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ORIGINAL ARTICLE

Apoptosis-Mediated Inhibition of Human Gastric Cancer Cell Proliferation by Cirsilineol

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#Equal contribution

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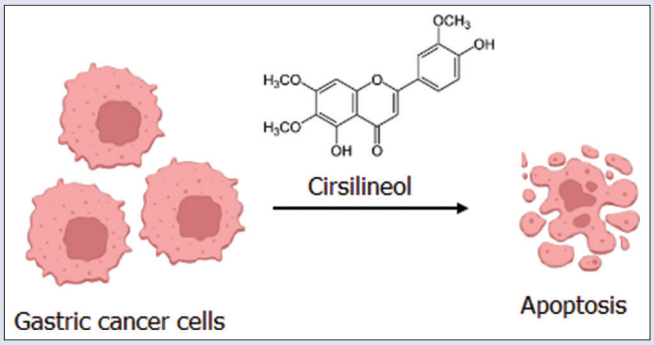
ABSTRACT

Background: Flavonoids constitute one of the best-characterized groups of plant secondary metabolites with enormous pharmaceutical potential. A flavone type of plant flavonoid, cirsilineol, has been reported to exhibit proapoptotic effects against malignant human cells. **Objectives:** The present study was designed to investigate the antiproliferative effects of cirsilineol against human gastric cancer cells. **Materials and Methods:** Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and colony formation assays. Apoptosis was detected by acridine orange/ethidium bromide (AO/EB) and annexin V/propidium iodide (PI) assay. Protein expression was examined by western blotting analysis. **Results:** The results showed cirsilineol inhibits the proliferation of human gastric cancer cells. The IC_{50} of cirsilineol against human gastric cancer cells (BGC-823, SGC-7901, and MGC-803) ranged from 8 to 10 μM. Nonetheless, cirsilineol exhibited comparatively lower antiproliferative effects against normal GES-1 cells. The IC_{50} of cirsilineol against normal GES-1 cells was found to be 120 μM. Colony formation assay showed that cirsilineol suppressed the colony formation of BGC-823 and MGC-803 cells in a dose-dependent manner. Acridine orange and ethidium bromide (AO/EB) staining showed that cirsilineol induced apoptosis in BGC-823 and MGC-803 cells. The percentage of apoptosis increased from 7.4% in control to 40.5% in BGC-823 cells and from 6.56% in control to 33.53% in MGC-803 cells at 8 μM cirsilineol. Western blotting showed cirsilineol caused an increase in Bax and cleaved caspase-3 and a decrease in Bcl-2 expression in both BGC-823 and MGC-803 cells. **Conclusion:** Together, the results are indicative of the proapoptotic and antitumor potential of cirsilineol against gastric cancer cells, suggestive of its possible therapeutic significance in future.

Key words: Anticancer, apoptosis, cirsilineol, flavonoid, gastric cancer

SUMMARY

- Cirsilineol inhibits gastric cancer viability in a dose-dependent manner.
- Cirsilineol inhibits colony formation of gastric cancer cells.
- Cirsilineol induces apoptotic cell death in gastric cancer cells.
- Cirsilineol enhances Bax and downregulates Bcl-2 expression in gastric cancer cells.



Abbreviations used: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide AO/EB: acridine orange/ethidium bromide PVDF: polyvinylidene fluoride

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INTRODUCTION

Gastric cancer, most frequently originating from the gastric mucosa, is one of the serious burdens of human health at the global level.[1] This malignancy is ranked as the fifth most common human cancer with 1 million annual cases worldwide.[2] In 2018, around 0.738 million people died of gastric cancer across the globe, making it the second leading cause of cancer-related mortalities.[3] Despite recent therapeutic advancements, the 5-year survival rate of gastric cancer continues to remain considerably low, ranging between 20% and 30% throughout the world.[4] The major reasons for the higher lethality of gastric cancer include the difficulty of its prognosis, metastasis, multidrug resistance, and disease recurrence.[5] There is thus an urgent need for the exploration and formulation of efficient preventive and therapeutic modalities against this devastating disorder.

Flavonoids constitute a class of plant secondary metabolites with vast pharmacological potential. These metabolites have been reported to exhibit numerous beneficial effects on human health.[6] They have been shown to possess antioxidant, antimicrobial, antimutagenic, cardioprotective, neuroprotective, and anti-inflammatory effects.[7-9] A number of studies have described the anticarcinogenic potential of several flavonoids derived from natural products against different types of human cancers.[10-12] The flavonoid molecules inhibit the growth of human cancer cells by tinkering with a diverse array of cell signaling pathways.[13] The anticancer role of flavonoids is largely exerted via cell cycle arrest and/or apoptosis.[14] The proapoptotic effects of several plant flavonoids are well established.[15] Besides, flavonoids have been suggested to emerge as lead molecules in cancer prevention and chemotherapy.[13]

Cirsilineol is a plant-based trimethoxy and dihydroxy flavone type of flavonoid secondary metabolite [Figure 1a], which is reportedly

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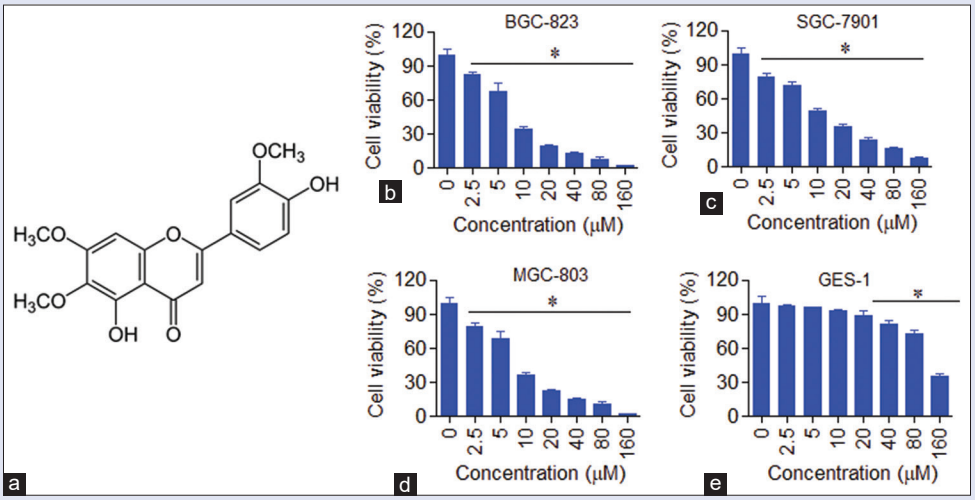

Figure 1: Cirsilineol inhibited the proliferation of gastric cancer cell lines. (a) Molecular structure of cirsilineol. MTT assay showing the viability of (b) BGC-823, (c) SGC-7901, and (d) MGC-803, and (e) normal gastric epithelial cell line (GES-1). Three replicates were used for conducting the experiments. Results are expressed as mean ± SD (*$P < 0.05$ for untreated vs. treated cells) MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, SD = standard deviation

found in *Artemisia vestita* and *Teucrium gnaphalodes*.[16] A number of studies have shown that cirsilineol exhibits remarkable antioxidant, anti-inflammatory, and anticancer potential.[17] Recently, it has been reported to halt proliferation of squamous lung carcinoma cells through reactive oxygen species (ROS)-mediated apoptosis.[18] Moreover, cirsilineol has been shown to possess gastroprotective effects against hydrochloric acid/ethanol-induced ulceration in rat models *in vivo*.[19] However, there is limited knowledge about the antiproliferative effects of cirsilineol on human gastric cancer cells. Therefore, herein, we investigate the antiproliferative effects of cirsilineol against human gastric cancer cells and explore the possible underlying molecular mechanisms.

MATERIALS AND METHODS

In vitro cell culture and treatment

Three different gastric cancer cell lines (BGC-823, MGC-803, and SGC-7901) and normal gastric epithelial cell line (GES-1) were procured from American Type Culture Collection (Manassas, Virginia, United States). Culturing of cell lines was carried out in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% Fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO_2 in a humidified incubator.

Cirsilineol was purchased from M/s Sigma-Aldrich, and its stock solutions were prepared in dimethyl sulfoxide (DMSO). Cirsilineol was diluted with DMSO to make the working treatment concentrations.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

The gastric cancer cell lines (BGC-823, MGC-803, and SGC-7901) and GES-1, gastric epithelial cells, were administered varying doses of cirsilineol in 96-well plates using an initial density of 3×10^4 cells per well. The cells were cultured for 24 h at 37°C with 5% CO_2. This was followed by addition of 10 μL of 2.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for another 4 h. Next, 200 μL of DMSO was added to dissolve the formazan crystals. Finally, absorbance was taken at 570 nm using enzyme-linked immunosorbent assay (ELISA) plate reader to determine cell viability.

Colony formation assay

For the assessment of their colony formation, 5×10^3 BGC-823 and MGC-803 cancer cells differentially treated with cirsilineol were cultured in six-well plates for a period of 15 days at 37°C. Afterward, the colonies formed were fixed with 70% ethanol and then stained using 0.2% crystal violet solution. After washing with Phosphate buffered saline (PBS), the colonies were examined and counted under an inverted microscope.

Acridine orange and ethidium bromide assay

BGC-823 and MGC-803 cancer cells were treated with 0, 4, or 8 μM cirsilineol at 37°C for 24 h. Next, the cells were collected through centrifugation. Then, PBS washing and subsequent fixation in 70% ethanol were carried out. The cells were then mixed with acridine orange and ethidium bromide (AO/EB) dual staining solution and subsequently assessed under a fluorescent microscope (Tokyo, Japan) for the assessing their morphology.

Annexin V/propidium iodide assay

The apoptosis of cirsilineol-treated BGC-823 and MGC-803 cancer cells was also investigated using the annexin V-FITC/propidium iodide (PI) staining assay. Following their treatment with cirsilineol, the cells were fixed with 4% paraformaldehyde and then subjected to dual annexin V-FITC/PI staining (Sigma-Aldrich). Finally, the cells were subjected to flow cytometry to examine the percent cell apoptosis.

Western blotting

After the total protein was extracted from cirsilineol-treated BGC-823 and MGC-803 cancer cells with Radioimmunoprecipitation assay buffer (RIPA) lysis and extraction buffer (Thermo Fisher Scientific), Braford assay was used for the quantification of protein lysates. The latter were equally loaded and separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. The proteins were transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Merck-Millipore) and the latter were blocked using 5% skim milk. The membrane was then incubated with the primary antibodies Bax (Santa Cruz Biotechnology (sc-7480); Santa Cruz, CA, USA), Bcl-2 (9664; Cell Signaling Technology), and Actin (sc-58673; Santa-Cruz, CA, USA) at 4°C overnight (dilution for all antibodies

1:1000). The proteins were then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (sc-2357-CM; Santa Cruz, CA, USA) for an hour. At last, chemiluminescence was used for the detection of specific protein bands. β-actin was used as an endogenous control in the western blot analysis.

Statistical analysis

Experiments were carried out in triplicates and the data were represented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Student's *t*-test were used for evaluating the significance of the results. *P* values <0.05 were considered to be statistically significant. GraphPad Prism software was used for performing the statistical analyses.

RESULTS

Gastric cancer cell proliferation was inhibited by cirsilineol

Three different gastric cancer cell lines (BGC-823, SGC-7901, and MGC-803) and normal GES-1 cells were administered with grades of cirsilineol doses ranging from 0 to 160 μM for 24 h to evaluate the antiproliferative effects of cirsilineol by MTT assay. It was found that cirsilineol [Figure 1a] treatment inhibited the growth of gastric cancer cell lines significantly ($P < 0.05$) and in a dose-dependent manner [Figure 1b–d]. The Half-maximal inhibitory concentration of cirsilineol against the human gastric cancer cells (BGC-823, SGC-7901, and MGC-803) ranged from 8 to 10 μM [Table 1]. Nonetheless, cirsilineol exhibited comparatively lower antiproliferative effects against normal GES-1 cells [Figure 1e]. The IC_{50} of cirsilineol against normal GES-1 cells was found to be 120 μM [Table 1]. These results suggest antiproliferative effects of cirsilineol against human gastric cancer cells.

Cirsilineol suppressed gastric cancer cell colony formation

To analyze the effect of cirsilineol treatment on the clonogenic capability of gastric cancer cells, BGC-823 and MGC-803 cancer cells were treated with 0, 4, or 8 μM cirsilineol. Colony formation of both the gastric cancer cell lines was found to decrease significantly ($P < 0.05$) with increase in treatment concentrations [Figure 2]. At 8 μM cirsilineol concentration, the colony percent cell formation was found to be less than 20% of control for both BGC-823 and MGC-803 cancer cells, suggestive of the antiproliferative effects of cirsilineol.

Cirsilineol induces apoptosis in gastric cancer cells

To unveil the possible mechanism for the antiproliferative effects of cirsilineol against gastric cancer cells, BGC-823 and MGC-803 cancer cells were treated with 0, 4, or 8 μM cirsilineol. The cells were then subjected to AO/EB double staining and subsequently examined under a fluorescent microscope. It was found that with increasing treatment concentrations, the number of BGC-823 and MGC-803 cells positively stained with EB increased, reflective of induction of cell death [Figure 3]. The green, orange, and red colors were representative of normal, early, and late apoptotic cells. To gain further support, the levels of apoptosis of BGC-823 and MGC-803 cancer cells differentially treated with cirsilineol were analyzed using Annexin V-FITC/PI staining followed by flow cytometry. The results indicated that percent cancer cell apoptosis increased with an increase in the treatment concentration of cirsilineol. The percentage of apoptosis increased from 7.4% in control to 40.5% in BGC-823 cells and from 6.56% in control to 33.53% in MGC-803 cells at 8 μM cirsilineol [Figure 4]. Taken together, these results suggest that involvement of apoptosis in cirsilineol induces antiproliferative effects on human gastric cancer cells.

Table 1: Effects of cirsilineol cells on the viability of normal and gastric cancer cell lines, expressed as IC_{50}

Cell line	IC_{50} (μM)
BGC-803	8
SGC-7901	10
MGC-803	8
GES-1	120

Cirsilineol modulated Bax/Bl-2 and caspase pathways in gastric cancer cells

Western blot analysis was performed to specifically assess the apoptotic pathways targeted by cirsilineol in gastric cancer cells. The results showed that treatment of BGC-823 and MGC-803 cancer cells with cirsilineol *in vitro* significantly ($P < 0.05$) increased the expression levels of Bax and cleaved caspase-3 protein [Figure 5]. On the other hand, cirsilineol treatment was shown to significantly ($P < 0.05$) repress the expression of Bcl-2 protein in a concentration-dependent manner *in vitro*.

DISCUSSION

Recent improvements in the cancer screening modalities, along with the advancement of therapeutic procedures have led to a slight decline in the overall incidence and mortality of gastric cancer. Nevertheless, it is still a major global health concern and accounts for the third highest number of cancer-based deaths worldwide.[20,21] The disease management particularly becomes challenging at advanced stages of tumorigenesis, and thus necessitates identification of novel therapeutic agents.[22] In this direction, the researchers are actively engaged in the search for natural products with anticancer potential against human gastric cancer. Moreover, identification of natural product-based anticancer therapeutic agents is gaining more and more support because of their free availability and being relatively safe on normal human cells.[23] Cirsilineol, a plant-based flavanone, has been shown to display a number of pharmacological and health benefits.[23] It has been reported to show immunomodulatory properties in suppressing the onset of inflammatory bowel disease through selective inhibition of interferon-gamma (IFN-γ) signaling pathway.[24] Again, this molecule reportedly minimizes Lipopolysaccharide (LPS)-mediated inflammation both *in vitro* and *in vivo* by targeting the TLR-4/NF-kB/IKK signaling pathway.[25] Additionally, cirsilineol exhibits protective function against ovalbumin-induced allergic rhinitis via modulation of inflammatory and oxidative stresses.[26] Moreover, the compound is also known for its gastroprotective function.[19] The antiproliferative action of cirsilineol has also been explored against human cancer cells by induction of apoptosis.[27] In one of the recent studies, the molecule was shown to suppress proliferation of human squamous lung cancer cells through ROS-driven apoptosis.[18] In the present study, cirsilineol was found to inhibit the growth of human gastric cancer cells, while it showed marginal effects on normal cell viability. In coherence with the previous studies, the gastric cancer cells were shown to be inducted with apoptosis under cirsilineol administration. The aberrant cellular programming of apoptosis is one of the major factors responsible for extended survival and malignant behavior of human cancer cells, and restoration of the same has been reported to emerge as a vital lead in cancer management in future.[28,29] As per the current study results, cirsilineol modulated the expression of Bax and Bcl-2 proteins as well as augmented caspase-3 cleavage to signal the

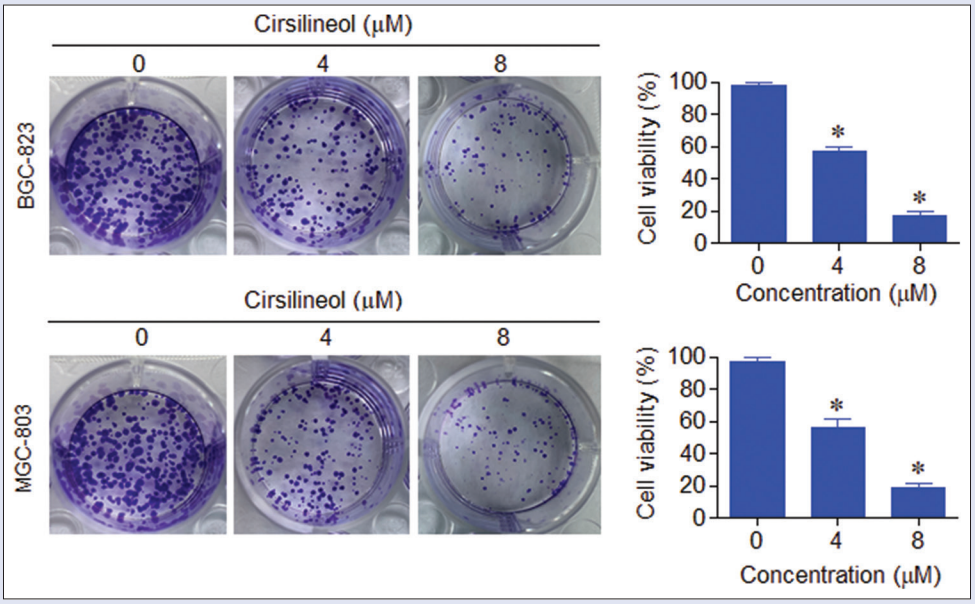

Figure 2: Cirsilineol suppressed gastric cancer cell clonogenicity. Clonogenic colony formation assay of BGC-823 and MGC-803 gastric cancer cells treated with 0, 4, or 8 μM cirsilineol. The assay was performed in triplicates, and results are given as mean ± SD ($P < 0.05$ for untreated vs. treated cells) SD = standard deviation

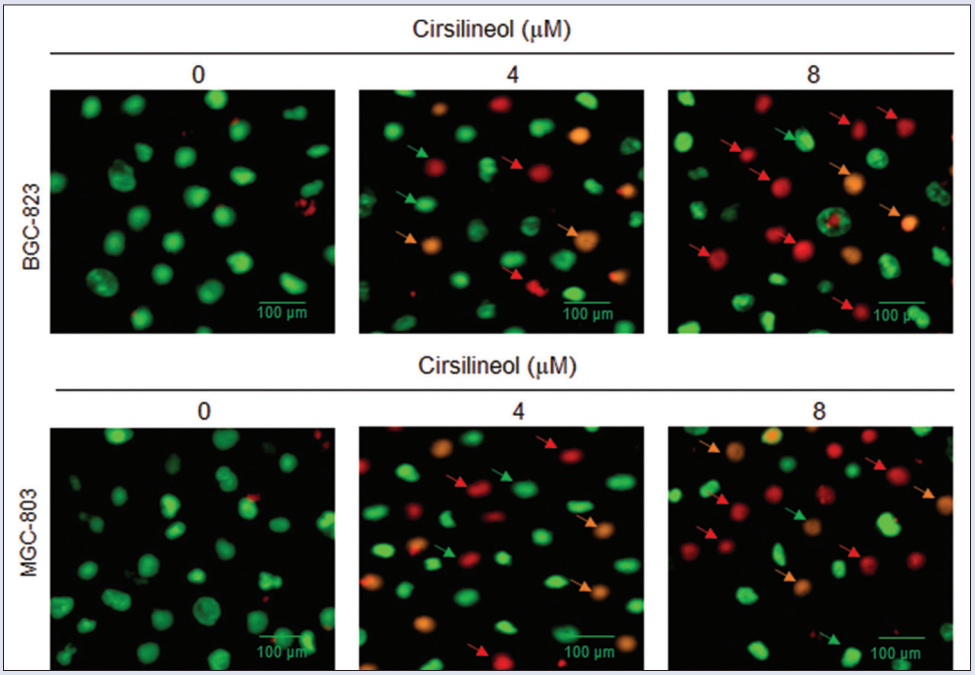

Figure 3: Cirsilineol induced apoptosis in gastric cancer cells *in vitro*. AO/EB staining analysis of BGC-823 and MGC-803 gastric cancer cells treated with 0, 4, or 8 μM cirsilineol. Green arrows correspond to the normal cells, while orange and red arrows represent the early and late apoptotic cells, respectively AO/EB = acridine orange/ethidium bromide

induction of apoptosis in gastric cancer cells. Bax and Bcl-2 belong to the Bcl-2 subfamily of apoptosis-related proteins, whose expression markedly acts as a switch for cell apoptosis, and the relay is exerted through the caspase protein cascade.[30] Summing up, the results indicate that cirsilineol has proapoptotic effect on human gastric cancer cells, which highlights its possible therapeutic utility against the growth and progression of the deadly disease of gastric cancer. However, further studies directed at the evaluation of anticancer effects of cirsilineol against more gastric cancer cells lines and under *in vivo* conditions are urgently required.

CONCLUSION

The results of the present study are conclusive of the antiproliferative potential of cirsilineol against gastric cancer cells. Administration of

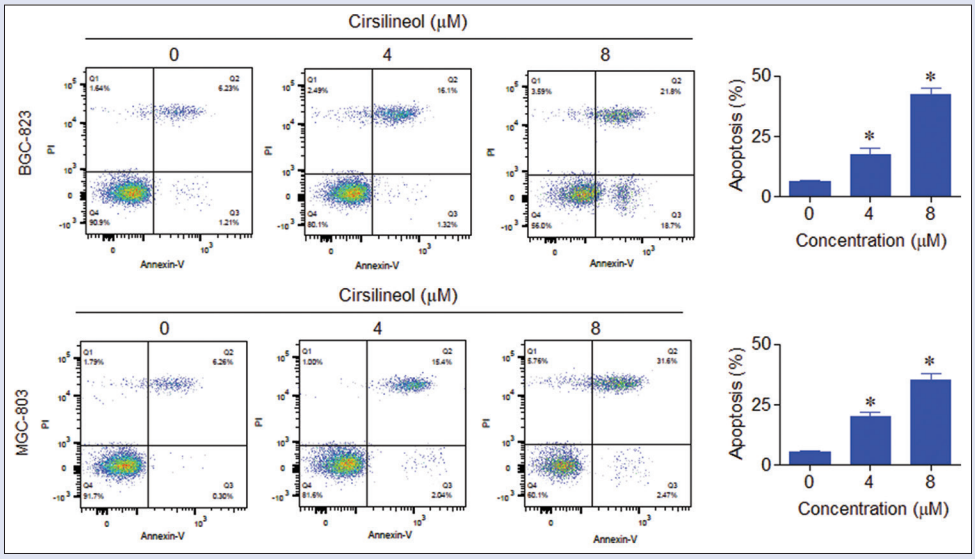

Figure 4: Analysis of gastric cancer cell apoptosis induction by cirsilineol using Annexin V-FITC/PI double staining. BGC-823 and MGC-803 gastric cancer cells treated with 0, 4, or 8 μM cirsilineol were doubly stained with an equimolar staining solution of Annexin V-FITC and PI, following which flow cytometry was conducted to determine the relative percentage of cell apoptosis. The apoptosis assay was performed in triplicates, and results are given as mean ± SD (*P < 0.05 for untreated vs. treated cells) PI = propidium iodide, SD = standard deviation

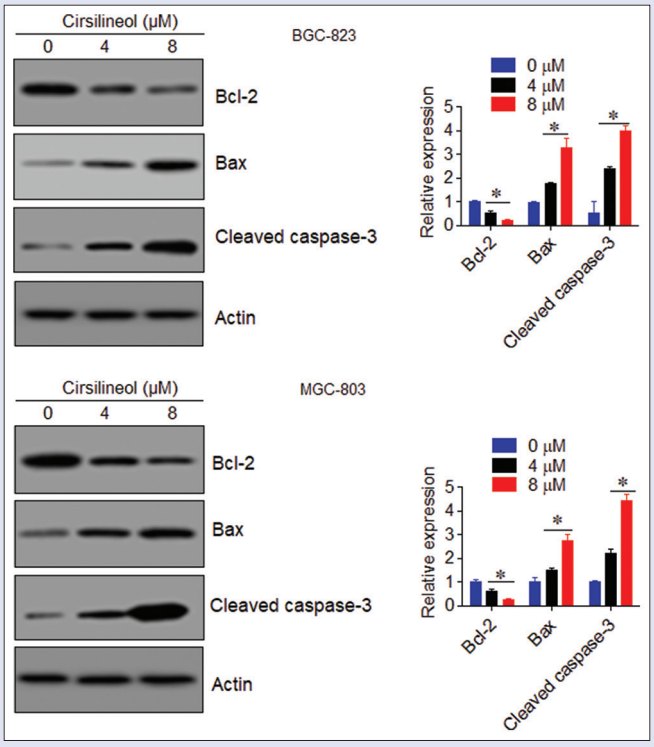

Figure 5: Cirsilineol modulated Bax/Bcl-2 and cleaved caspase-3 expression levels in gastric cancer cells. Western blotting of Bax, Bcl-2, and cleaved caspase-3 proteins from BGC-823 and MGC-803 gastric cancer cells treated with 0, 4, or 8 μM cirsilineol. The expression analyses were performed using three replicates, and results are presented as mean ± SD (*P < 0.05 for untreated vs. treated cells) SD = standard deviation

cirsilineol decreased the viability of gastric cancer cells *in vitro* selectively. Apoptosis was found to be induced in gastric cancer cells supplemented with cirsilineol. The antiproliferative efficacy of cirsilineol might be improved through semi-synthetic chemistry approaches to possibly use it as a lead chemotherapy molecule against gastric cancer in future.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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