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Piperine Inhibits Cell Proliferation and Induces Apoptosis of Human Gastric Cancer Cells by Downregulating Phosphatidylinositol 3-Kinase (PI3K)/Akt Pathway

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Data Interpretation D
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Background: Piperine has been reported to inhibit proliferation and induce apoptosis in various cancer cells. This study aimed to explore the efficacy and underlying mechanism of piperine in human gastric cancer.

Material/Methods: MTT assay was performed to examine the effect of piperine (concentrations of 0–300 μM) on the proliferation of human gastric cancer SNU-16 cells and normal human gastric epithelial GES-1 cells. Flow cytometry and Western blot were used to determine cell apoptosis and the expression level of protein (Cyto C, cleaved PARP, cleaved caspase-3, Bax, Bcl-2, Bad, Bcl-xl, PI3K, pPI3K, Akt, and pAkt), respectively. To further investigate the anti-tumor mechanism of piperine in SNU-16 cells, we used a small-molecule Akt activator SC79 in this study. The *in vivo* mechanism of piperine against gastric cancer was evaluated using a xenograft tumor model.

Results: The results showed that piperine inhibited proliferation and induced apoptosis of SNU-16 cells. Piperine up-regulated the protein expression of Bax, Bad, Cyto C, cleaved PARP, and cleaved caspase-3, but downregulated the protein expression of Bcl-2, Bcl-xl, pPI3k, and pAkt. However, SC79 reversed the function of piperine on the apoptosis-related proteins. An *in vivo* study revealed that, compared with the control group, the tumor volume of mice treated with piperine was significantly reduced. Piperine enhanced cleaved caspase-3 expression but decreased Ki-67 expression in a dose-dependent manner. Moreover, the nontoxicity effect of piperine was confirmed by H&E staining analysis in kidney and heart tissues of mice.

Conclusions: Our findings suggest that piperine inhibits proliferation and induces apoptosis of human gastric cancer cells through inhibition of the PI3K/Akt signaling pathway.

MeSH Keywords: **Apoptosis • Phosphatidylinositol 3-Kinase • Piper nigrum • Stomach Neoplasms**

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Background

Gastric cancer is one of the most common malignancies worldwide, with high morbidity and mortality rates [1]. It has been reported that the 5-year overall survival rate in early gastric cancer patients exceeds 90%, while the median overall survival rate in patients with metastatic gastric cancer is rarely more than 1 year [2]. Currently, several risk factors for gastric cancer progression have been well-defined, such as *Helicobacter pylori* infection, radiation and smoking [3–5]. As the main therapy strategies for gastric cancer, surgery, chemotherapy and radiotherapy have unsatisfactory therapeutic effects, including poor prognosis and tumor recurrence [6]. Therefore, it is of great urgency to find out better anti-cancer agents with greater efficacy and fewer adverse effects for the treatment of gastric cancer.

Piperine (Figure 1A), an alkaloid extracted from the genus *peperum*, has been widely used for daily consumption and is commonly used in traditional medicine [7]. Recent studies have shown that piperine has anti-oxidation [8], anti-depression [9], anti-tumor [10], and anti-inflammation [11] effects. A study conducted by Shaheer et al. has revealed that piperine pretreatment can suppress proliferation and activate apoptosis, thereby enhancing radiosensitization of colon cancer cells [12]. One recent study has demonstrated that piperine-induced apoptosis activates caspase-3, caspase-9, and cleaved PARP via the JNK/p38 MAPK pathway in ovarian cancer [10]. However, the role and underlying mechanism of piperine in gastric cancer remain unclear.

The present study used human gastric cancer SNU-16 cells to explore the mechanism underlying the effect of piperine on proliferation, apoptosis, and invasion of SNU-16 cells *in vitro* and *in vivo*, thereby providing a safe and potential anti-cancer agent for the treatment of gastric cancer.

Material and Methods

Cell culture

Human gastric cancer SNU-16 cells and gastric epithelial GES-1 cells were obtained from the Cell Biology Institute of the Chinese Academy of Sciences. The SNU-16 and GES-1 cells at a suitable concentration were cultured in RPMI 1640 media (Gibco, Eggenstein, Germany) supplemented with 10% fetal bovine serum (Gibco, USA), penicillin (100 U/ml), and streptomycin (100 mg/ml) in a humidified atmosphere of 5% CO₂ in room air at 37°C. The culture medium was changed regularly every 2 days.

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) assay was performed to investigate

cell viability. SNU-16 and GES-1 cells were seeded onto 96-well plates at a density of 5×10³ cells per well. After 24 h of incubation at 37°C, culture medium was added with piperine (5, 10, 20, 50, 100, 200, and 300 μM; Sigma, USA) or left untreated (control) for 48 h. Next, MTT solution was added to each well and incubated at 37°C for 4 h. Later, dimethyl sulfoxide (DMSO; Sigma, USA) was added to each well to stop the reaction, and the absorbance was measured at 450 nm.

Cell apoptosis determination

SNU-16 cells were exposed to piperine (50, 100, and 150 μM) or left untreated (control) for 48 h in 6-well culture plates containing 5×10⁵ cells per well. In addition, SNU-16 cells were pretreated with caspase inhibitor Z-VAD-FMK (20 μM) for 1 h, followed by addition of 150 μM of piperine. Then, cell suspensions were prepared by trypsinization (Sino-American Hua Mei Biotechnology Company, Beijing, China) and were centrifuged at 3000×g for 5 min at 4°C. After that, the cell pellet was resuspended in 500 μl binding buffer and the apoptosis rate was examined by annexin V-FITC/PI (fluorescein isothiocyanate/propidium iodide) dual staining apoptosis detection kit (Nanjing KeyGEN Biotech. Co. Ltd., China) according to the instructions of the manufacturer. Then, flow cytometric analysis was performed using a Becton-Dickinson FACS Calibur flow cytometer (Franklin Lakes, NJ, USA), and data were analyzed by Cell Quest software. Before treatment with 150 μM of piperine for 48 h, SNU-16 cells were treated with SC79 (a small-molecule Akt activator, Sigma, USA) or not [13] for a final concentration of 10 μM for 1 h. All experiments were performed 3 times.

Caspase-3 activity assay

Caspase-3 activity in cell lysates was determined using a caspase-3 activity kit (Beyotime Institute of Biotechnology, Nantong, China). Activity levels were normalized to total protein levels of the corresponding cell pellet and expressed as percentage of treated cells to that of control.

Western blot analysis

SNU-16 cells were treated with piperine (50, 100, and 150 μM) or left untreated (control). After 48 h, cells were harvested with trypsinization and washed with cold PBS (pH 7.2–7.4), followed by lysis in ice-cold lysis buffer for 30 min and centrifugation at 12 000×g for 10 min at 4°C to remove cell debris. The total protein concentration was quantified by BCA protein assay kit (Pierce Life Science, Rockford, IL, USA). The same amount of each protein lysate (15 μl) was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (KeyGEN, Nanjing, China). Then, the membranes were

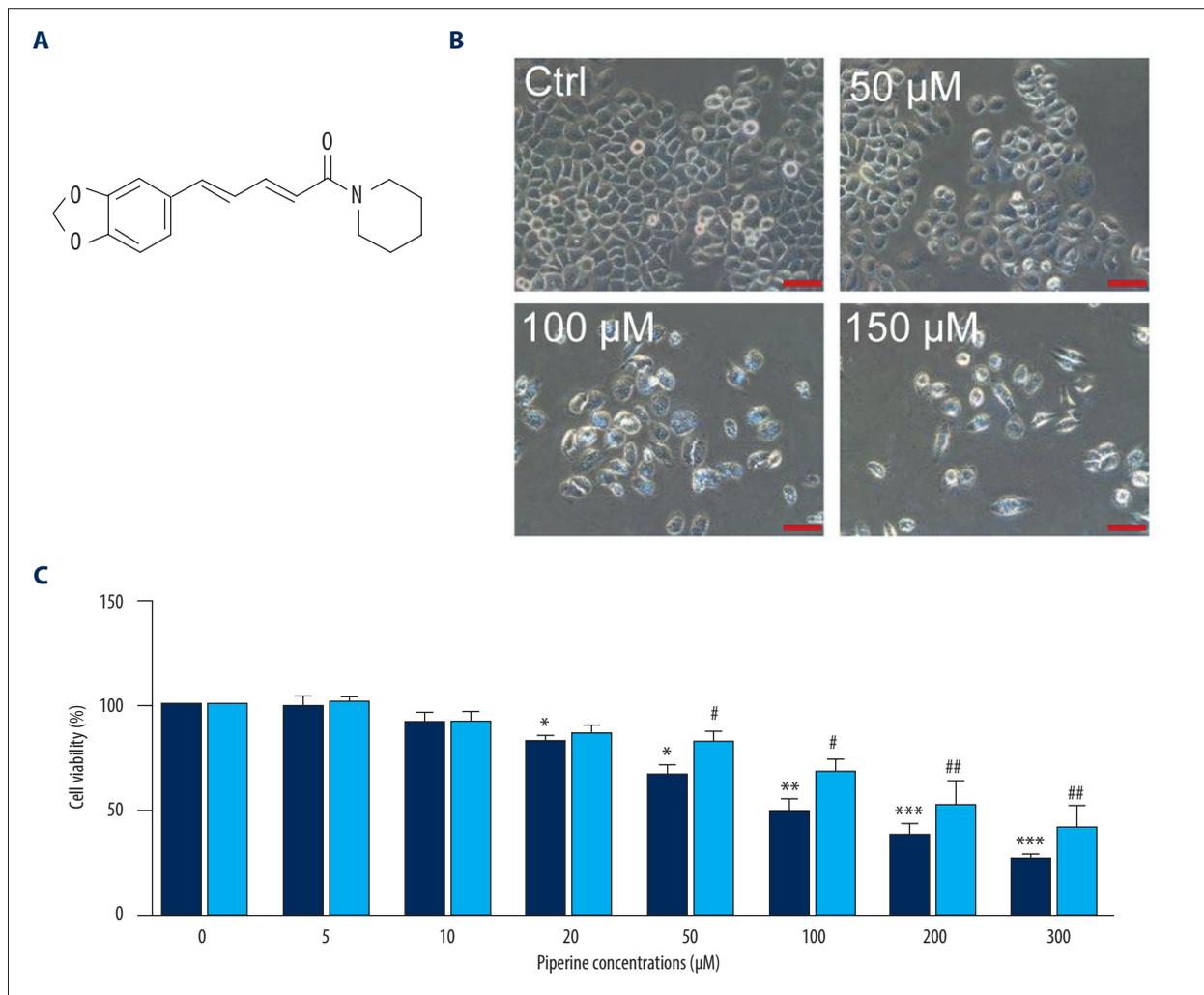


Figure 1. Inhibitory effect of piperine on the growth of human gastric cancer SNU-16 cells. **(A)** The structure of piperine. **(B)** Morphological view of live and dead SNU-16 cells treated with 50–150 μM of piperine. Photomicrographs were taken by inverted phase-contrast microscope (scale bar=50 μm). **(C)** Growth inhibition rates of SNU-16 cells and normal human gastric epithelial GES-1 cells treated with piperine at indicated concentrations for 24 h. Data are presented as the mean \pm SD of at least 3 independent experiments, * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ compared with control SNU-16 cells. # $P<0.05$ and ## $P<0.01$ compared with control GES-1 cells.

blocked with 5% non-fat milk for 2 h and washed 3 times in Tris-buffered saline containing Tween 20 (TBST). To probe for cytochrome c (Cyto C), cleaved PARP, cleaved caspase-3, Bax, Bcl-2, Bad, Bcl-xl, PI3K, pPI3K, Akt, pAkt, and GAPDH (Boster Biological Technology, Inc., Wuhan, China), the membranes were incubated at 4°C with the relevant antibodies overnight. After 3 washes in TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies at room temperature for 1 h. Signals were detected by ECL Plus Chemiluminescence kit (Beyotime Biotechnology, Nanjing, China) on X-ray film. The bands were visualized by Gel-Pro 32 software (Media Cybernetics, Inc., Rockville, MD, USA) and GAPDH as a loading control.

In addition, SC79 10 μM was added or not to the medium of SNU-16 cells for 1 h before piperine 150 μM was treated, and the procedures of western blot analysis were performed as described. All the experiments were done in triplicate.

Tumor xenograft model

A total of 30 BALB/c nude mice weighing 20–21 g were purchased from Hangzhou Ziyuan Experimental Animal Technology Co. and maintained in specific pathogen-free conditions. All animal work was performed at the Second Affiliated Hospital of Wenzhou Medical University. The mice were housed with free access to a commercial diet and water under specific pathogen-free conditions. SNU-16 cells (5×10^6 cells in a volume of

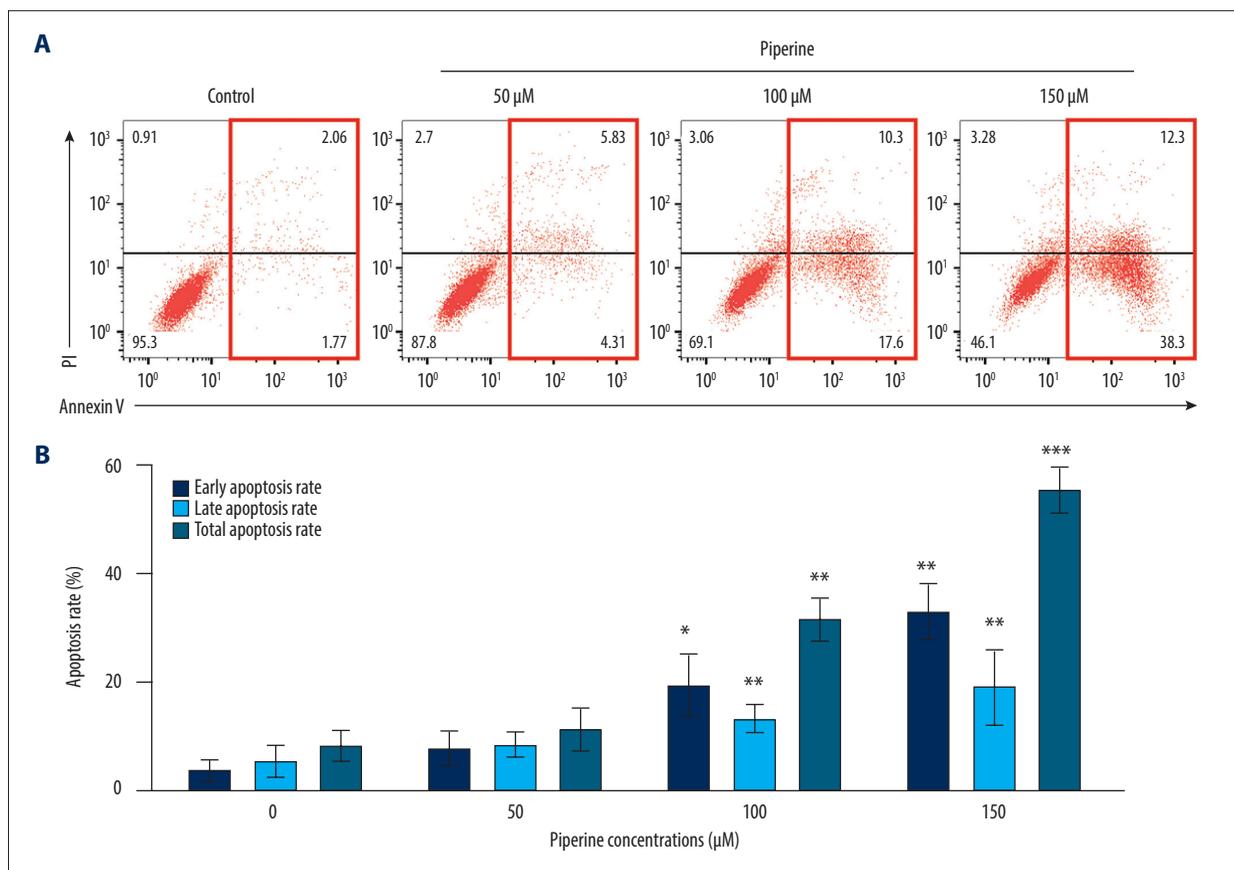


Figure 2. Piperine induces the apoptosis of SNU-16 cells. **(A)** SNU-16 cells were exposed to piperine (50, 100, 150 μM) for 24 h. Percentage of cell apoptosis was determined by annexin V/PI staining and flow cytometry. **(B)** The apoptotic rates of SNU-16 cells induced by piperine. Data are presented as the mean±SD of at least 3 independent experiments, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with control cells.

0.1 mL) were implanted in the groin of BALB/c mice. When tumors reached a volume of 100-200 mm³ after 12 days, mice were randomly divided into 3 groups (10 mice in each group): a control group (1% DMSO, once a day), a low-dose group (30 mg/kg of piperine, once a day), and a high-dose group (60 mg/kg of piperine, once a day). The body weight and tumor volume of mice were measured every 2 days. Tumor volume was calculated by the following formula: $(\text{length} \times \text{width}^2) / 2$. On day 30, all mice were sacrificed (dislocation of cervical vertebra) under anesthesia using pentobarbital sodium (35 mg/kg). Then, their serum samples were aliquoted into sterile tubes and centrifuged at 1100×g for 10 min at room temperature for subsequent analysis. The alanine transaminase (ALT) and aspartate transaminase (AST) activities were determined by VetScan analyzer (Abaxis, Inc.). As previously described [14], immunohistochemistry (IHC) staining was used to determine the expression of Ki-67 and cleaved-CASP3, and hematoxylin and eosin (H&E) staining was used to examine the histology of the harvested hearts and kidneys of mice. In addition, relative hepatic indexes were tested. Animal experiments were approved by the Ethics Committee of Wenzhou Medical University (wydw 2020-0799).

Statistical analysis

Data are presented as mean±standard deviation. All data were analyzed by SPSS 20.0 (SPSS Inc., Chicago, IL, USA). We used one-way ANOVA followed by Dunnett's post hoc test when comparing more than 2 groups of data, and one-way ANOVA and non-parametric Kruskal-Wallis test followed by Dunn's post hoc test when comparing multiple independent groups. P values less than 0.05 were regarded as statistically significant.

Results

Piperine inhibited the proliferation and induced the apoptosis of SNU-16 cells

MTT was performed to evaluate the effect of piperine on the proliferation of SNU-16 cells and GES-1 cells. Compared with the untreated cells, piperine inhibited the growth of SNU-16 and GES-1 cells in a dose-dependent manner (Figure 1B). Phase-contrast photomicrographs of SNU-16 cells indicated

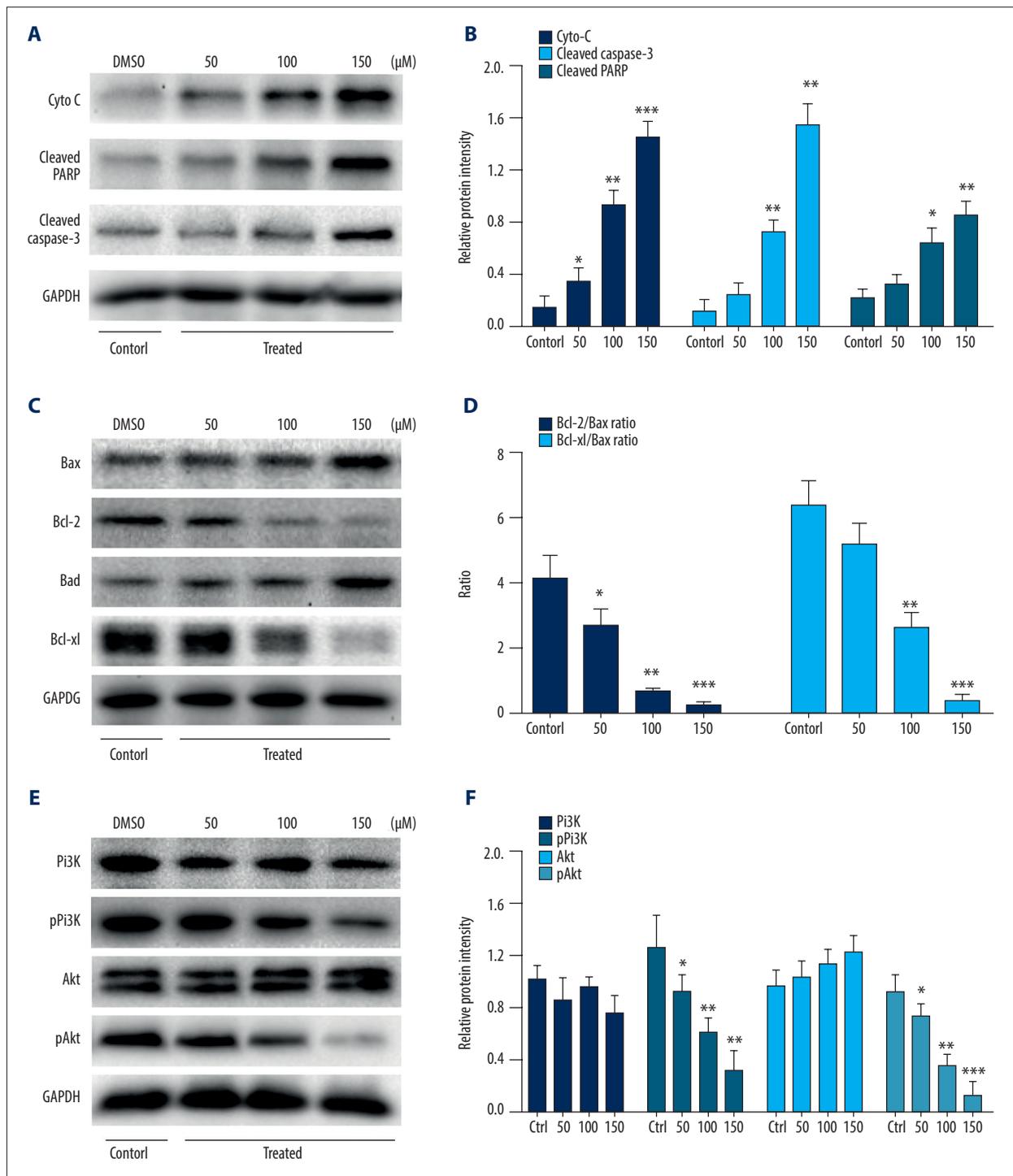
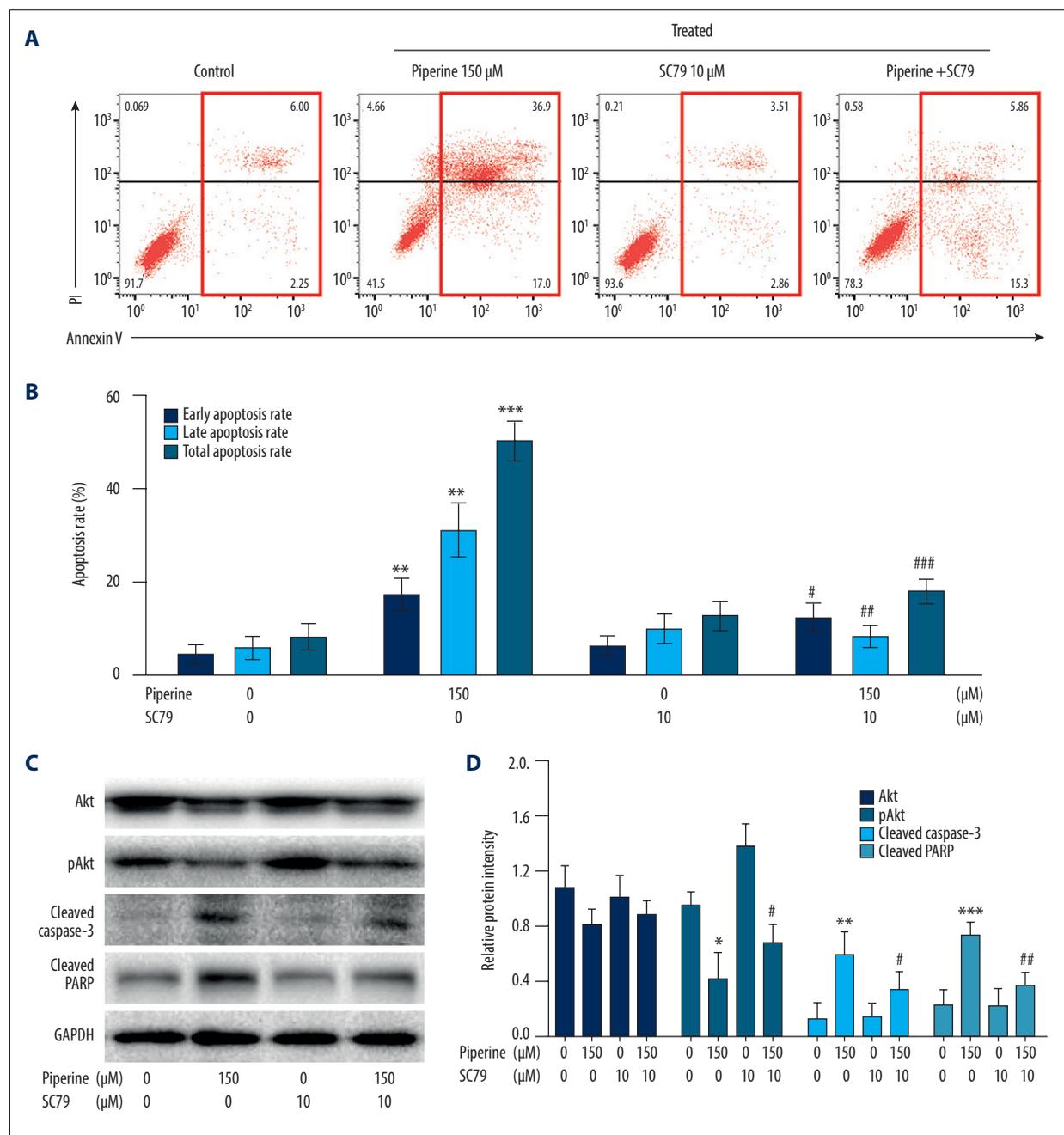


Figure 3. Effect of piperine on changes of apoptosis-related proteins. SNU-16 cells were treated with piperine (0, 50, 100, and 150 µM) for 18 h before analysis. GAPDH was used as loading control. **(A)** Western blot bands for Cyto C, cleaved caspase-3, and cleaved PARP proteins. **(B)** Quantification of Cyto C, cleaved caspase-3, and cleaved PARP proteins. **(C)** Expression of Bcl-2, Bax, Bad, and Bcl-xl proteins was determined by western blot. **(D)** Quantification of Bcl-2/Bax, Bax, Bad, and Bcl-xl proteins. The Bcl-2/Bax and Bcl-xl/Bad ratio were downregulated by piperine treatment in SNU-16 cells. **(E)** Expression of pPI3K, PI3K, Akt, and pAkt proteins after piperine treatment was determined by western blot. **(F)** Quantification of pPI3K, PI3K, Akt and pAkt proteins. Data was presented as the mean ±SD of at least 3 independent experiments, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with control cells.

that cells treated with piperine became round and finally detached (Figure 1C). Then, annexin V/PI staining was used to detect whether piperine can induce apoptosis in SNU-16 cells. Figure 2 shows that the percentage of apoptotic cells is dose-dependently increased after piperine treatment. Apoptotic cells in early and late stages increased significantly at the dose of 150 μM , and the total percentage of apoptotic cells in 150 μM of piperine was up to 50.6%. Together, these data demonstrated that piperine was cytotoxic to gastric cancer cells.

Piperine promoted apoptosis via activation of caspase-3 and regulation of Bcl-2 family proteins

To examine whether piperine induces SNU-16 cell apoptosis by activating caspase enzyme, caspase-3 activity and Cyto C level were measured by MTT and western blot. Figure 3A and 3B showed that piperine significantly upregulated cleaved caspase-3 expression and augmented Cyto C level. Piperine also increased caspase-3 activity in SNU-16 cells in a dose-dependent manner and induced cell apoptosis. However, co-treatment of caspase-3 inhibitor Z-VAD-FMK blocked cell apoptosis



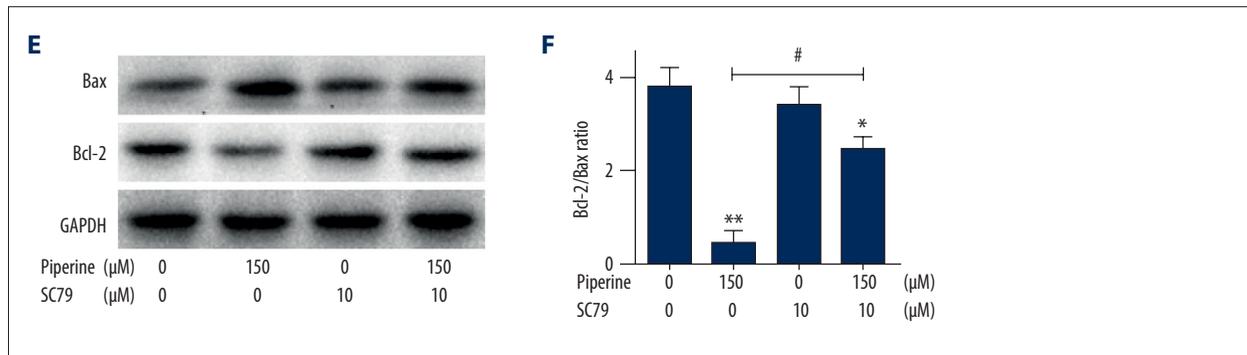
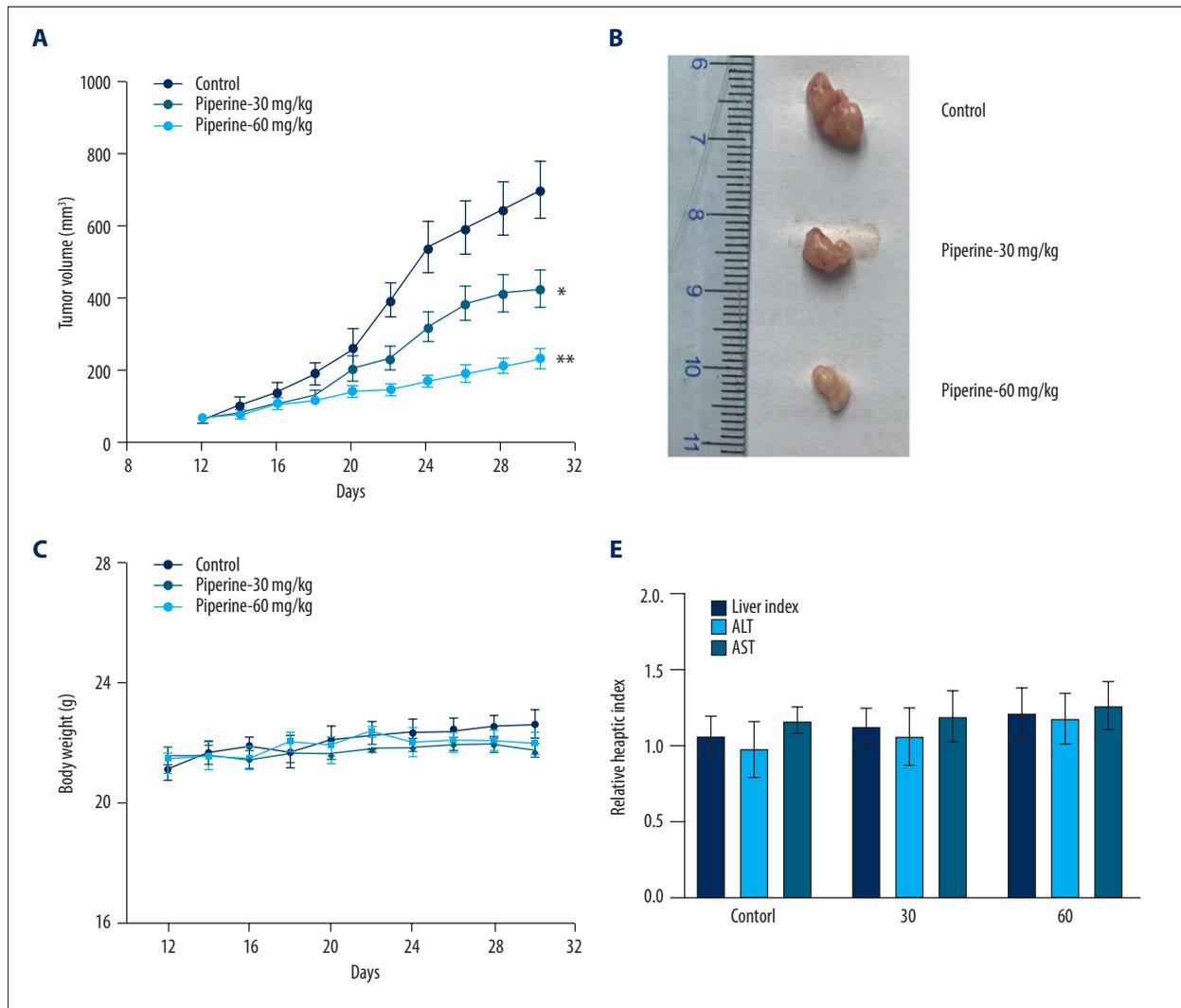


Figure 4. The efficacy of Akt activator (SC79) on piperine-induced apoptosis. SNU-16 cells were determined after treatment with piperine for 24 h in the presence or absence of SC79 (10 μM). (A) Apoptosis detection with annexin V/PI double staining in different groups by flow cytometry. (B) Column bar graph of mean cell florescence for early apoptotic, late apoptotic, and total apoptotic cells. (C) Expression of pAkt, Akt, cleaved caspase3, and cleaved PARP proteins in the presence or absence of SC79. (D) Quantification of pAkt, Akt, cleaved caspase3, and cleaved PARP proteins. (E) Expression of Bcl-2 and Bax proteins after piperine and/or SC79 treatment. (F) Quantification of Bcl-2 and Bax proteins. The Bcl-2/Bax ratio was significantly upregulated in the presence of SC79. Data are presented as the mean±SD of at least 3 independent experiments, * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with control cells; # $P<0.05$, ## $P<0.01$ and ### $P<0.001$ compared with the piperine alone group.



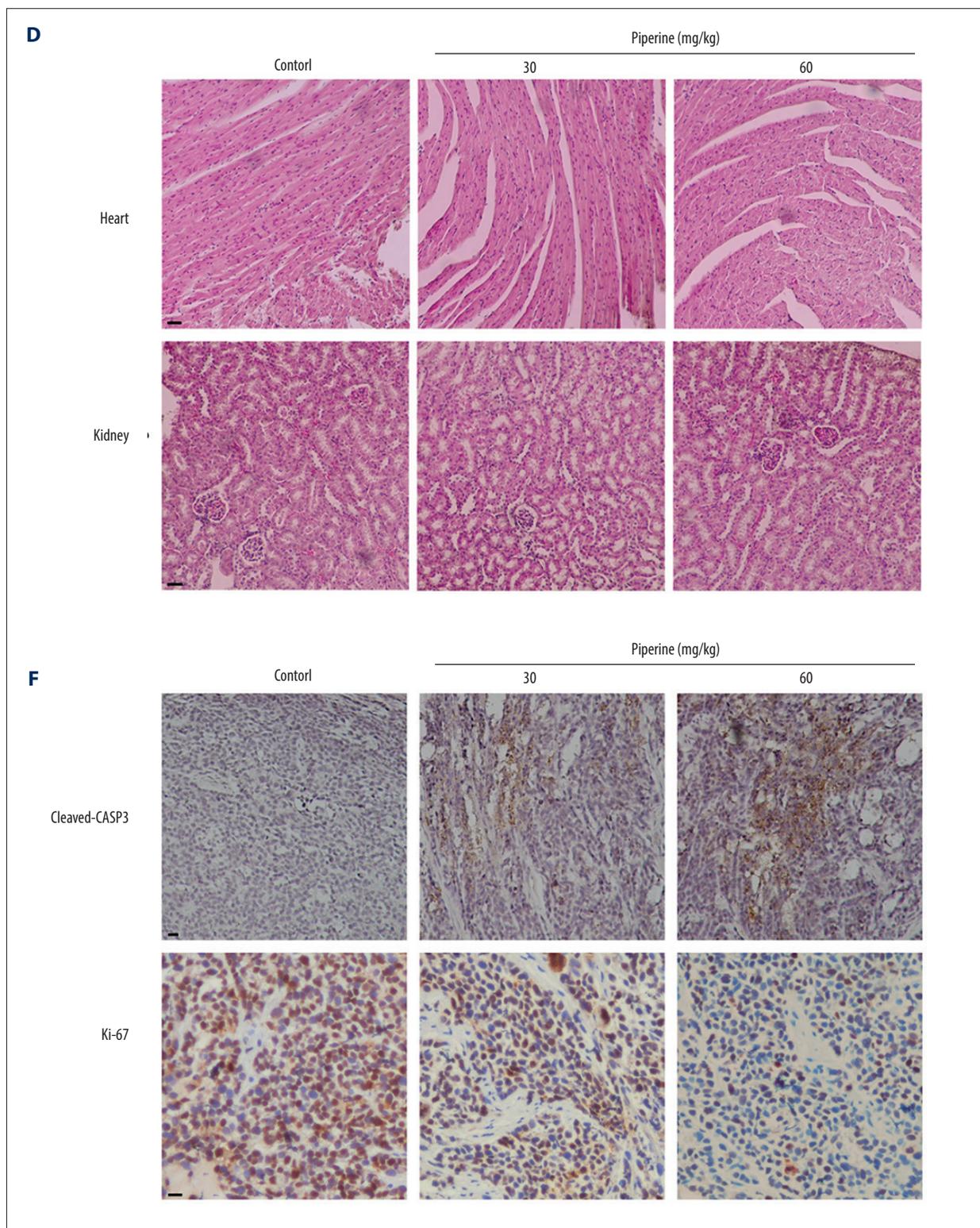


Figure 5. Piperine inhibited SNU-16 xenograft tumor growth *in vivo*. Gastric cancer SNU-16 (A) tumor volume and (B) harvested tumor in mice after treatment with piperine. (C) Weight change curves of animals in different groups. (D) The histology of heart and kidney *in vivo* (scar bar=50 μ m). (E) The values of relative hepatic indexes. (F) Expression of caspase-3 and Ki-67 after piperine treatment *in vivo* (scar bar=40 μ m). Data are presented as the mean \pm SD, * $P<0.05$ and ** $P<0.01$ compared with control group.

(Supplementary Figure 1). As a major indicator of apoptosis, PARP is the substrate of caspase-3, and the activation of cleaved caspase-3 can induce cleavage of PARP during cell apoptosis [15]. As expected, cells treated with piperine showed dose-dependent enhancement of cleaved PARP level (Figure 3A, 3B).

Since Bcl-2 family proteins play a crucial role in balancing apoptosis [16], western blot analysis was performed to analyze the expression of Bax, Bad, Bcl-2, and Bcl-xl. Results showed that piperine led to downregulated expression of Bcl-2 and Bcl-xl and upregulated expression of Bax and Bad ($P < 0.05$) (Figure 3C, 3D). These results revealed that piperine promoted cell apoptosis mainly through the intrinsic (mitochondria) pathway.

Piperine induced apoptosis through inhibition of PI3K/Akt pathway

To investigate the relationship between the PI3K/Akt signaling pathway and piperine-induced apoptosis, western blot analysis was utilized to measure the expression level of related proteins (PI3k, pPI3k, Akt, pAkt). Compared with the control group, treatment with piperine (50, 100, and 150 μM) dose-dependently decreased the expression of pPI3K and pAkt in SNU-16 cells. However, no obvious change was observed in the PI3K and Akt expression (Figure 3E, 3F).

To further investigate the association between cell apoptosis and the PI3K/Akt signaling pathway, a small-molecule Akt activator SC79 was used. Annexin V/PI staining assay showed that SC-79 (10 μM) pretreatment abolished the pro-apoptotic effect of piperine (Figure 4A, 4B). Compared with the piperine alone group, SC79 downregulated the protein expression of cleaved caspase-3 and cleaved PARP but upregulated Bcl-2 and p-Akt protein expression (Figure 4C–4F). Taken together, these findings indicated that piperine facilitated apoptosis through inhibition of the PI3K/Akt signaling pathway in SNU-16 cells.

Piperine inhibited SNU-16 xenograft tumor growth *in vivo*

To further analyze the antitumor effect of piperine, a xenograft tumor model was established by transplanting SNU-16 cells into the groins of mice. Compared with the control group, significant inhibition of the growth of xenograft tumors was noticed after piperine (30 and 60 mg/kg) treatment (Figure 5A, 5B). Interestingly, there was no significant change of body weight in the control group and piperine-treated groups, suggesting that piperine exhibited no obvious toxicity in the 18-day treatment (Figure 5C). In addition, the nontoxicity effect was confirmed by H&E staining of the kidneys and hearts of mice (Figure 5D). Furthermore, the liver indexes and serum liver makers ALT and AST were tested, and there were no significant changes among the 3 groups (Figure 5E). As additional markers of growth and apoptosis, Ki-67 and cleaved caspase-3

expression were investigated using IHC staining. Piperine resulted in a significant reduction in Ki-67 expression and increase in cleaved caspase-3 expression (Figure 5F). Overall, these data show that piperine has anti-tumor activity and high safety *in vivo*.

Discussion

Due to environmental pollution caused by industrial production, human living habits, and other factors, the incidence of various cancers is increasing year by year [17]. To date, researchers have devoted great efforts in the search for drugs that maximize efficacy and minimize adverse effects in the treatment of cancers [18]. Several studies have shown that piperine, the major active chemical ingredient in pepper, has anti-cancer, anti-inflammatory, and analgesia effects, which makes it of great interest in cancer treatment [19,20]. Piperine has been reported to have anti-cancer effects in various tumors, but little is known about its function on human gastric cancer.

In the present study, we investigated the impact of piperine on gastric cancer both *in vitro* and *in vivo*, and found that piperine inhibited proliferation and induced apoptosis of SNU-16 cells in a dose-dependent manner through inhibition of the PI3K/Akt pathway and activation of the mitochondria-mediated pathway. Piperine also dose-dependently reduced tumor volume in mice, with high safety.

Mitochondria are important organelles that participate in the regulation of cell apoptosis [21]. As members of the Bcl-2 family, pro-apoptotic proteins (Bax and Bad) and anti-apoptotic proteins (Bcl-2 and Bcl-xl) play a significant role in modulating mitochondrial membrane permeability [22]. Chan et al. proved that high expression of Bax could destroy mitochondrial integrity and induce cell apoptosis [23]. We speculated that the pro-apoptotic effect of piperine in gastric cancer has a relationship with the mitochondrial signaling pathway. To test this idea, western blot analysis was performed to determine the relationship between piperine and pro-apoptotic and anti-apoptotic Bcl-2 members. We found that piperine downregulated Bcl-2 and Bcl-xl protein expression, but upregulated Bax and Bad protein expression.

Fujitani et al. reported that oxidative stress caused by accumulation of excessive reactive oxygen species (ROS) could significantly limit the growth of cancer cells [24]. In animal cells, mitochondria have long been recognized as central players in ROS-dependent apoptotic cell death [25]. Mitochondrial dysfunction under the mediation of apoptotic stimulation factors such as ROS can promote Cyto C release, thereby resulting in either caspase-dependent or caspase-independent apoptosis [26]. Caspases play a vital part in the process of

cell apoptosis, among which caspase-3 is a cell death protease that is often activated [27]. The change in Bax/Bcl-2 ratio can initiate caspase signaling, and when caspases is activated, it can destroy the cellular structure and eventually lead to cell death [28]. Cleaved PARP is a major indicator of cell apoptosis, and activation of caspase-3 during apoptosis leads to cleavage of PARP [29]. In this study, we found that the expression levels of Cyto C, cleaved caspases-3, and cleaved PARP were significantly increased after piperine treatment, revealing that a decreased ratio of Bcl-2/Bax and Bcl-xl/Bad greatly upregulated the expression of Cyto C, cleaved caspase-3, and cleaved PARP, and eventually induced apoptosis.

The PI3K/Akt signaling pathway plays a significant role in regulating proliferation and apoptosis of cells [30]. Akt, also known as protein kinase B (PKB), is one of the major downstream targets of PI3K [31]. The PI3K/Akt signaling pathway regulates the progression of various cancers by increasing the anti-apoptotic activity of Akt. Phosphorylation of Akt usually increases the expression of Bcl-2 and Bcl-xl and decreases Bax and Bad expression. Recently, many researches have reported that Akt overexpression is related with tumor progression and poor prognosis in human gastric cancer, suggesting that the PI3K/Akt pathway is a potential target for the treatment of gastric cancer [32,33]. Our data indicated that the diminished Bcl-2/Bax and Bcl-xl/Bad ratio and the enhanced Cyto C, cleaved PARP, and cleaved caspase-3 expression were mediated by downregulating the PI3K/Akt pathway after piperine treatment, but cell apoptosis induced by piperine was attenuated after combination treatment of Akt activator SC79. As mention before, piperine can induce cell death via the JNK/p38 MAPK-mediated apoptotic pathway in ovarian cancer [10]. In addition, Cheng et al. suggested that high levels of ROS can modulate the PI3K/AKT pathway, thus regulating cell apoptosis

and metabolism [34]. Nevertheless, the relationship between piperine and the JNK/p38 MAPK pathways remains unclear and warrants further study.

SNU-16 cells were implanted in mice to detect the *in vivo* anti-tumor effect of piperine on tumor growth. Compared with the control group, piperine (30 and 60 mg/kg) markedly inhibited the growth of xenograft tumors, while no significant change of body weight was observed. Yoo et al. reported similar tumor growth suppression in A375SM and A3775P cells in human melanoma in nude mice administered piperine at 50 and 100 mg/kg for 4 weeks [35]. Additionally, H&E staining results showed that piperine had no toxic effects on the kidneys and hearts of mice, and there were no significant changes in ALT and AST of mice in the 3 groups, revealing the high safety of piperine. We also found that piperine could induce apoptosis of xenograft HCC tissues, manifested by reduced Ki-67 expression and augmented cleaved caspase-3 expression.

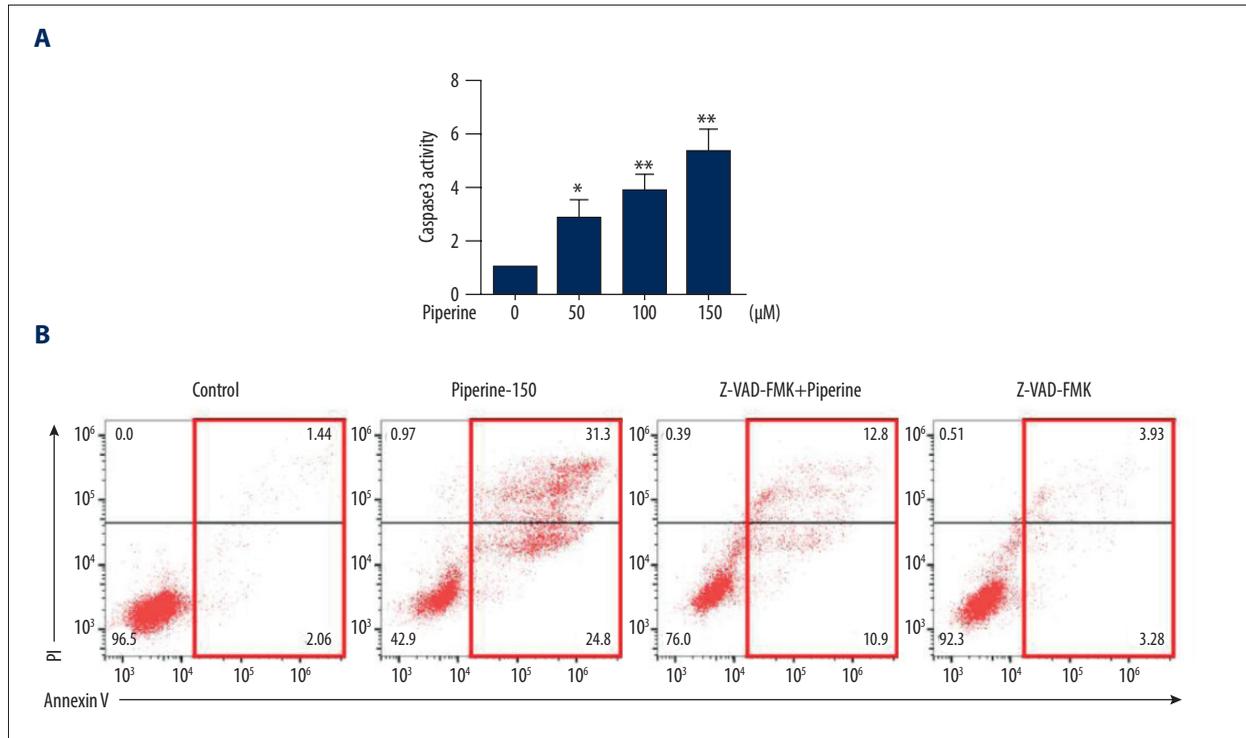
Conclusions

Piperine inhibits proliferation and induces apoptosis in human gastric cancer, which is associated with the upregulated protein expression of Bax, Bad, Cyto C, cleaved PARP, and cleaved caspase-3, and the downregulated protein expression of Bcl-2 and Bcl-xl. In addition, the anti-cancer mechanism of piperine in the gastric cancer cell line SNU-16 may be realized by inhibiting the PI3K/Akt pathway. In summary, piperine may be an effective chemotherapeutic agent against gastric cancer.

Conflicts of interest

None.

Supplementary Data



Supplementary Figure 1. (A) Piperine increased caspase-3 activity in SNU-16 cells. Cells were exposed to piperine at indicated concentrations for 20 h. Caspase-3 activity was measured using a substrate kit (n=3) * $P < 0.05$ and ** $P < 0.01$ compared to control group. (B) The apoptotic rates of SNU-16 cells induced by piperine. Apoptosis detection with Annexin V/PI double staining in different groups by flow cytometry. Z-VAD-FMK (20 μM) pretreated for 1 h. Data are presented as the mean ± SD of at least 3 independent experiments.

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