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Antitumor Effects of Glychionide-A Flavonoid in Human Pancreatic Carcinoma Cells Are Mediated by Activation of Apoptotic and Autophagic Pathways, Cell Cycle Arrest, and Disruption of Mitochondrial Membrane Potential

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Pancreatic cancer has high incidence and low survival rates around the globe, mainly due to late diagnosis and unavailability of efficient chemotherapeutic agents. In the present study, the anticancer potential of glychionide-A was examined against PANC-1 pancreatic cancer cells.





Material/Methods: CellTiter-Glo Luminescent Cell Viability Assay Kits were used for assessment of cell viability. Electron microscopy and DAPI staining were used for the detection of apoptosis and autophagy, respectively. Annexin V/PI staining was used for determination of apoptotic cell percentage. Cell cycle distribution and ROS and MMP levels were determined by flow cytometry. Protein expression was examined by Western blot analysis.

Results: The results revealed that glychionide-A significantly inhibited the proliferation of the PANC-1 pancreatic cancer cells (IC_{50} , 14 μ M). However, minimal toxicity was observed against the normal hTRET-HPNE pancreatic cells (IC_{50} , 100 μ M). The anticancer activity of glychionide-A against the PANC-1 cells was found to be due to induction of autophagy and apoptosis. Glychionide-A prompted apoptosis and autophagy and was also associated with alteration in apoptosis- (Bax, Caspase 9 and Bcl-2) and autophagy- (LC3I, II, Beclin 1 and p62) related protein expression. Glychionide-A also caused the arrest of PANC-1 cells in the G₂/M phase of the cell cycle. The percentage of PANC-1 cells in G₂ phase increased from 19.5% to 49.4% upon treatment with glychionide-A. Finally, glychionide-A caused an increase in the level of ROS and decline in MMP levels of the PANC-1 pancreatic cancer cells.

Conclusions: In brief, these results reveal that glychionide-A significantly inhibits the growth of pancreatic cancer cells via inducing apoptosis and autophagy, and could prove valuable in the chemotherapeutic treatment of pancreatic cancer. Therefore, further research is needed, especially more advanced *in vivo* experiments.

MeSH Keywords: **Apoptosis • Autophagy • Cell Cycle • Pancreatic Neoplasms**

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Background

Pancreatic cancer causes many deaths in developing countries and has been reported to be one of the deadliest neoplasms worldwide [1]. Pancreatic cancer causes an estimated 331 000 deaths annually, accounting for 4% of all cancer-related mortality, and is considered to be 7th leading cause of cancer-related deaths across the globe [2]. With just a 5% five-year survival rate, it is a devastating cancer [3]. Therefore, markers for early detection, exploration, and identification of efficient chemotherapeutic agents and post-therapeutic monitoring are greatly needed. Plants have been utilised by humans for the preparation of drugs in the past and are still used for the isolation of drugs to cure many human diseases [4]. Generally, plants create a huge array of metabolites to overcome stress and to adapt to challenging environmental conditions. These chemical entities, often referred to as secondary metabolites, have been shown to exhibit medicinal properties and have been used for the treatment of diseases as deadly as cancer [5]. For example, taxol and camptothecins are among the common anticancer agents of plant origin [6]. These secondary metabolites have been chemically classified into different groups, and flavonoids form an important group with tremendous pharmacological potential [7]. They have been found to exhibit a range of bioactivities such as anti-inflammatory, antioxidant, antimicrobial, and anticancer activity [8]. Glychionide-A is a flavonoid extracted in its pure form from several plant species, including *Glycyrrhiza glabra* [9]. It has been found to inhibit the growth of cancer cells [10], but its antiproliferative effects have not been examined against pancreatic cancer. Herein, we for the first time report the anticancer activity of glychionide-A against pancreatic cancer cells. The results showed that glychionide-A can halt the growth of pancreatic cancer cells. Our results suggest that Glychionide-A may serve as a beneficial metabolite that can be used in the development of chemotherapy for pancreatic cancer.

Material and Methods

Chemicals and other reagents

Glychionide-A (purity >98%; determined by high-performance liquid chromatography), 3-(4, 5-dimethyl-2-thiazolyl)-2, and 5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Annexin V-FITC and propidium iodide were purchased from Wuhan Boster Biological Technology (Wuhan, China). Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium were purchased from HyClone (Logan, UT, USA). Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Tianjin HaoYang Biological Manufacture Co. (Tianjin, China). Horseradish peroxidase-labeled anti-mouse and anti-rabbit

secondary antibodies and all other antibodies were purchased from Cell Signalling Technology (MA, USA). Cell culture plasticware was purchased from BD Biosciences (San Jose, CA, USA).

Cell lines and culturing conditions

The pancreatic cancer cell line PANC-1 and normal hTRET-HPNE pancreatic cells were procured from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium in a CO₂ incubator (Thermo Scientific) at 37°C with 98% humidity and 5% CO₂.

Cell viability assay

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay. The wells of a 96-well plate were seeded with 2×10⁴ PANC-1 pancreatic normal hTRET-HPNE pancreatic cells per well, incubated overnight, and then treated with increasing doses (0–100 μM) of glychionide-A for different periods of time. After incubation, MTS solution was added to the cells according to the manufacturer's guidelines, and then absorbance was measured at 490 nm using an ELISA plate reader (ELX 800; Bio-Tek Instruments, Inc., Winooski, VT, USA).

Transmission electron microscopy (TEM)

For electron microscopy, the glychionide-A-treated (0, 7, 14, and 28 μM) cells were fixed in a solution of 4% glutaraldehyde in 0.05 M sodium cacodylate, postfixed in 1.5% OsO₄, and dehydrated in alcohol. They were then prepared for flat embedding in Epon 812 and then observed using a Zeiss CEM 902 electron microscope.

Apoptosis assay

For apoptosis detection, the pancreatic cancer PANC-1 cells (0.6×10⁶) were grown in 6-well plates. After an incubation period of around 12 h, the PANC-1 cells were subjected to glychionide-A treatment (0, 7, 14, and 28 μM) for 24 h at 37°C. As the cells sloughed off, 25-μl cell cultures were put onto glass slides and subjected to staining with DAPI. The slides were covered with cover slips and examined with a fluorescent microscope. The annexin V/PI staining of the glychionide-A-treated PANC-1 cells was carried out as described previously [11].

Cell cycle analysis

The distribution of pancreatic cancer cells in different cycle phases was assessed by flow cytometry after PI staining, following the method reported in the literature. In brief, the pancreatic cancer cells were grown in 6-well plates and treated

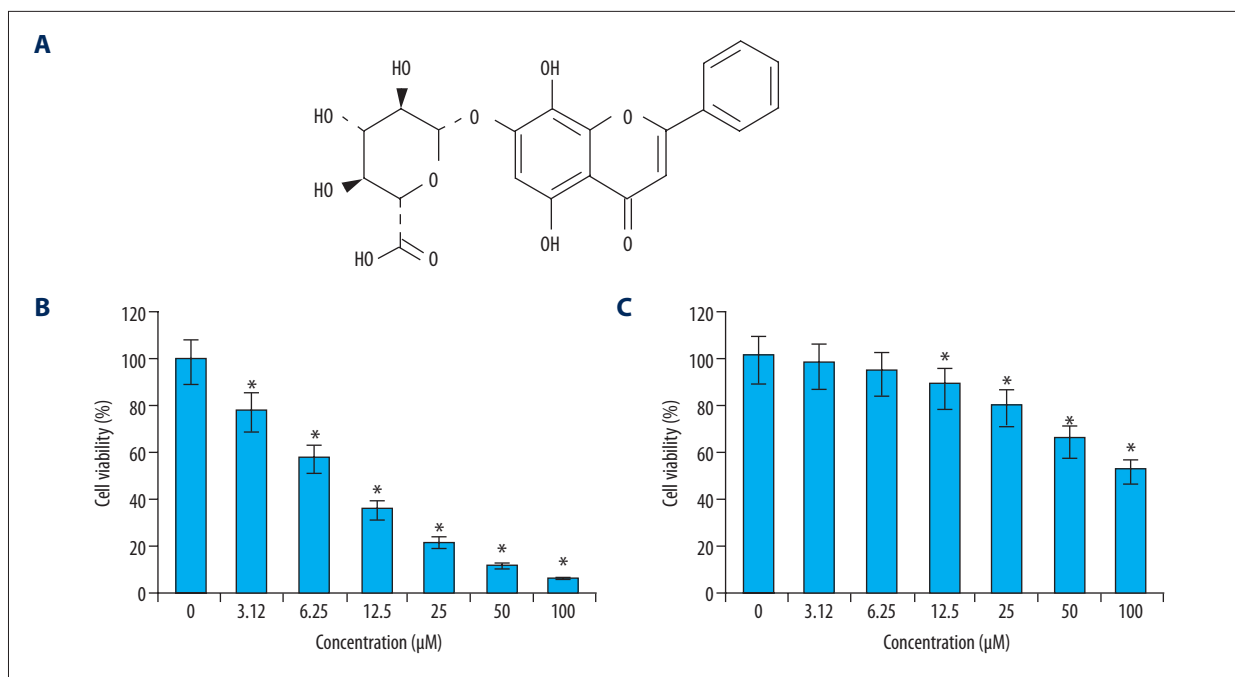


Figure 1. (A) Chemical structure of glychionide-A and (B) effect of glychionide-A on viability of PANC-1 and (C) normal hTRET-HPNE pancreatic cells as determined by the MTS cell viability assay. The experiments were performed in triplicate and are shown as mean \pm SD (* $p < 0.01$).

with glychionide-A (0, 7, 14, and 28 μ M) for 24 h. The cells were then collected and washed with PBS, followed by fixation in ethanol (70%). After overnight incubation at 4°C, the cells were subjected to PI staining and flow cytometry.

Determination of the reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) levels

For determination of the ROS and MMP levels, the pancreatic PANC-1 cells were treated with 0, 7, 14, or 28 μ M concentrations of glychionide-A for 24 h, then the ROS and MMP levels in the PANC-1 cells were determined as described previously [12].

Western blot analysis

To determine the expression of the selected proteins in the glychionide-A-treated (0, 7, 14, and 28 μ M) PANC-1 cells, the cells were lysed with RIPA buffer, and the protein content of each lysate was estimated by BCA assay. The samples were then loaded on the SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for 24 h. After this, the membranes were incubated with HRP-conjugated secondary antibody for 50 min at 25°C. Enhanced chemiluminescence reagent was used to visualise the protein bands.

Statistical analysis

All results are depicted as mean \pm standard deviation (SD) from at least 3 independent experiments. The differences between groups were analysed by one-way ANOVA with GraphPad Prism 7 software. Statistical significance was set at * $P < 0.01$.

Results

Glychionide-A inhibits the growth of pancreatic cancer cells

The effects of glychionide-A (Figure 1A) on the proliferation of the PANC-1 pancreatic cancer was investigated by MTS cell viability assay, showing that glychionide-A exerts antiproliferative effects on the PANC-1 pancreatic cancer cell lines. The IC_{50} of 14 μ M was observed for the PANC-1 cell line (Figure 1B). However, minimal toxicity was observed against the normal hTRET-HPNE pancreatic cells (IC_{50} 100 μ M) (Figure 1C). Moreover, it was found that the anticancer effects of glychionide-A on the pancreatic cancer cells were concentration-dependent.

Glychionide-A induces both autophagy and apoptosis in pancreatic cancer cells

The effects of glychionide-A on the PANC-1 pancreatic cancer cells were examined by electron microscopy, showing that

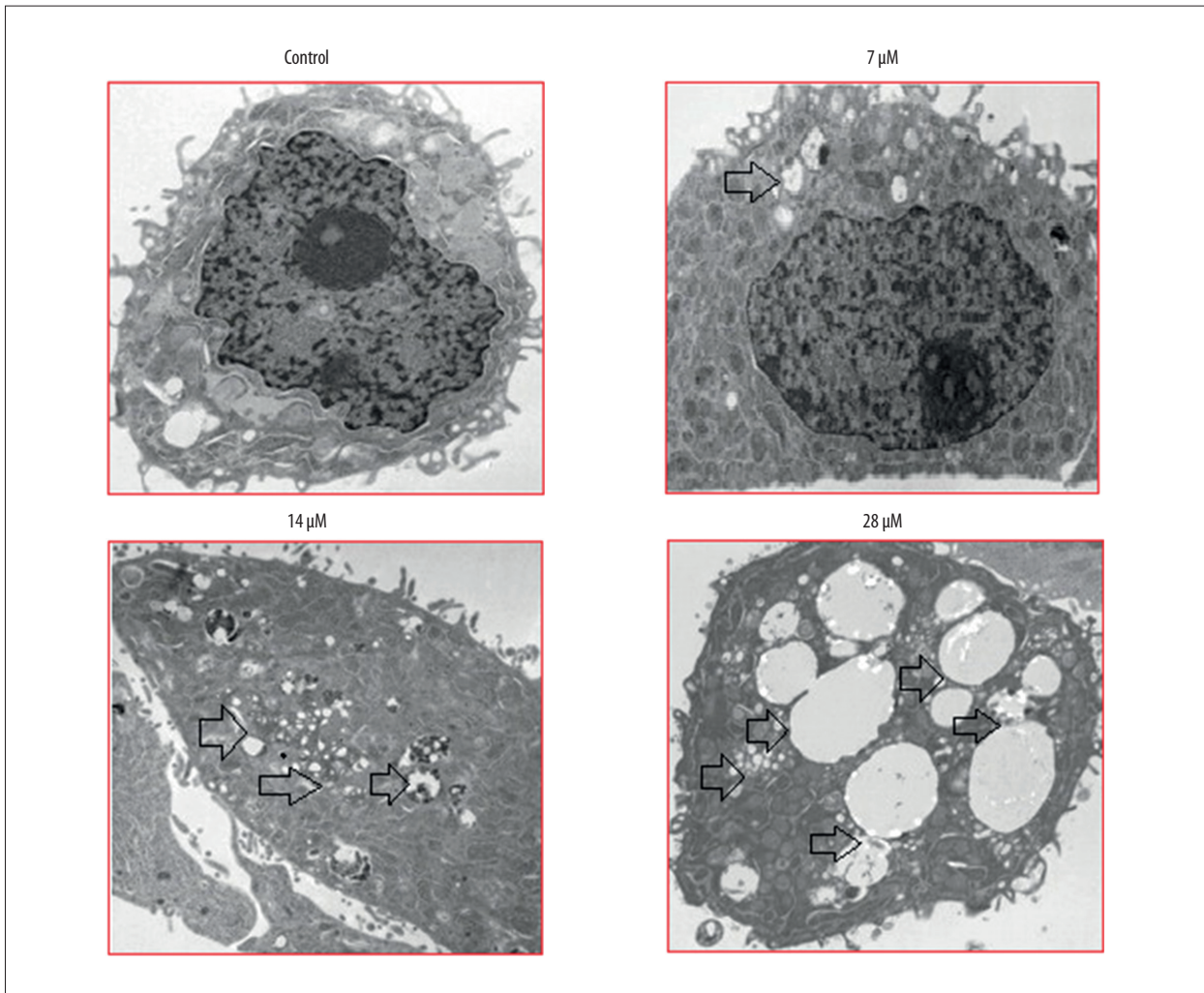


Figure 2. Electron microscopy images of glychionide-A treated PANC-1 cells showing induction of autophagy. The experiments were repeated in triplicate (arrows depict autophagosomes).

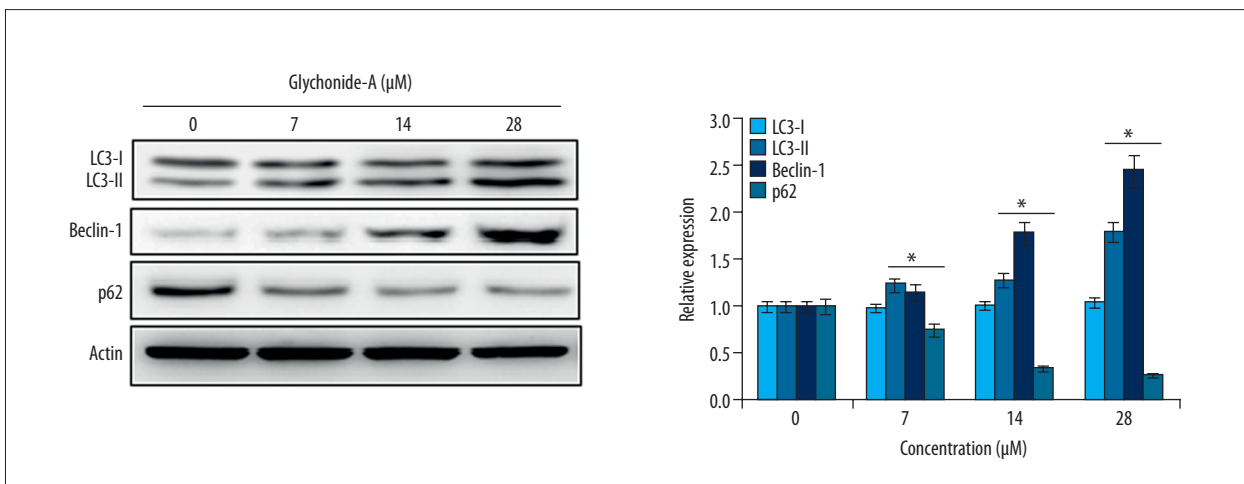


Figure 3. Effect of glychionide-A on the autophagy-related protein expression levels in PANC-1 pancreatic cancer cells as revealed by Western blot analysis. The experiments were repeated in triplicate.

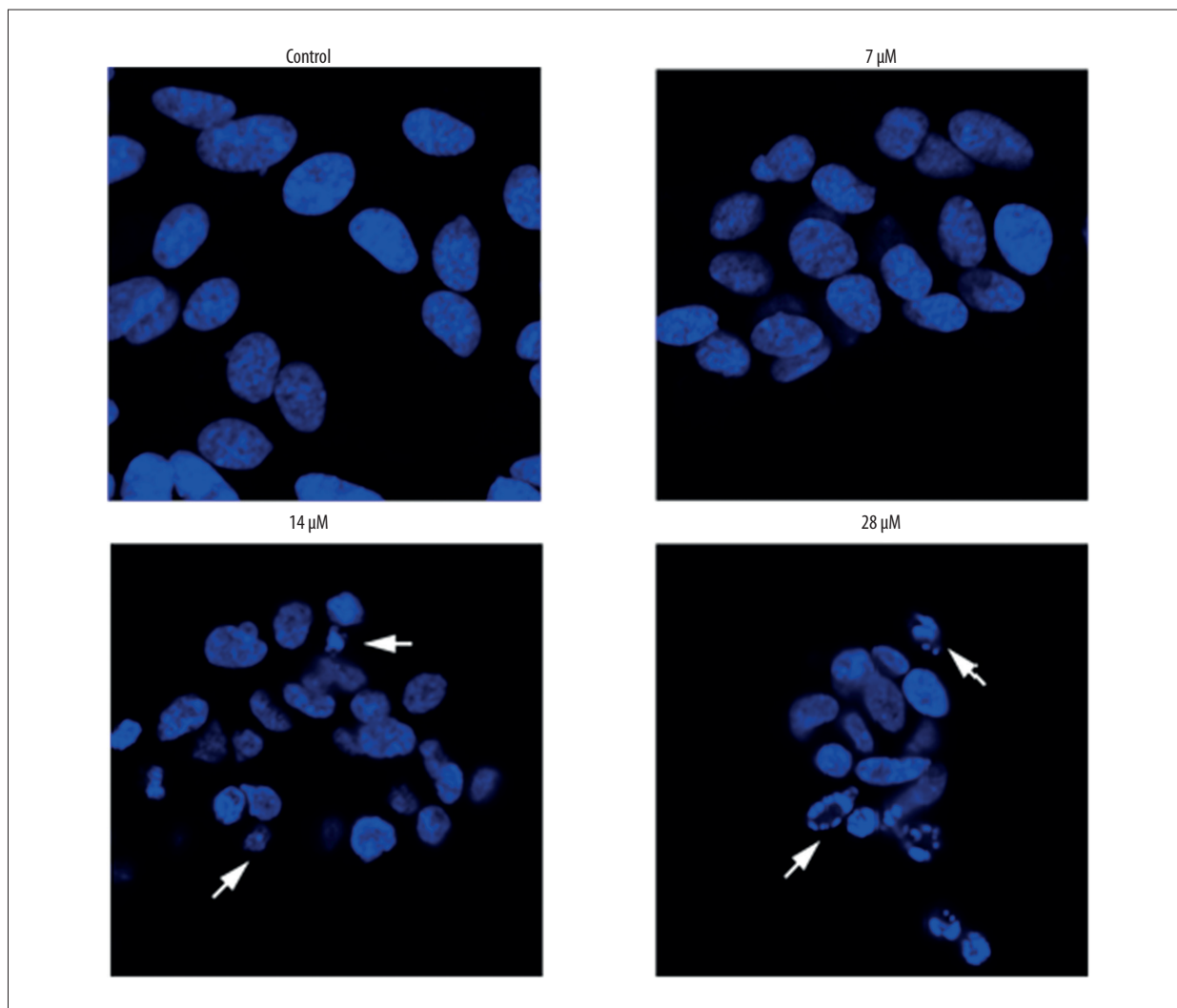


Figure 4. DAPI staining images showing induction of apoptosis by glychionide-A levels in PANC-1 pancreatic cancer cells. The experiments were performed in triplicate.

glychionide-A triggered the formation of autophagosomes in the PANC-1 pancreatic cancer cells, indicative of autophagy (Figure 2). In addition, glychionide-A also caused shrinkage of the nuclei of the PANC-1 cells, suggestive of apoptosis. For the confirmation of autophagy, the expression of autophagy-associated proteins was examined, and it was found that glychionide-A increased the levels of Beclin-1 and LC3-II and suppressed p62 expression. However, no effects were observed on the expression of LC3-I (Figure 3). Apoptosis was confirmed by DAPI staining, which showed remarkable changes in the nuclear morphology of the PANC-1 cells (Figure 4). Annexin V/PI staining showed that the percentage of apoptotic PANC-1 cells increased from 2.5% in control to 46.25% at 28 μM concentration of glychionide-A (Figure 5). The apoptosis was further confirmed by the increased expression of Bax, and Caspase-9 decreased expression of the Bcl-2 in PANC-1 cells (Figure 6).

Glychionide-A causes G_2/M arrest of pancreatic cancer cells

The effect of glychionide-A on distribution of PANC-1 pancreatic cancer in different cell cycle phases was assessed by flow cytometry. It was found that glychionide-A caused a remarkable increase in the percentage of the PANC-1 cells in G_2 phase of the cell cycle. The percentage of PANC-1 cells in the G_2 phase increased from 19.5% to 49.4% upon treatment with glychionide-A (Figure 7). These results clearly indicate that glychionide-A induces G_2/M cell cycle arrest of the pancreatic cancer cells.

Glychionide-A disrupts the MMP levels in PANC-1 cells

Next, we explored whether glychionide-A has an effect on the MMP levels of the PANC-1 cells treated with various

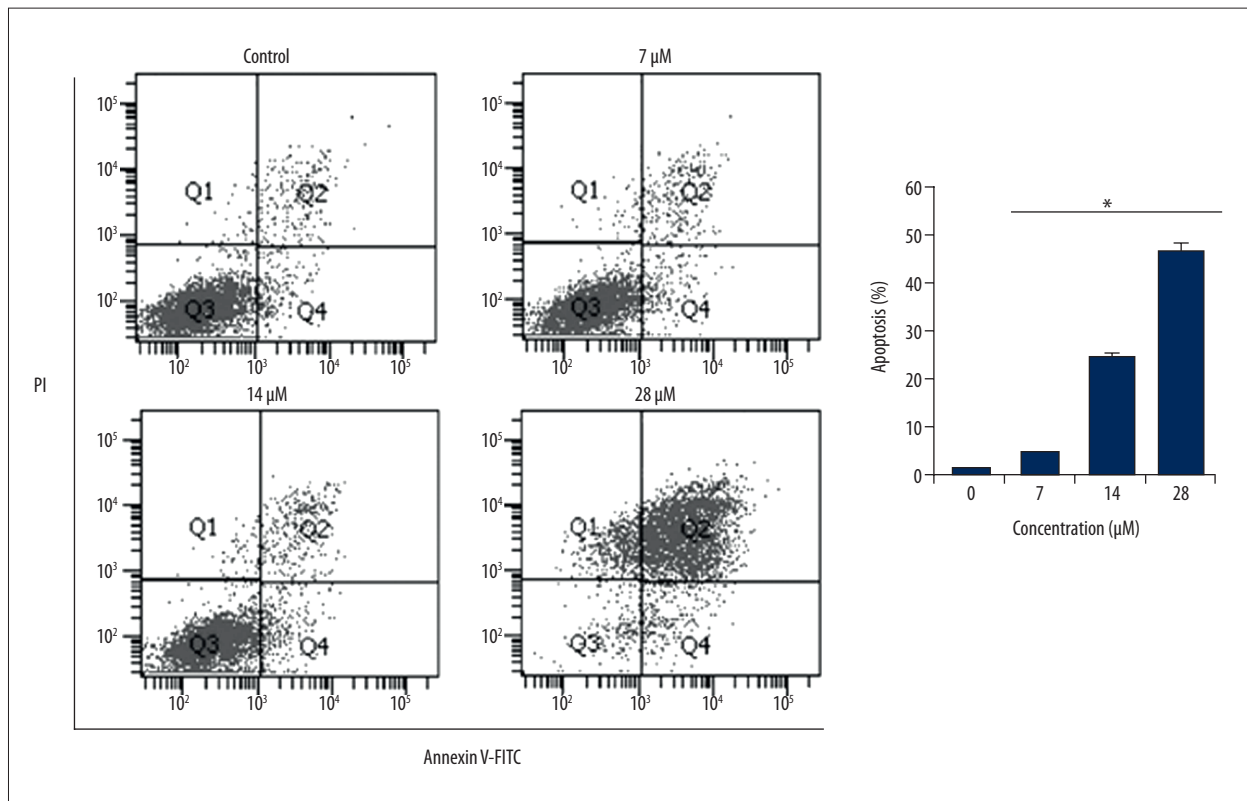


Figure 5. Estimation of the apoptotic cell populations in glychionide-A treated PANC-1 cells as depicted by annexin V/PI staining. The experiments were performed in triplicate.

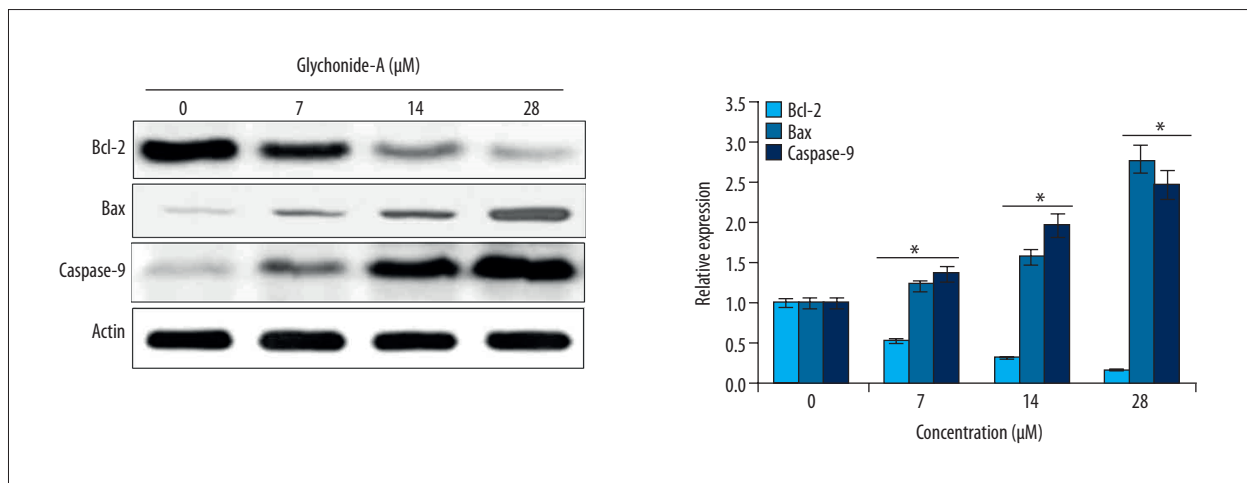


Figure 6. Effect of glychionide-A on the expression of Caspase, 9Bax, and Bcl-2 protein levels in PANC-1 pancreatic cancer cells as depicted by Western blot analysis. The experiments were repeated in triplicate.

concentration of glychionide-A. We found that glychionide-A treatment caused a sharp decline in the MMP levels of the PANC-1 cells. The MMP levels decreased from 100% in controls to about 33% in controls (Figure 8A)

Glychionide-A causes generation of ROS in PANC-1 cells

The effect of glychionide-A was also investigated on the ROS levels of the PANC-1 pancreatic cells at 0, 7, 14, and 28 μM concentrations by flow cytometry. It was found that glychionide caused significant ($p < 0.01$) increases in the levels of ROS in

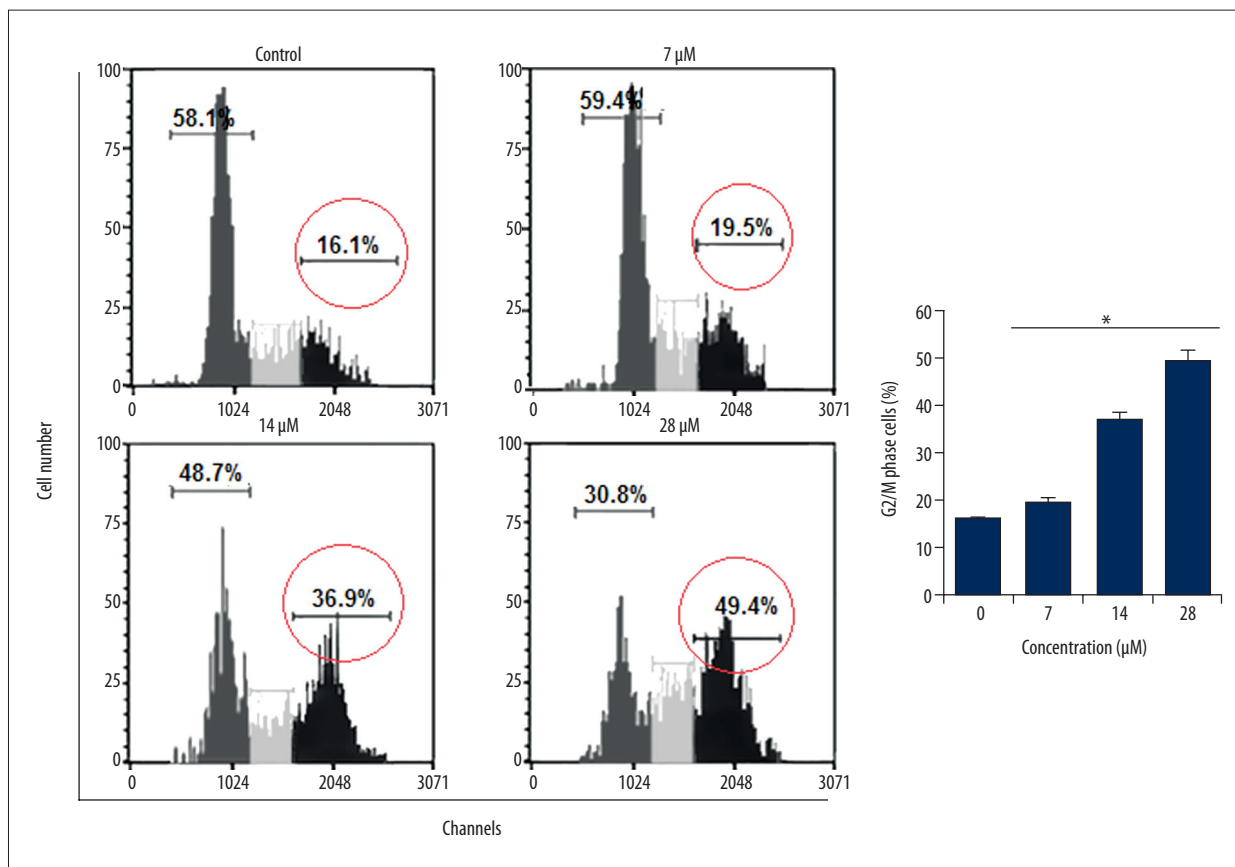


Figure 7. Effect of glychionide-A on cell cycle as revealed by flow cytometry. The experiments were performed in triplicate.

the PANC-1 cells. The ROS levels increased from 100% in controls to about 185% at 28 μM of glychionide-A (Figure 8B)

Discussion

Pancreatic cancer is ranked as the 7th most frequent type of cancer worldwide. The 5-year survival rate is just 5%; therefore, there is an urgent need to look for novel treatment options to reduce the high incidence and improve the 5-year survival rate [2,3]. The chemotherapeutic agents used for the management of pancreatic cancer are generally inefficient and exhibit severe adverse effects on the overall health of patients [13]. Plant-derived anticancer agents have received remarkable attention in the recent past due to their minimal toxic effects. As such, more and more plant-derived natural products are being evaluated against cancer cells for their anticancer activity [14]. Herein, the anticancer effects of glychionide-A were examined against human cancer cell lines. It was found that glychionide-A caused a considerable decline in the viability of pancreatic cancer cells. Several flavonoids have been shown to inhibit the proliferation of cancer cells. For example, kaempferol inhibits proliferation of glioblastoma cells [15]. Autophagy is a vital process that triggers death of harmful cells and survival of

normal cells [16]. Similarly, apoptosis eliminates the harmful cells from the body [17]. In the present study, investigation of the mechanism of action of glychionide-A revealed that it prompts both autophagy and apoptotic cell death of PANC-1 pancreatic cancer cells. This was also associated with changes in the expression of autophagy and apoptosis-related protein expression. In addition, glychionide-A caused arrest of PANC-1 at the G2/M checkpoint and thereby halted growth. Previous studies have indicated that several of the anticancer molecules induce autophagy and apoptosis of cancer cells [18]. The induction of the autophagy and apoptosis are often associated with increase in the ROS and disruption of MMP [19]. ROS has been implicated in the induction of both autophagy and apoptosis in cancer cells; for example, Silibinin, a natural flavonoid, has been reported to induce both autophagy and apoptosis via ROS-dependent disruption of mitochondrial membrane potential [20]. Here, we observed that glychionide-A significantly increased ROS and decreased MMP levels of the PANC-1 pancreatic cancer cells, indicative of ROS-mediated apoptosis and autophagy. We found that glychionide-A inhibited the growth of xenografted tumors, showing the potential of glychionide-A in treatment of pancreatic cancer.

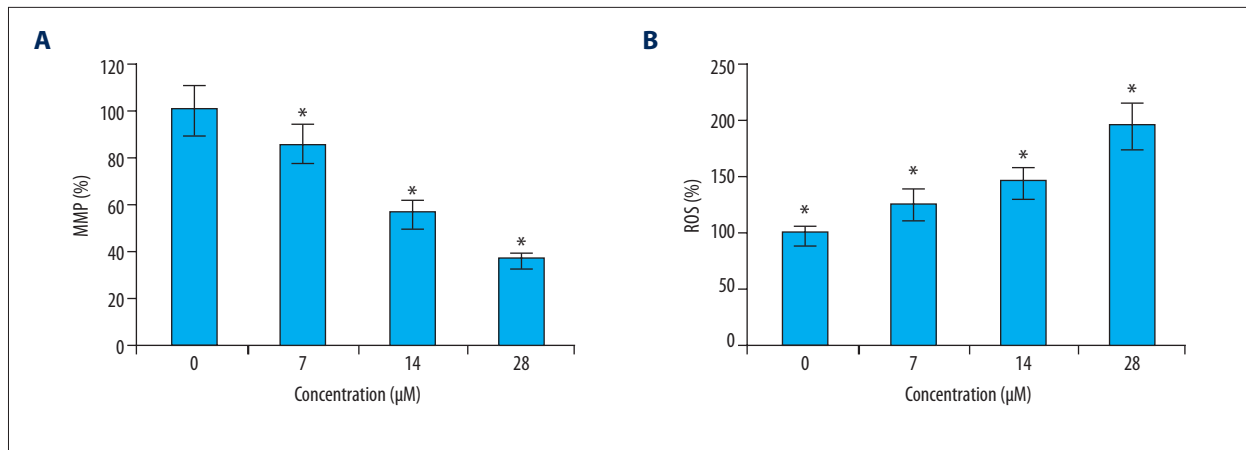


Figure 8. Effect of Glychionide-A on the (A) MMP and (B) ROS levels in PANC-1 pancreatic cancer cells depicted by flow cytometry. The experiments were performed in triplicate.

Conclusions

Glychionide-A inhibits the proliferation of pancreatic cancer cells by autophagy and apoptotic cell death. It can also induce cell cycle arrest and disruption of the mitochondrial membrane potential. As such, glychionide-A could prove to be an important chemotherapeutic agent and warrants further investigation.

Conflict of interest

None.

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