

Original Research Article

Effect of 3,3'-Biisofraxidin on Apoptosis of Human Gastric Cancer BGC-823 Cells

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Abstract

Purpose: To study the effect of 3,3'-biisofraxidin from *Sarcandrae Herba* on the proliferation of BGC-823 cells and the possible mechanisms.

Methods: Cell Counting Kit-8 (CCK-8), flow cytometry, Western blot and xenograft assays were used to determine the effects of 3,3'-biisofraxidin on the proliferation, apoptosis, apoptotic proteins and xenograft of BGC-823 cells.

Results: 3,3'-Biisofraxidin significantly ($p < 0.01$) inhibited the proliferation of BGC-823 cells (concentrations: 10 - 40 μM ; cell viability: 30.45 - 76.68 % in CCK-8 assay) with half maximal inhibitory concentration (IC_{50}) value of 20.35 μM and induced the apoptosis of BGC-823 cells (concentrations: 10, 20 and 40 μM ; apoptotic cells: 11.92, 20.10 and 33.64 % in flow cytometry assay), compared with the control (cell viability: 99.73 %; apoptotic cells: 5.18 %). 3,3'-Biisofraxidin (10, 20 and 40 μM in vitro; 40 mg/kg in vivo) significantly ($p < 0.05$ or 0.01) down-regulated the expressions of anti-apoptotic proteins (*Bcl-2*, *Bcl-xl* and *Survivin*) and up-regulated the expressions of pro-apoptotic proteins (*Smac*, *caspase-3*, *caspase-7* and *caspase-9*), compared with the control. Moreover, the release of cytochrome c from the mitochondria to the cytoplasm was significantly ($p < 0.01$) promoted in vitro, compared with the control. 3,3'-Biisofraxidin (40 mg/kg) significantly ($p < 0.05$ or 0.01) inhibited the growth of tumor in xenograft assay, compared with the control.

Conclusion: 3,3'-Biisofraxidin significantly induces the apoptosis of BGC-823 cells in vitro and in vivo through the mitochondria-mediated apoptotic pathway, and therefore has a potential to be developed into an anti-gastric cancer drug.

Keywords: *Sarcandrae Herba*, Gastric cancer, 3,3'-Biisofraxidin, Mitochondria-mediated apoptosis, Cell Counting Kit-8, Xenograft

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INTRODUCTION

Sarcandrae Herba (SH), a traditional Chinese herbal medicine, is from *Sarcandra glabra* (Thunb.) Nakai (Chloranthaceae). In recent decades, the anti-cancer activity of SH have been widely reported. SH can be used to prevent and treat 5-FU-induced thrombocytopenia [1]. SH extracts can inhibit the growth of tumor *in vivo*, and the mechanism is related to cell cycle arrest

and inhibition of pro-apoptotic proteins (*Bcl-2* and *Bax*) [2,3]. It is reported that *Sarcandrolide F* and *Sarcandrolide H* from SH exhibit cytotoxicity against the HL-60 cell line with IC_{50} values of 0.03 and 1.2 μM [4] and SGP-2 from SH inhibits the migration and proliferation of human osteosarcoma cells [5]. Currently, constituents from traditional Chinese herbal medicines have been regarded as a most valuable resource for developing novel and advanced therapies for

cancer [6-8]. The chemical constituents of SH have drawn our attention due to its wide anti-cancer activity.

Gastric cancer is a common cancer in the world and over 70 % of gastric cancer occurs in developing countries [9-11]. Currently, gastric cancer is a notable cancer burden and needs to be urgently prevented and controlled in China [12,13]. 3,3'-Biisofraxidin is a biscoumarin isolated from anticancer fraction from SH [14] and its effect on the proliferation and apoptosis of BGC-823 cells remains unclear. In this work, we determined the effect of 3,3'-biisofraxidin on the proliferation of BGC-823 cells and the possible mechanisms *in vitro* and *in vivo* using CCK-8, flow cytometry, western blot and xenograft assays.

EXPERIMENTAL

Plant material

Sarcandrae Herba (SH) was obtained from Chengdu international trade city in 2013 and identified by Jian-Tao Wu. A voucher specimen (voucher no. 2013154XAJTUSM) was stored in the School of Medicine, Xi'an Jiaotong University for future reference.

Chemicals and reagents

Fetal bovine serum (FBS) and RPMI-1640 medium were provided by Invitrogen (Carlsbad, CA, USA). Cell Counting Kit-8 (CCK-8), Enhanced BCA Protein Assay Kit and dimethylsulfoxide (DMSO) were provided by Beyotime (Shanghai, China), while Annexin V-FITC/PI apoptosis assay kits were provided by STANDARDS (Shanghai, China). Also, Polyvinylidene difluoride (PVDF) was provided by Millipore (Bedford, MA, USA). Primary antibodies against Bcl-2, Bcl-xl, Survivin, Bax, Smac, cytochrome c, caspase-3, caspase-7, caspase-9, β -actin and horseradish peroxidase (HRP)-conjugated secondary antibody were provided by Abcam (Cambridge, UK), Cell Signaling Technology (Beverly, MA, USA) and Jackson Immuno Research Laboratories (West Grove, Pennsylvania, USA). In addition, analytical grade reagents and 300 - 400 mesh silica gel were provided by Qingdao Haiyang Chemical Co. Ltd (Qingdao, China).

Animals

Nude mice, 5 - 6 weeks old, were provided by the SLRC Laboratory Animal Company (Shanghai, China) and kept under specific pathogen-free conditions in a biological cabinet.

The animals were appropriately maintained, and all actions were strictly in accordance with international ethical guidelines of National Institutes of Health Guide concerning the Care and Use of Laboratory Animals [15]. Experiments were performed with the approval of the Animal Experimentation Ethics Committee of School of Medicine, Xi'an Jiaotong University (Protocol no. XAJTUSMAEEC2013).

Preparation of 3,3'-biisofraxidin

SH (100 kg) was extracted with 95 % ethanol, and the ethanol solution was concentrated under high vacuum to obtain crude extract. Then the crude extract was chromatographed over 300 - 400 mesh silica-gel column eluting with systemic gradient of cyclohexane-ethyl acetate. Fractions including 3,3'-biisofraxidin were combined and its solvent was evaporated to yield a mixture (56 g), which was purified by silica-gel column eluting with gradient of chloroform-methanol to obtain target analyte (2.6 g). The chemical structure and purity of target analyte were identified and analyzed using nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC). In addition, 3,3'-biisofraxidin was dissolved in 0.5 % of DMSO to obtain different concentrations of 3,3'-biisofraxidin for different assays.

Cell culture

BGC-823 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with FBS (10 %), penicillin (100 U/mL) and streptomycin (100 U/mL) at 37 °C in a humidified atmosphere of 5 % CO₂ and 95 % air [7]. BGC-823 cells were sub-cultured till reaching logarithmic phase of growth, and then assays were carried out on the re-cultured BGC-823 cells.

Cell growth assay

The effect of 3,3'-biisofraxidin on the proliferation of BGC-823 cells was evaluated using CCK-8 assay. Briefly, cells were seeded on 96 well cultured plates at a cell density of 2×10^4 with RPMI-1640 medium. After 24 h of incubation, cells were treated with 10, 15, 20, 25, 30, 35 and 40 μ M of 3,3'-biisofraxidin and with 0.5 % of DMSO (control). After 48 h, CCK-8 was added into each well and cells were cultured at 37 °C in 5 % CO₂ and 95 % air for another 3 h. Then the optical density (OD) of each well was measured at 450 nm using a micro-plate reader (Bio Rad, Model 680) [16].

Apoptosis analysis

After the treatment with 10, 20, and 40 μM of 3,3'-biisofraxidin and with 0.5 % of DMSO (control) for 48 h, BGC-823 cells were harvested and washed thrice with phosphate buffer and saline (PBS). Then the washed BGC-823 cells were re-suspended in staining buffer and stained with Annexin V-FITC/PI, and then BGC-823 cells were analyzed by flow cytometry [17].

Western blot analysis

After the treatment with 10, 20, and 40 μM of 3,3'-biisofraxidin and with 0.5 % of DMSO (control) for 48 h, total proteins of BGC-823 cells were extracted. The concentration of total proteins was determined using an Enhanced BCA Protein Assay Kit. Then total proteins (40 μg) was separated by SDS/PAGE and transferred electrophoretically onto a PVDF membrane. After blocking with 5 % fat-free milk, the membranes were incubated with primary antibodies overnight at 4 °C and then washed with Tris buffered saline-Tween (TBS-T) [18]. The washed membrane was further incubated with HRP-conjugated secondary antibody for 1 h at room temperature in TBS-T [7]. Immediately, anti- and pro-apoptotic proteins were detected by chemiluminescence. In addition, β -actin was picked as internal control to assess protein loading.

Nude mouse tumor xenograft model

BGC-823 cells (2×10^6 cells/nude mouse) were subcutaneously injected in the right flank of nude mice to establish xenograft model [19]. When tumor size reached 2-3 mm in diameter, nude mice were randomly divided into two groups ($n = 10$) and given the following treatments: the control group was treated with 0.5 % of DMSO

and the 3,3'-biisofraxidin group was treated with 40 mg/kg of 3,3'-biisofraxidin once a day for 20 days by intraperitoneal injection. The tumor sizes (width and length) and body weight of nude mice were measured every 5 days using vernier caliper and electronic scales, and the tumor volumes were calculated using the formula: $\text{volume} = \text{width}^2 (\text{mm}^2) \times \text{length} (\text{mm}) \times 0.5$ [20]. Finally, nude mice were killed immediately, and the tumor tissues were collected for western blot analysis.

Statistical analysis

All data are presented as mean \pm standard deviation (SD). Two-tailed Student's t-test and one-way ANOVA were separately used to analyze the differences between two groups and among 3 or more groups. Differences were recognized as statistically significant at $p < 0.05$. All data were analyzed using SPSS 21.0 (IBM SPSS Statistics, USA).

RESULTS

Identification and purity of 3,3'-biisofraxidin

The target analyte was identified as 3,3'-biisofraxidin according to ^{13}C -NMR data of target analyte [14] and its chemical structure is shown in Figure 1. The purity of 3,3'-biisofraxidin was more than 94 %, determined by area normalization method of HPLC.

Effects of 3,3'-biisofraxidin on the proliferation of BGC-823 cells

The effect of 3,3'-biisofraxidin on the proliferation of BGC-823 cells was evaluated using CCK-8 assay.

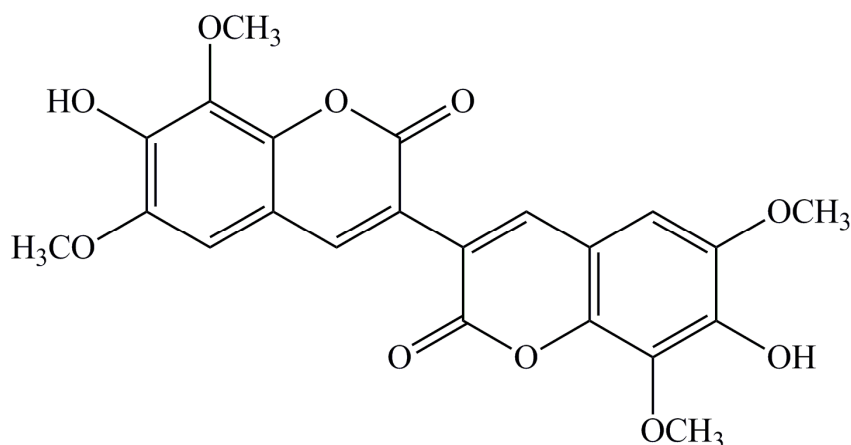


Figure 1: Chemical structure of 3,3'-biisofraxidin

As shown in Figure 2, after the treatment with 10, 15, 20, 25, 30, 35 and 40 μM of 3,3'-biisofraxidin, the proliferation of BGC-823 cells (cell viability: 30.45 - 76.68 %) was significantly ($p < 0.01$) inhibited, compared with the control (cell viability: 99.73 %), and the half maximal inhibitory concentration (IC_{50}) value was 20.35 μM .

Effects of 3,3'-biisofraxidin on the apoptosis of BGC-823 cells

According to the results of CCK-8 assay, 3,3'-biisofraxidin significantly inhibited the proliferation of BGC-823 cells, and then the flow

cytometry analysis was used to study whether the anti-proliferative activity of 3,3'-biisofraxidin against BGC-823 cells was associated with apoptosis. As shown in Figure 3, after the treatment with 10, 20 and 40 μM of 3,3'-biisofraxidin for 48 h, the apoptosis of BGC-823 cells (apoptotic cells: 11.92, 20.10 and 33.64 %) was significantly ($p < 0.01$) induced, compared with the control (apoptotic cells: 5.18 %). Therefore, the anti-proliferative activity of 3,3'-biisofraxidin against BGC-823 cells was associated with apoptosis.

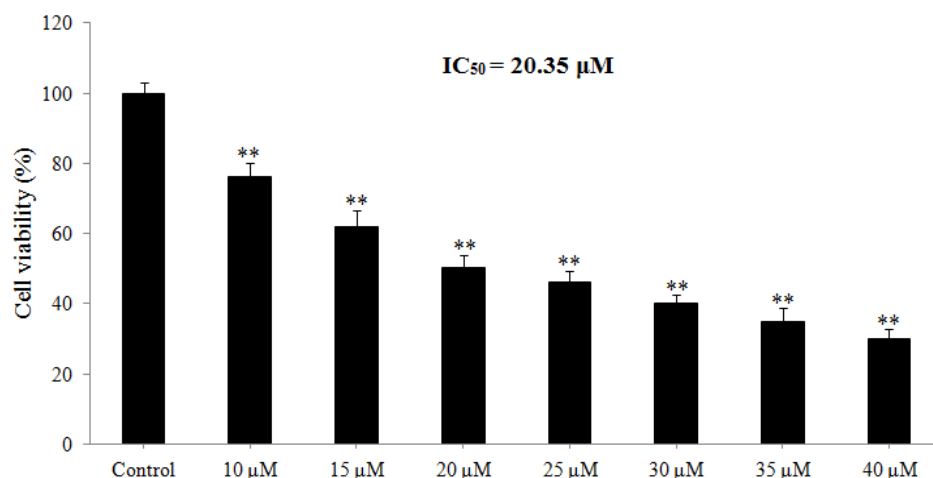


Figure 2: Anti-proliferative activity of 3,3'-biisofraxidin against BGC-823 cells; $**p < 0.01$, compared with the control

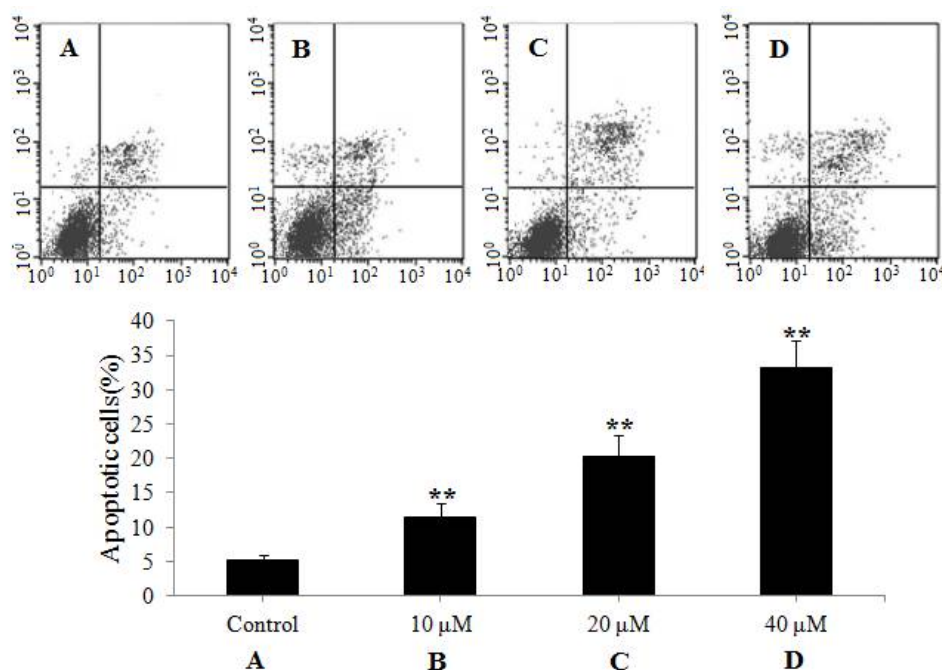


Figure 3: Inducing effects of 3,3'-biisofraxidin on the apoptosis of BGC-823 cells; $**p < 0.01$, compared with the control

Effects of 3,3'-biisofraxidin on the expressions of apoptotic proteins

To explore the pro-apoptotic mechanisms of 3,3'-biisofraxidin on BGC-823 cells, western blot was used to study the effect of 3,3'-biisofraxidin on the expressions of anti-apoptotic proteins (Bcl-2, Bcl-xl and Survivin) and pro-apoptotic proteins (Bax, Smac, cytochrome c, caspase-3, caspase-7 and caspase-9) in BGC-823 cells. As shown in Figures 4, 5 and 7, after the treatment with 10, 20 and 40 μM of 3,3'-biisofraxidin, the expressions of Bcl-2, Bcl-xl and Survivin were

significantly ($p < 0.05$ or 0.01) inhibited and the expressions of Smac, caspase-3, caspase-7 and caspase-9 were significantly ($p < 0.05$ or 0.01) increased, compared with the control, whereas the expression of Bax was not significantly affected by the treatment. Meanwhile, the release of cytochrome c from the mitochondria to the cytoplasm was significantly ($p < 0.01$) promoted (Figure 6), compared with the control. In addition, after the treatment with 10 μM of 3,3'-biisofraxidin, the expression of Bcl-xl was not significantly affected.

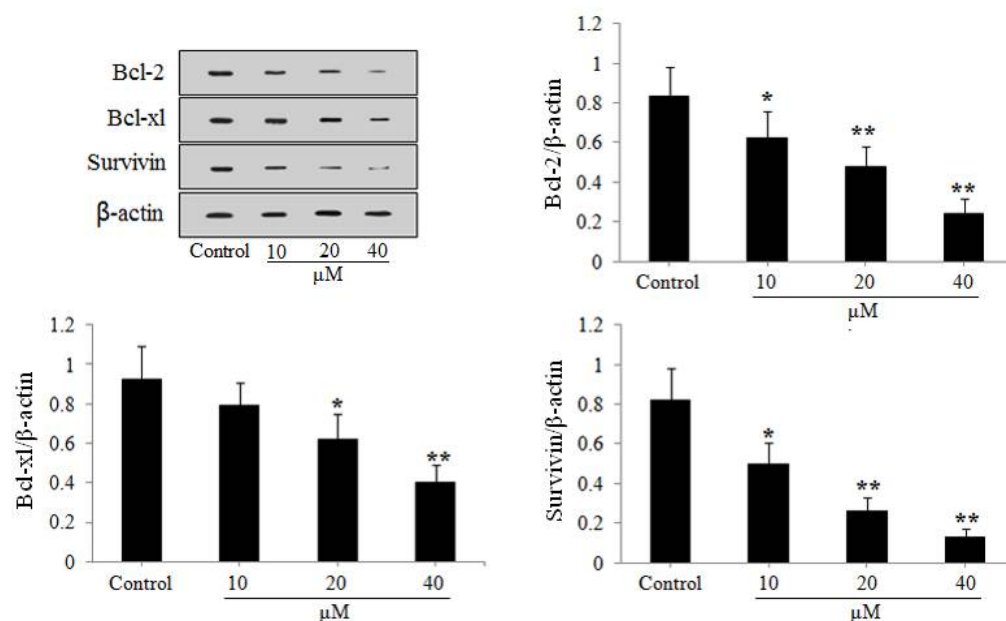


Figure 4: Effects of 3,3'-biisofraxidin on the expressions of anti-apoptotic proteins (Bcl-2, Bcl-xl and Survivin) in BGC-823 cells; * $p < 0.05$, ** $p < 0.01$, compared with the control

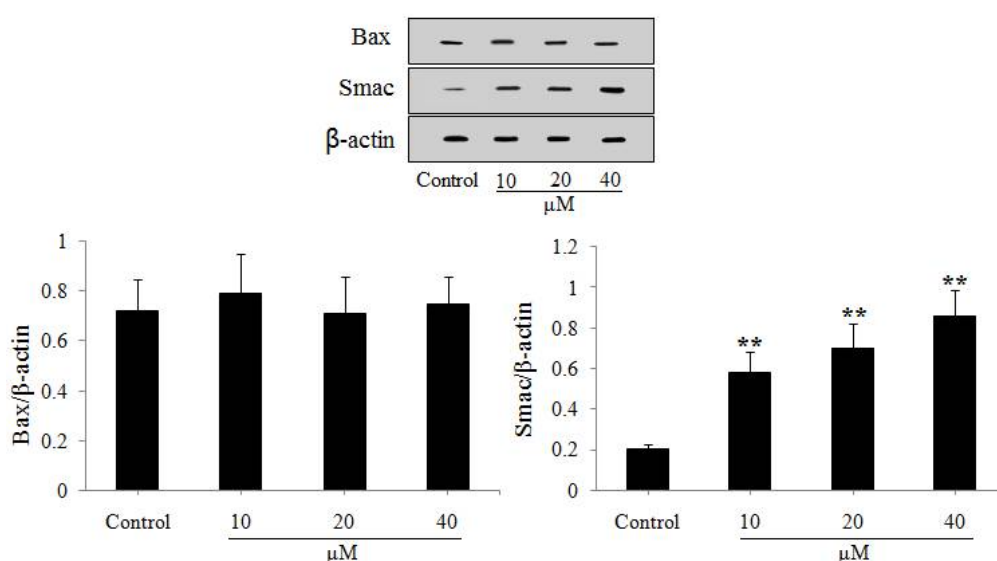


Figure 5: Effects of 3,3'-biisofraxidin on the expressions of pro-apoptotic proteins (Bax and Smac) in BGC-823 cells; ** $p < 0.01$, compared with the control

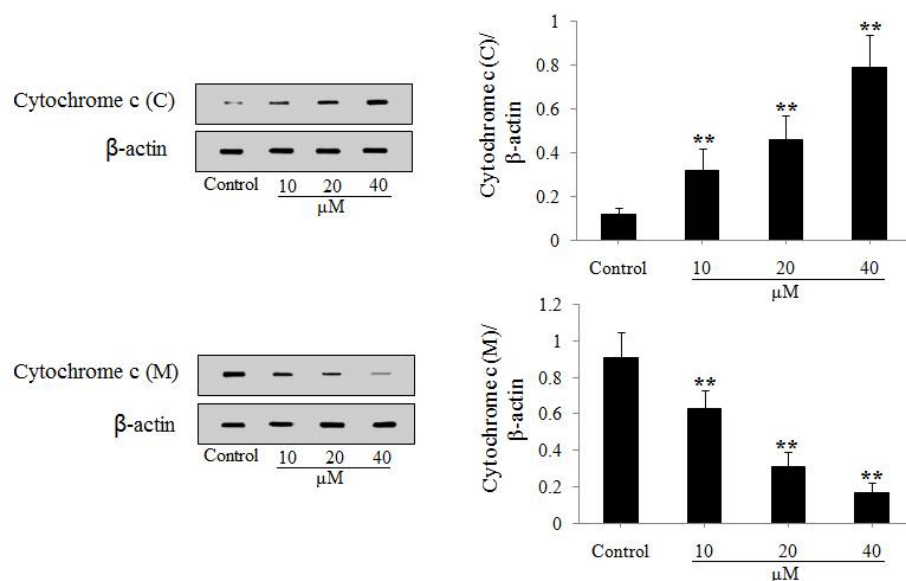


Figure 6: Effects of 3,3'-biisofraxidin on the release of cytochrome c from the mitochondria to the cytoplasm in BGC-823 cells; Cytochrome c (M) and Cytochrome c (C) represented cytochrome c in the mitochondria and the cytoplasm, respectively; ** $p < 0.01$, compared with the control

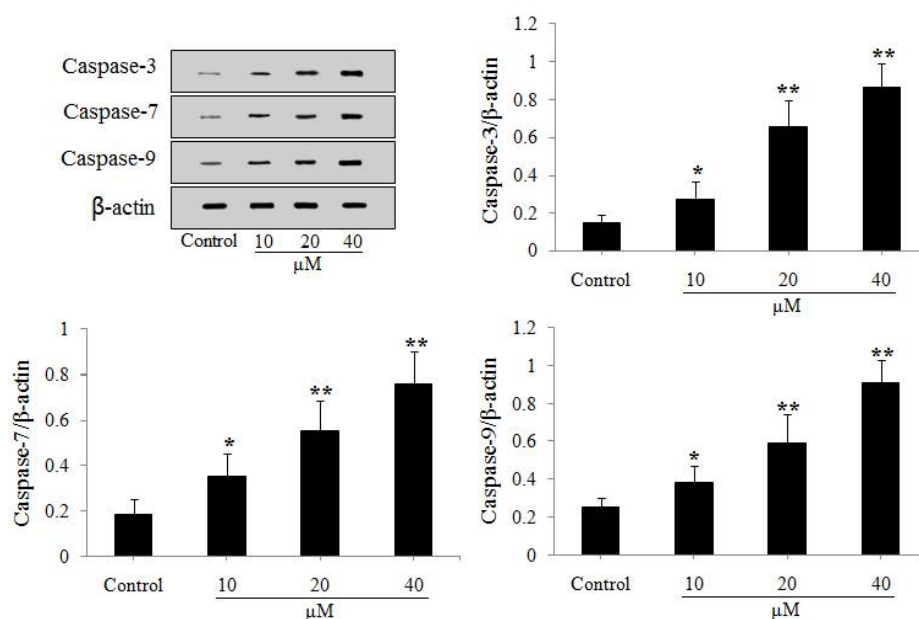


Figure 7: Effects of 3,3'-biisofraxidin on the expressions of pro-apoptotic proteins (caspase-3, caspase-7 and caspase-9) in BGC-823 cells; * $p < 0.05$, ** $p < 0.01$, compared with the control

Effect of 3,3'-biisofraxidin on BGC-823 xenograft model

The BGC-823 xenograft model was used to further study the activity of 3,3'-biisofraxidin on BGC-823 cells *in vivo*. As shown in Figure 8, after the treatment with 40 mg/kg of 3,3'-biisofraxidin once a day for 20 days, the growth of BGC-823 xenograft tumor was significantly ($p < 0.05$ or 0.01) inhibited during the observation period, compared with the control, whereas the

growth of body weight of nude mice was not significantly affected by the treatment. Moreover, the results of western blot analysis of tumor tissue suggested that the expressions of Bcl-2, Bcl-xl and Survivin were significantly ($p < 0.01$) inhibited (Figure 9), and the expressions of Smac, caspase-3, caspase-7 and caspase-9 were significantly ($p < 0.01$) increased (Figure 10), compared with the control.

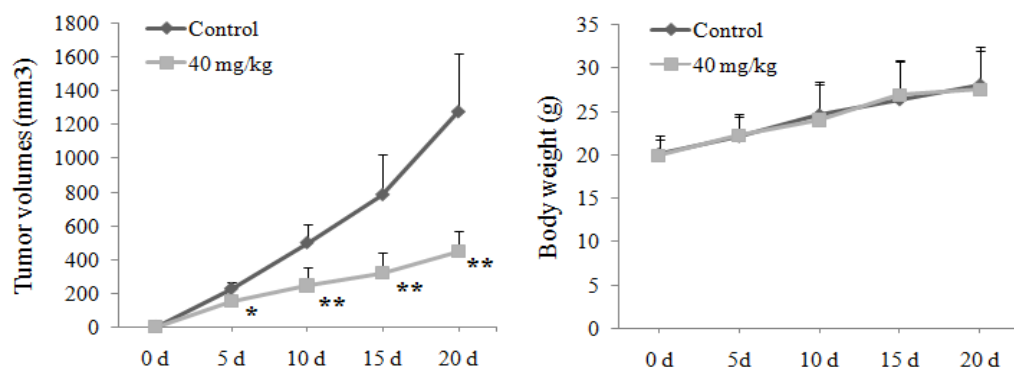


Figure 8: Effects of 3,3'-biisofraxidin on the tumor volumes and body weight of nude mice in BGC-823 xenograft model; * $p < 0.05$, ** $p < 0.01$, compared with the control

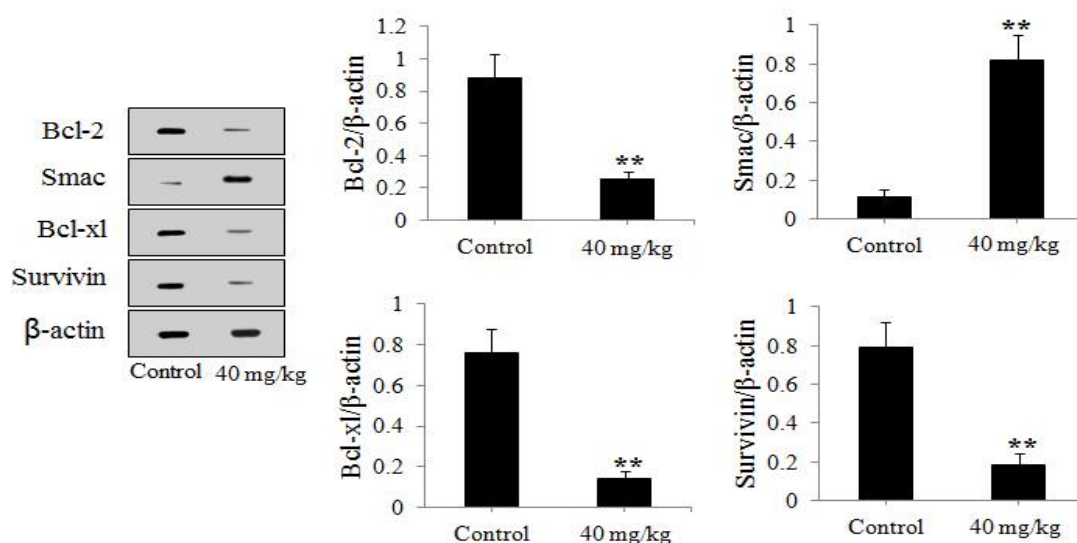


Figure 9: Effects of 3,3'-biisofraxidin on the expressions of anti-apoptotic proteins (Bcl-2, Bcl-xl and Survivin) and pro-apoptotic protein (Smac) in BGC-823 xenograft model; ** $p < 0.01$, compared with the control

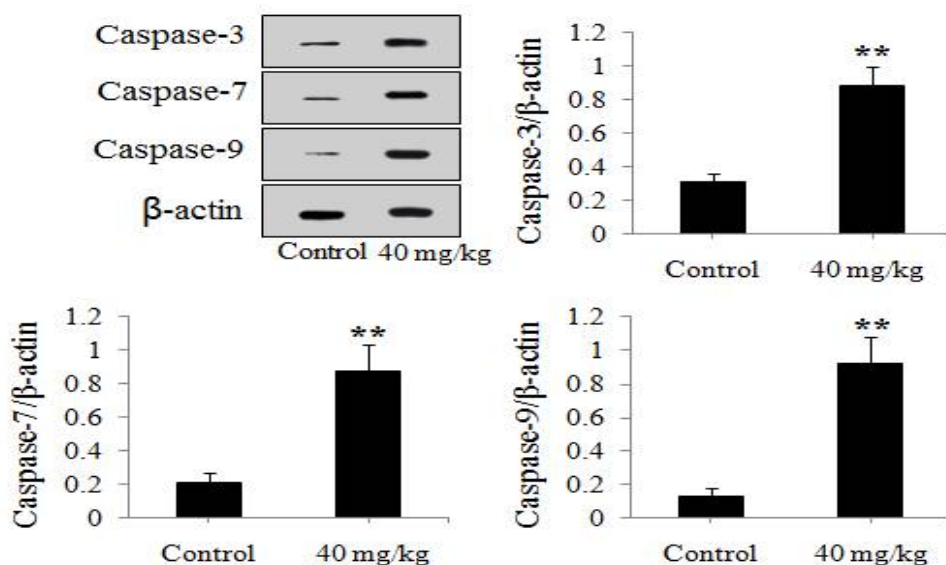


Figure 10: Effects of 3,3'-biisofraxidin on the expressions of pro-apoptotic proteins (caspase-3, caspase-7 and caspase-9) in BGC-823 xenograft model; ** $p < 0.01$, compared with the control

DISCUSSION

In the present study, we demonstrated that 3,3'-biisofraxidin inhibited the proliferation of BGC-823 cells and the mechanism was related to mitochondria-mediated apoptosis *in vitro* and *in vivo* using CCK-8, flow cytometry, western blot and xenograft assays for the first time.

BGC-823 cells are commonly used to study anti-gastric cancer activity of drugs [21,22]. The mitochondria-mediated apoptosis is an important pathway in inducing apoptosis of cancer cells. The anti-apoptotic proteins (Bcl-2, Bcl-xl, Survivin, etc.) and pro-apoptotic proteins (Bax, Smac, cytochrome c, caspase-3, caspase-7, caspase-9, etc.) play important roles in mitochondria-mediated apoptotic pathway [23]. If a drug can induce apoptosis of cancer cells via mitochondria-mediated apoptotic pathway, the down-regulation of the expression levels and activities of anti-apoptotic proteins (Bcl-2, Bcl-xl, Survivin, etc) and the up-regulation of the expression levels and activities of pro-apoptotic proteins (Bax, Smac, cytochrome c, caspase-3, caspase-7, caspase-9, etc.) can be observed [24-26]. The relationships among mitochondria-mediated apoptosis-related proteins are very complex. Bcl-2 protein inhibits the releases of cytochrome c and Smac from the mitochondria to the cytoplasm, but Bax inhibits the function of Bcl-2 [27,28]. The released cytochrome c along with ATP, procaspase-9 and apoptotic protease activating factor-1 (Apaf-1) form apoptosome in the cytoplasm and then the caspase recruitment domain (CARD) of procaspase-9 and Apaf-1 are activated. Subsequently, the effective caspases (caspase-3, caspase-7, etc.) were activated and the apoptosis of cancer cells is induced [29,30]. Survivin inhibits the function of the activated CARD of procaspase-9 and Apaf-1 and the activation of effective caspases, but Smac inhibits the function of Survivin [31]. In conclusion, the mitochondria-mediated apoptosis of cancer cells results from the interactions between anti-apoptotic proteins (Bcl-2, Bcl-xl, Survivin, etc.) and pro-apoptotic proteins (Bax, Smac, cytochrome c, caspase-3, caspase-7, caspase-9, etc.).

The results of CCK-8 assay suggested that 3,3'-biisofraxidin significantly inhibited the proliferation of BGC-823 cells with the IC₅₀ value of 20.35 μ M and the results of flow cytometry analysis indicated that the anti-proliferative activity of 3,3'-biisofraxidin on BGC-823 cells was related to apoptosis. Moreover, the results of western blot indicated that after treatment with

3,3'-biisofraxidin, the expressions of anti-apoptotic proteins (Bcl-2, Bcl-xl and Survivin) were significantly down-regulated, the expressions of pro-apoptotic proteins (Smac, caspase-3, caspase-7 and caspase-9) were significantly up-regulated and the release of cytochrome c from the mitochondria to the cytoplasm was significantly promoted. Further, tumor xenograft model in nude mouse confirmed that 3,3'-biisofraxidin had a significant inhibitory effect on BGC-823 xenograft tumor *in vivo* by significantly down-regulating the expressions of Bcl-2, Bcl-xl and Survivin and up-regulating the expressions of Smac, caspase-3, caspase-7 and caspase-9.

CONCLUSION

The findings of the study indicated that 3,3'-biisofraxidin induced the apoptosis of BGC-823 cells *in vitro* and *in vivo* and the mechanism was associated with mitochondria-mediated apoptosis. However, studies about further pro-apoptotic mechanisms of 3,3'-biisofraxidin need to be examined in future work.

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