others[2-5]. Classically, cardenolides are used to treat congestive heart failure and arrhythmia[6-8]. Additionally, certain cardenolides extracted from some plants or animals have been demonstrated to be capable of blocking tumor cell proliferation through regulation of cell signal transduction[9-15].

Currently, gastric cancer is one of the leading malignancies in China. However, the treatment outcome is not satisfactory because early diagnosis of gastric cancer remains difficult and most patients have already developed metastatic lesions when diagnosed[16].

Basic fibroblast growth factor (bFGF) has been shown to be a multifunctional growth factor for tumor development[17-20], and it exerts its biological effects mainly through interaction with its high-affinity receptor, fibroblast growth factor receptor-1 (FGFR1)[21-24]. Compiling evidence has demonstrated that bFGF signaling is involved in the development of gastric cancer[25,26].

Nuclear factor-kappa B (NF-κB) is a ubiquitous dimeric transcription factor that plays pivotal roles in regulating the expression of genes encoding cytokines and chemokines that are involved in tumor proliferation, angiogenesis, and synthesis of anti-apoptotic proteins[27,28]. It has been documented that NF-κB can mediate bFGF signaling[29] and some types of cardiac glycosides can block the activation of NF-κB[30,31]. As a result, we hypothesize that cardiac glycosides may suppress gastric tumor growth *via* a decrease in NF-κB activity and blocking of the bFGF signaling pathway. In the present study, we attempted to test this hypothesis in an *in vitro* cell culture model.

## MATERIALS AND METHODS

### Plant material
Latex of *Antiaristoxicaria* (Pers.) *Lesch* collected in Lingshui county of Hainan Province, China in November 2005 was identified with the assistance of Professor Zhunian
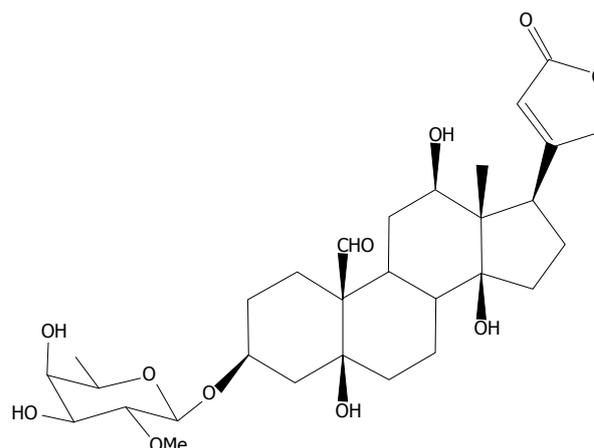


**Figure 1 The structure of toxicarioside A.**

Wang at the Institute of Crops Genetic Resources, Chinese Academy of Tropical Agricultural Sciences. The specimen was numbered as No. AN200511.

### Chemicals and reagents
Rabbit-anti human bFGF and FGFR1 were purchased from Santa Cruz (Santa Cruz, CA, United States). Rhodamine (TRITC)-conjugated mouse anti-rabbit immunoglobulin G (IgG), fluorescein isothiocyanate (FITC)-conjugated mouse anti-rabbit IgG, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypan blue and pyrrolidinedithiocarbamate (PDTC) were obtained from Sigma (Sigma Aldrich, St Louis, MO, United States). Fetal bovine serum (FBS), RPMI 1640 medium and trypsin were procured from Gibco (Gibco, Carlsbad, CA, United States).

### Extraction and isolation of toxicarioside A
With 95% EtOH, 4.0 L of the latex of *Antiaristoxicaria* were extracted thrice at room temperature and filtered. The combined extract was evaporated in vacuo to yield a syrup (263.8 g), which was fractionated sequentially with petroleum ether, EtOAc, and n-BuOH. The EtOAc fraction (8.68 g) that showed potent cytotoxic activity in the bioassay was passed through pressure-reduced column chromatography using step-wise elution with CHCl$_3$-MeOH (50:1, 20:1, 10:1, 5:1, 2:1, 1:1 and 0:1, v/v), generating seven corresponding fractions, A1-A7. Fraction A7 (2.55 g) was further separated on silica gel column chromatography, from which compound 1 (788.1 mg) was eluted with CHCl$_3$-MeOH (14:1, v/v). On the basis of spectral data and chemical analyses, compound 1 was defined as toxicarioside A (Figure 1).

### Cell culture
Human gastric cancer cell line (SGC-7901) was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/mL streptomycin at 37 ℃ in a humidified atmosphere with 5% CO$_2$. Cells at the logarithmic phase were

used for experiments.

## Proliferation assay

MTT assay and trypanblue staining were used to determine the growth and viability of SGC-7901 cells. For the MTT assay, SGC-7901 cells in logarithmic growth were trypsinized and harvested and then the cells were seeded onto a 96-well plate. After 24 h, fresh RPMI 1640 medium containing different concentrations of toxicarioside A (0.5, 1.5, 4.5, 9.0 μg/mL) was added at 100 μL per well, respectively, and 6 replicate wells were used for each of the concentrations. After incubation for different time intervals, 10 μL of MTT (5 mg/mL) was added to each well and the cells were further incubated at 37 ℃ for 4 h. The supernatant was then removed and 100 μL DMSO was added into each well. Absorbance (A value) at a wavelength of 490 nm was measured with a Bio-TekEXL808 microplate reader (Bio-Rad, Hercules, CA, United States). For trypanblue staining, SGC-7901 cells were trypsinized and seeded into 24-well plates at a density of $0.5 \times 10^4$/mL. After 4.5 μg/mL toxicarioside A was added, the cells were collected and counted using trypan blue staining under an inverse light microscope for 3 consecutive days.

## Invasion and migration assay

Invasion assays were performed in a 24-well Transwell chamber (Corning, Lowell, MA, United States) as previously described[32]. Briefly, each Transwell chamber was coated with 15 μg Matrigel, $5 \times 10^4$ cells were seeded to pre-coated filters in 200 μL of serum-free medium containing different concentrations of toxicarioside A (0.5, 1.5, 4.5, 9.0 μg/mL) in triplicate. The lower parts of the chambers were filled with 500 μL of RPMI 1640 medium containing 10% FBS. After incubation in a 5% $CO_2$ humidified incubator at 37 ℃ for 24 h, the cells on the upper surface were gently removed with a cotton swab, and the filters were fixed with 95% alcohol for 15-20 min and stained with hematoxylin-eosin for 15 min. The number of cells on the lower surface of the filters was quantified under a microscope. The same procedures were followed for the migration assay except the Transwell chambers were not coated with Matrigel.

## Immunofluorescence staining

To detect the expression of bFGF as well as its receptor FGFR1 in SGC-7901 cells, the rabbit antibody (1:100) against bFGF and FGFR1 were used. The antigenic sites were localized by TRITC-conjugated mouse anti-rabbit IgG and FITC-conjugated mouse anti-rabbit IgG, and images of antigenic sites were captured under a laser scanning confocal microscope (FV500, Olympus, Tokyo, Japan).

## RNA extraction and reverse transcription polymerase chain reaction

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's protocols. Reverse transcription polymerase chain reaction was carried out using pairs of primers (Invitrogen) as follows for semiquantitative assessment: bFGF (NM_002006.4) sense, 5'-AAG AGC GAC CCT CAC ATC AA-3'; anti-sense, 5'-TCG TTT CAG TGC CAC ATC CGT CAA TA CC-3', yielding a 225 bp product; FGFR1 (M34641) sense, 5'-CTT CTGT TTC AG-3'; anti-sense, 5'-TCC ACA ATG CAG GTG TAG TT-3', yielding a 354 bp product. β-actin (NM_001101) sense, 5'-GTT GCG TTA CAC CCT TTC TT-3', anti-sense, 5'-CGA AGG CTC ATC ATT CAA AA-3', yielding a 443 bp product. The products were separated by electrophoresis on a 1.5% agarose gel and visualized under UV using the gel documentation system (Bio-Rad Gel Doc1000, Bio-Rad). The mRNA levels of bFGF, FGFR1 were calculated based on the denstometric values of the specific bFGF, FGFR1 bands after adjustment with that of the β-actin band.

## Western blotting analysis

This was performed as previously described with minor modifications[33]. Cells were homogenized and separated by sodium dodecyl sulfate-polyacrylamide gel (12.5%) electrophoresis followed by electrophoretic transfer of proteins from the gel to a nitrocellulose membrane blot (Bio-Rad). The blot was incubated with a rabbit anti-bFGF antibody (1:500) or a rabbit anti-FGFR1 antibody (1:500) at 4 ℃ overnight, followed by incubation with the corresponding horseradish peroxidase-conjugated anti-biotin antibody (1:2000) at room temperature for 1 h. The immunoreactive signals were visualized with enhanced chemiluminescence reagents (Pierce, Rockford, IL, United States).

## Electrophoretic mobility shift assay

To determine NF-κB activation, electrophoretic mobility shift assay (EMSA) was conducted essentially as described previously[34]. In brief, nuclear proteins (10 μg) were incubated with the reaction buffer for 20 min at room temperature, followed by incubation with oligonucleotide containing the consensus sequence for the NF-κB-DNA binding site (5'-AGAGTGGGAATT TC-CACTCA-3')[35] (synthesized by Invitrogen, Shanghai, China). The reaction mixture was separated in a nondenaturing polyacrylamide gel (6%) that was later stained by SYBR Green EMSA staining solution from Molecular Probes (Invitrogen) with continuous, gentle agitation for about 20 min, protected from light. The gel was then washed in 150 mL of $dH_2O_2$ and the stained nucleic acids were visualized and the image documented under UV using the gel documentation system (Bio-Rad Gel Doc1000).

## Statistical analysis

All data are expressed as mean ± SE. For a comparison between two groups, the Student's *t* test was performed. For comparisons among multiple groups, an ANOVA was carried out, followed by a Student-Newman-Keuls test. Differences were considered significant when $P < 0.05$.

| Table 1 The inhibition rates of human gastric cancer cell line cells treated with different concentrations of toxicarioside A for different time intervals | | | | |
|---|---|---|---|---|
| **Groups** | **24 h** | | **48 h** | |
| | *A* value | Inhibitory rate (%) | *A* value | Inhibitory rate (%) |
| Control | 0.879 ± 0.048 | 0.00 ± 0.00 | 0.932 ± 0.036 | 0.00 ± 0.00 |
| Toxicarioside A (μg/mL) | | | | |
| 0.5 | 0.793 ± 0.062 | 9.79 ± 7.63 | 0.752 ± 0.073 | 19.13 ± 5.38 |
| 1.5 | 0.646 ± 0.041 | 27.83 ± 4.78[a] | 0.596 ± 0.113 | 36.19 ± 3.81[a] |
| 4.5 | 0.528 ± 0.078 | 40.18 ± 3.32[a] | 0.443 ± 0.056 | 49.32 ± 5.17[b] |
| 9.0 | 0.352 ± 0.092 | 61.84 ± 6.61[b] | 0.301 ± 0.049 | 66.94 ± 7.03[b] |

Data representative of six independent experiments were expressed as mean ± SE. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

| Table 2 Effect of toxicarioside A on cell migration and invasion | | | | | |
|---|---|---|---|---|---|
| **Groups** | **Dose (μg/mL)** | **Migration** | | **Invasion** | |
| | | Cell number | Inhibition rate (%) | Cell number | Inhibition rate (%) |
| Control | 0.0 | 69.40 ± 6.38 | 0.00 ± 0.00 | 65.60 ± 7.20 | 0.00 ± 0.00 |
| Toxicarioside A | 0.5 | 62.50 ± 8.90 | 10.01 ± 9.78 | 57.80 ± 4.32 | 10.89 ± 8.64 |
| | 1.5 | 54.80 ± 7.30 | 22.38 ± 10.64[a] | 49.70 ± 5.68 | 24.03 ± 9.06[a] |
| | 4.5 | 41.60 ± 5.88 | 39.58 ± 11.62[a] | 36.40 ± 7.94 | 44.68 ± 9.19[a] |
| | 9.0 | 35.80 ± 8.32 | 48.13 ± 10.12[b] | 30.10 ± 6.46 | 54.38 ± 8.17[b] |

Data representative of three independent experiments were expressed as mean ± SE. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

## RESULTS

### Effect of toxicarioside A on SGC-7901 cell proliferation

To assess the effect of toxicarioside A on the growth of gastric cancer, SGC-7901 cells were treated at various concentrations (0.5, 1.5, 4.5, 9.0 μg/mL) for 24-48 h and cell viability following these treatments was determined by MTT assays. As shown in Table 1, toxicarioside A reduced SGC-7901 cell viability in a time- and dose-dependent manner. Cell growth curves also showed that toxicarioside A significantly inhibited SGC-7901 cell growth as compared with the control (Figure 2).

### Effect of toxicarioside A on SGC-7901 cell migration and invasion

The results of Transwell cell migration and invasion are presented in Table 2 and Figure 3. Clearly, the addition of toxicarioside A to the medium in the upper chamber resulted in significant suppression of SGC-7901 migration and invasion in a dose-dependent manner at 1.5, 4.5 and 9.0 μg/mL, toxicarioside A inhibited SGC-7901 migration by 22.38% ± 10.64%, 39.58% ± 11.62% and 48.13% ± 10.12%, respectively ($P < 0.05$), and inhibited SGC 7901 invasion by 24.03% ± 9.06%, 44.68% ± 9.19% and 54.38% ± 8.17%, respectively ($P < 0.01$), as compared with the control group.

### Effect of toxicarioside A on bFGF and FGFR1 in SGC-7901 cells

At the protein level, the expression of bFGF and FGFR1 was predominantly detected in the cytoplasm of SGC-7901 cells and toxicarioside A significantly decreased this ex-
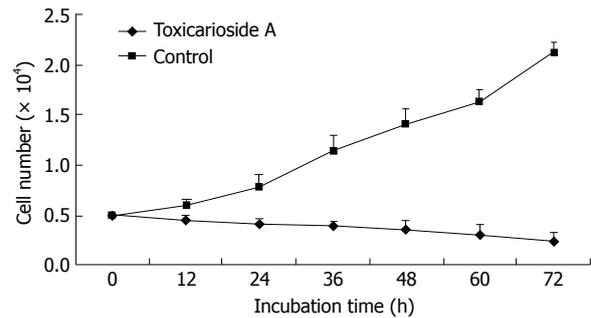


**Figure 2 Effect of toxicarioside A on the growth curve of human gastric cancer cell line cells.** Cells were plated in 24-well plates at a density of 1 × 10⁴/mL and treated with 4.5 μg/mL toxicarioside A for 72 h. The results shown are representative of three independent experiments.
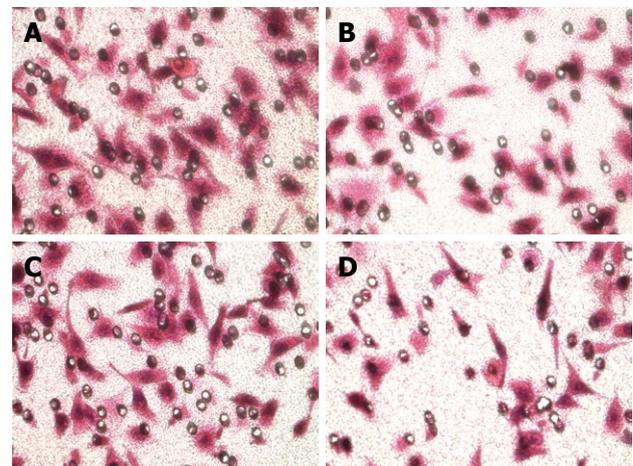


**Figure 3 Representative figures of cell migration and invasion in non-treated and toxicarioside A-treated human gastric cancer cell line cells.** A: Migration in the control group; B: Migration in the thetoxicarioside A-treated (4.5 μg/mL) group; C: Invasion in the control group; D: Invasion in the toxicarioside A-treated group.

pression, as assessed by immunofluorescence staining (Figure 4A) and Western blotting analysis (Figure 4C). At the mRNA level, the expression of bFGF and FGFR1 was decreased by toxicarioside A in a dose-dependent manner in SGC-7901 cells (Figure 4B).

### Effect of toxicarioside A on NF-κB-DNA binding activity in SGC-7901 cells

To determine the effect of toxicariosideA on NF-κB activation, the NF-κB-DNA binding activity was determined in both toxicarioside A-treated and control SGC-7901 cells by EMSA. As shown in Figure 5, after treatment with toxicarioside A at various concentrations for 48 h, the NF-κB-DNA binding activity was decreased in a dose-dependent manner as compared with the control group ($P < 0.05$ or $P < 0.01$, Figure 5).

### Effect of PDTC on toxicarioside A-induced downregulation of bFGF

To further determine whether NF-κB activation was necessary for bFGF expression, and was involved in toxicarioside A-induced downregulation of bFGF, a specific in-
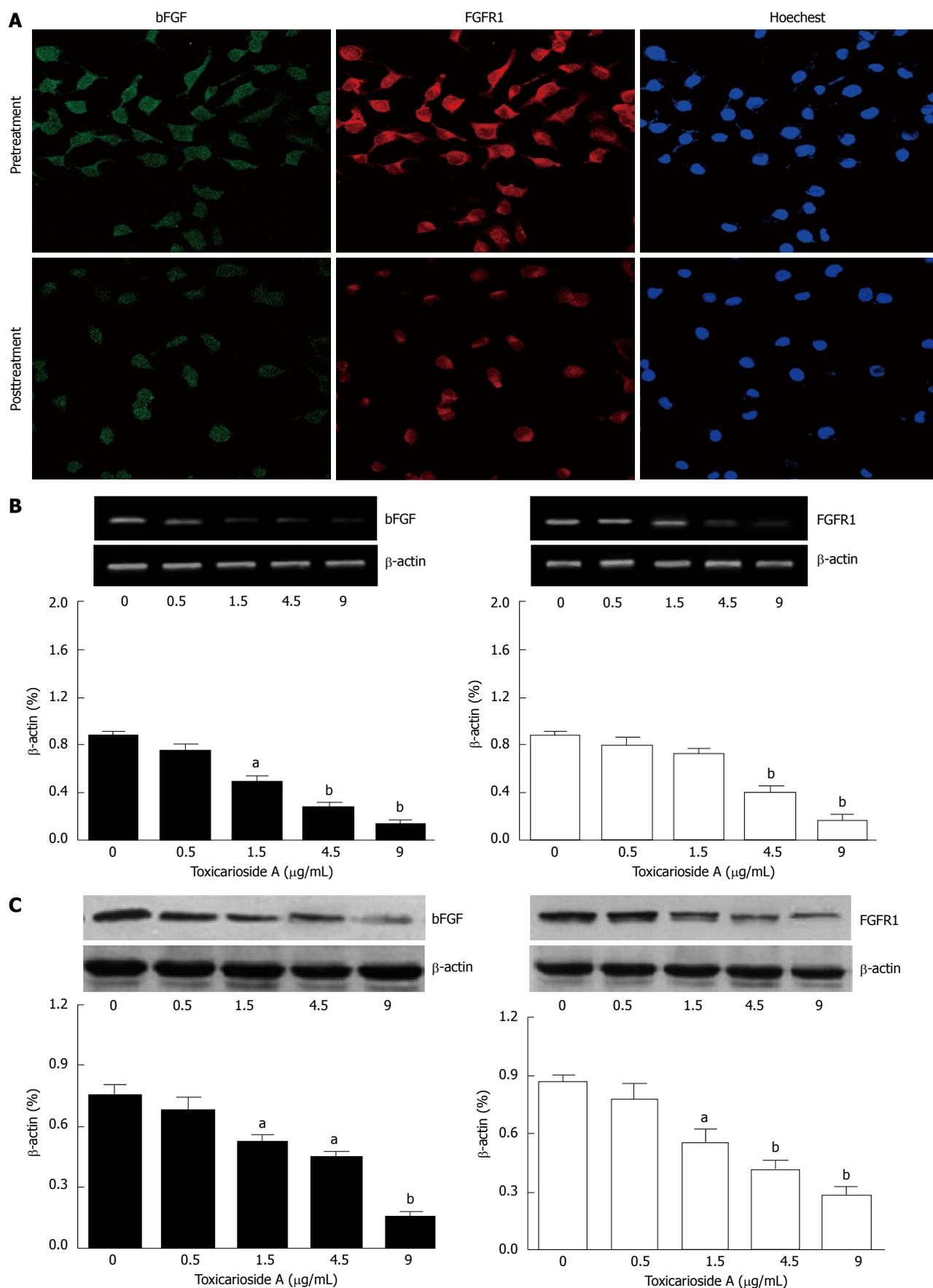
**Figure 4 Basic fibroblast growth factor and fibroblast growth factor receptor-1 expression in human gastric cancer cell line cells.** A: The expression of basic fibroblast growth factor (bFGF) and fibroblast growth factor receptor-1 (FGFR1) were detected using rhodamine and fluorescein isothiocyanate-conjugated mouse anti-rabbit immunoglobulin G in non-treated and toxicarioside A (4.5 μg/mL)-treated cells; B: bFGF and FGFR1 mRNA expression by reverse transcription polymerase chain reaction; C: bFGF and FGFR1 protein levels by Western blotting analysis. Results are depicted as mean ± SE of three independent experiments. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.
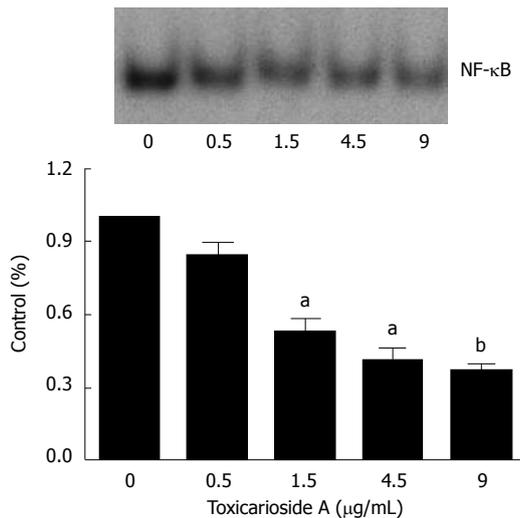
Figure 5 **Effect of toxicarioside A on nuclear factor-kappa B-DNA binding activity in human gastric cancer cell line cells.** After cells were incubated with various concentrations of toxicarioside A for 48 h, nuclear proteins were isolated and electrophoretic mobility shift assay was performed to determine nuclear factor-kappa B (NF-κB)-DNA binding activity. Results are depicted as mean ± SE of three independent experiments. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.



Figure 6 **Effect of inhibitor, pyrrolidinedithiocarbamate, on basic fibro-blast growth factor protein expression.** Administration of pyrrolidinedithiocar-bamate (PDTC) (50 μmol/L) reinforced the toxicarioside A (4.5 μg/mL)-induced downregulation of basic fibroblast growth factor (bFGF). Results are depicted as mean ± SE of three independent experiments. [a]$P < 0.05$ *vs* control group.

hibitor of NF-κB activation, PDTC, was used. As shown in Figure 6, PDTC treatment significantly blocked bFGF expression, which was potentized when both PDTC and toxicarioside A were added to SGC-7901 cells.

## DISCUSSION

*Antiaristoxicaria* (Pers.) *Lesch* (Moraceae) is widespread in the tropical rain forest of southeastern Asia, and is best known for its remedial properties against injuries due to poisoned arrows, darts and blowdarts[36]. The latex-sap and seeds of *Antiaristoxicaria* consists of a complex mixture of active cardenolide glycosides, from which several cardenolides have been isolated in our laboratory and other research groups[2-5]. Besides the classical effect of the cardenolides on inhibition of the ubiquitous cell surface $Na^+$, $K^+$-ATPase, the effect of cardiac glycosides on the growth of human malignant tumor cells has been reported in the recent past[11-15]. In the present work, we investigated the anti-cancer activity of toxicarioside A isolated from the latex of *Antiaristoxicaria*. Both the MTT assay and the growth curve analysis revealed that toxicarioside A resulted in inhibition of gastric cancer cell proliferation in a dose- and time-dependent manner. Malignant tumors are characterized by invasion and metastasis, an extremely complex process involving multi-steps. In this study, we assessed the migrating and invasive capabilities of SGC-7901 cells using the Transwell chamber assay. The results demonstrated that toxicarioside A not only suppressed cell motility, but also significantly reduced its ability to degrade the recombinant basement membrane in SGC-7901 cells.

To further investigate the molecular mechanism underlying the anti-tumor properties of cardenolides, we assessed the effect of toxicariosideA on bFGF expres-

sion in SGC-7901 cells. It is well known that bFGF, a regulatory factor secreted from cells, is involved in a variety of biological processes including cell differentiation, cell growth, migration, angiogenesis, and tumor formation[19,20]. The biological effect of bFGF is achieved mainly through interaction with its high-affinity receptor, FGFR1[21-24]. To elucidate whether the bFGF/FGFR1 signaling pathway was a target of toxicarioside A in gastric cancer cells, we evaluated changes in the expression of bFGF and FGFR1 in SGC-7901 cells after treatment with toxicarioside A at various concentrations. The results demonstrated that toxicarioside A down-regulated the expression of bFGF and FGFR1 at both mRNA and protein levels in SGC-7901 cells in a dose-dependent manner.
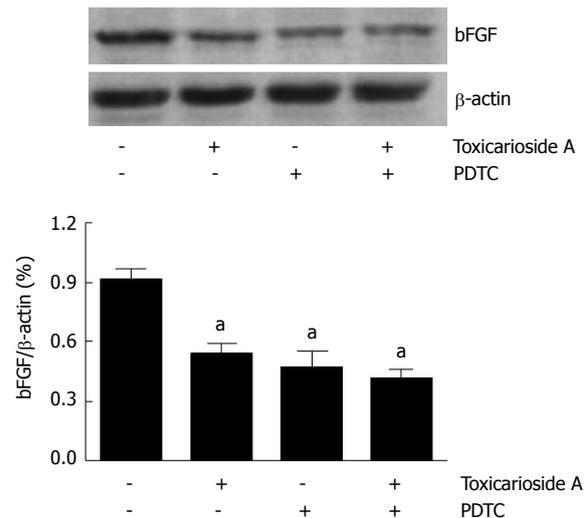
Next, we sought to investigate the molecules involved in the toxicarioside A-induced down-regulation of bFGF in SGC-7901 cells. The NF-κB signaling pathway is a central common regulator for the process of inflammation, viral replication, tumorigenesis, and apoptosis[37,38], and as a result has emerged as a potential target of numerous pharmaceutical agents[39,40]. Our results showed that toxicarioside A had an obvious suppressive effect on NF-κB-DNA binding activity in a dose-dependent manner, and treatment with an NF-κB specific inhibitor augmented the toxicarioside A-induced bFGF down-regulation in SGC-7901 cells, suggesting that the activated NF-κB may be partly necessary for bFGF expression in gastric cancer.

In summary, toxicarioside A weakened the abnormal activation of NF-κB to down-regulate the expression of bFGF, which in turn, interfered with bFGF/FGFR1 signal transduction subsequently leading to suppression ofproliferation, migration and invasion in SGC-7901 cells. Future research will focus on identification of new targets to provide the theoretical basis for the potential

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