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ORIGINAL ARTICLE

In vitro and *in vivo* human gastric cancer inhibition by Trifolirhizin is facilitated via autophagy, mitochondrial mediated programmed cell death, G2/M phase cell cycle arrest and inhibition of m-TOR/PI3K/AKT signalling pathway

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Summary

Purpose: *To investigate the anticancer effects of Trifolirhizin in SNU-5 human gastric cancer cells along with evaluation of its effects on autophagy, apoptosis, cell cycle phase distribution and m-TOR/PI3K signalling pathway.*

Methods: *The antiproliferative effect on gastric cancer cells was assessed by MTT assay. Autophagy was detected by electron microscopy and western blot. Apoptotic cell death was revealed by acridine orange (AO)/ethidium bromide (EB) and annexin V/propidium iodide (PI) staining using flow cytometry. Cell cycle analysis was carried out by flow cytometry. Protein expression was determined by immunoblotting. Xenografted mice models were used to evaluate in vivo the anticancer effects of Trifolirhizin.*

Results: *Trifolirhizin could significantly inhibit the proliferation of the gastric cancer cells. The anticancer activity of Trifolirhizin against the gastric cancer cells was found to be due to induction of autophagy and mitochondrial-mediated apoptosis. It was further observed that both apoptosis and autophagy-related protein expressions sere significantly altered. Further, it was found that Trifolirhizin could inhibit the m-TOR/PI3K/AKT signalling pathway. In vivo evaluation in xenografted mice indicated that Trifolirhizin inhibited significantly both tumor weight and tumor volume.*

Conclusions: *In conclusion, it can be safely stated that Trifolirhizin has the potential to be developed as a potent anticancer agent against gastric carcinoma provided further in depth evaluation of this compound is performed.*

Key words: *anticancer, apoptosis, autophagy, flow cytometry, gastric cancer, trifolirhizin*

Introduction

Cancer is the second major cause of mortality worldwide after cardiovascular disease. Although the incidence of gastric cancer has decreased in the last few decades owing to the progress made in the field of cancer research, the clinical outcomes are still unsatisfactory [1]. Compared to western countries the prevalence of gastric cancer is higher in Asian countries. In China alone around 0.4 million new cases of gastric cancer are reported annually [2]. In addition, around 70% of patients with gastric cancer are diagnosed for this devastating disease at advanced stages and thus are difficult to treat [3]. Moreover, gastric cancer shows high rate of recurrence and then the cancer cells develop resistance to anticancer drugs making it more difficult to manage [4]. Although a number of drugs have been developed for the treatment of cancers, they are mostly associated with a number of side effects which negatively affects the life of patients [5]. Hence, researches are being directed at identi-

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fying and developing anticancer agents that exhibit no or negligible side effects.

Plants have always inspired scientists to carry out research on them with their ability to synthesize a wide diversity of chemical scaffolds. Drugs such as podophyllotoxins and taxanes have plants as their source [6]. Many other lifesaving drugs such as the anti-malarial drug artemisinin comes from plant source [7]. Thus plants have served as a source of drugs in the past and will continue to serve in the future as many plant derived molecules are being screened for anticancer and many other activities [8]. In this study a natural product, Trifolirhizin, was screened for its anticancer activity against a panel of cancer cell lines. Trifolirhizin is an important flavonoid that has been isolated from several plant species including *Sophora flavescens* [9]. It has been reported to exhibit a number of bioactivities such as anti-inflammatory, anticancer and antimicrobial [10,11]. However, to the best of our knowledge, the anticancer effects of Trifolirhizin have not been reported against gastric cancer. This study was designed to investigate the effects of Trifolizhizin against human gastric cancer cells both *in vivo* and *in vitro*.

Methods

Cell lines and culturing conditions

The normal GES-1 and gastric cancer cell line SNU-5 were procured from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium in CO_2 incubator (Thermo Scientific) at 37°C with 98% humidity and 5% CO_2.

Cell viability assay

The antiproliferative effect of Trifolirhizin was assessed on gastric cancer cell line by MTT assay as described previously by Mosmann [14]. The gastric cancer cells were subjected to treatment with varied concentrations of Trifolirhizin and the proliferation rate was determined by taking absorbance at 570 nm via spectrophotometer.

Electron microscopy

The Trifolirhizin-treated gastric cancer cells were assessed by electron microscopy to examine autophagy. In brief, the gastric cancer cells were treated with 0, 12.5, 25 and 50 µM Trifolirhizin for 24 h. The cells were collected by trypsinization and subjected washed, was followed by fixation in glutaraldehyde (2%) in phosphate buffer (0.1 M). The cells were then post-fixed in osmium tetroxide (1%). This was followed by the treatment of the cells with ethanol and embed in resin. Thin section were then cut with the help of an ultramicrotome and subjected to electron microscopy.

Apoptosis assay

The effects of Trifolirhizin on the induction of apoptosis were determined by acridine orange (AO)/ethidium bromide (EB) staining as described earlier [15]. The cancer cells (0.6×10^6) were grown in 6-well plates. Following 12 h of incubation, the cells were subjected to Trifolirhizin treatment for 24 h at 37°C. The cell cultures were then centrifuged and the pellets were washed with phosphate buffered saline (PBS). Thereafter, the cells were AO/EB-stained, centrifuged and PBS-washed. Finally, the stained cells were examined by fluorescence microscopy. The percentage of the apoptotic cells was estimated by annexin V/propidium iodide (PI) staining as previously described [16].

Cell cycle analysis

The effects of Trifolirhizin distribution of the gastric cancer cells in different cycle phases was performed by flow cytometry after PI staining. In brief, the gastric cancer cells were grown in 6-well plates and treated with Trifolirhizin for 24 h. The cells then collected and PBS-washed, followed by fixation in ethanol (70%). After overnight incubation at 4°C, the cells were PI-stained and subjected to flow cytometry.

Western blotting

The protein expression was examined by western blotting. Following lysis of the gastric cancer cells in RIPA lysis buffer, the protein content of each lysate was estimated by bicinchoninic acid (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 24h. After this, the membranes were incubated with HRP-conjugated secondary antibody (1:1000) for 50 min at 25°C. Enhanced chemiluminescence reagent was used to visualize the protein bands.

In vivo study

The *in vivo* evaluation of Trifolirhizin was carried out in xenografted mice models. Male BALB/c nude mice were kept in the animal holding capacity facility. During the course of the study the National Institutes of Health standards were followed. In brief, the mice were injected with 5×10^6 SNU-5 cells sub-cutaneously at the left flank. The mice (n=12) of each group were injected intraperitoneally with DMSO (0.1%) dissolved Trifolirhizin and diluted with 100 µL normal saline at 25 mg/kg and taken as the day one of the experiment. Trifolirhizin was given to the mice thrice a week, while the control mice were given DMSO (0.1%) in normal saline only. At the end of 4 weeks, the mice were sacrificed and tumors were harvested for assessment of tumor growth and other investigations.

Statistics

All results were expressed as mean ± standard error (S.E.) from at least three independent experiments. The differences between groups were analyzed by one way ANOVA using GraphPad prism 7 software, and

the significance of difference was set as *p <0.05 and **p <0.01.

Results

Trifolirhizin selectively inhibits the growth of gastric cancer cells

The effects of Trifolirhizin on the proliferation of gastric cancer cells were examined on a panel of gastric cancer and normal gastric cell lines by MTT assay. It was found that that Trifolirhizin exerts antiproliferative effects on all SNU-5 gastric cancer cells line. The IC_{50} was defined as 25 μM for the SNU-5 cell line (Figure 1). However, the IC_{50} of Trifolirhizin was comparatively higher against the normal gastric GES-1 cells (IC_{50} 85 μM). In addition, it was noted that the anticancer effects of Trifolirhizin on the gastric cancer cells were concentration-dependent.

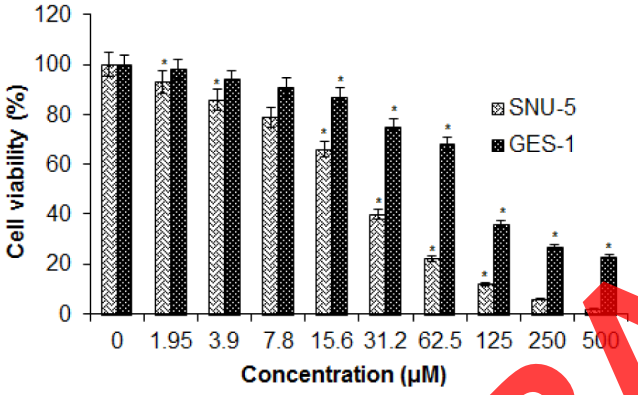

Figure 1. Effect of Trifolirhizin on the viability of the SNU-5 and GES-1 cells as determined by MTT assay. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01).

Trifolirhizin induces both autophagy and apoptosis in gastric cancer cells

The effects of Trifolirhizin on the SNU-5 gastric cancer cells were firstly evaluated by electron microscopy. It was found that Trifolirhizin

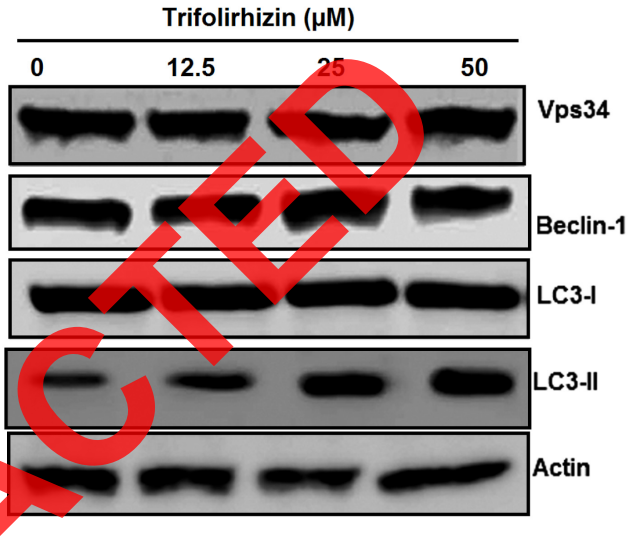

Figure 3. Effect of Trifolirhizin on the autophagy related protein expression as revealed by western blot analysis. The Figure shows that this molecule increases the expression of Vps34, LC3-II and Beclin-1. The experiments were performed in triplicate.

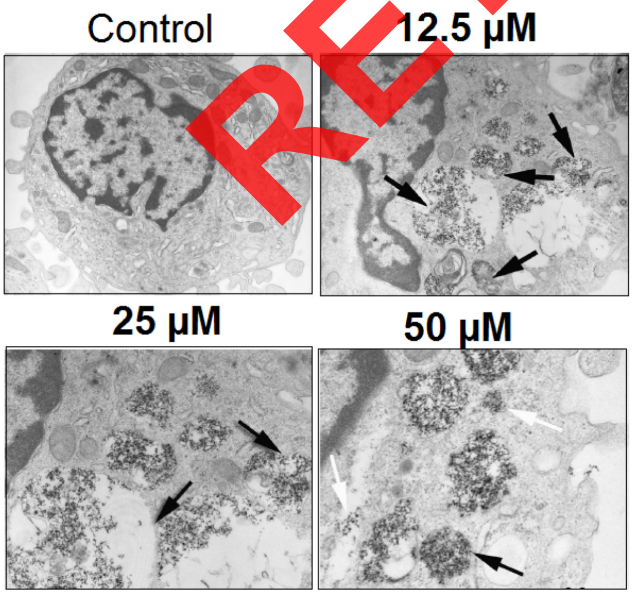

Figure 2. Electron microscopy images of Trifolirhizin treated SNU-5 cells showing induction of autophagy (arrows show autophagosomes). The experiments were performed in triplicate.

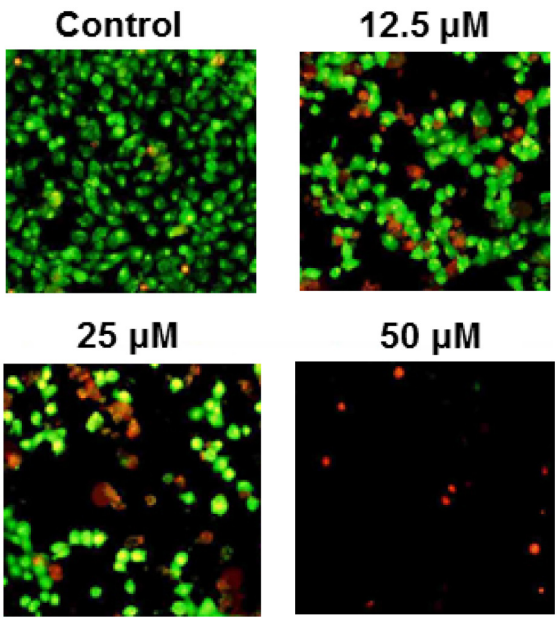

Figure 4. AO/EB staining images showing induction of apoptosis in a dose-dependent manner by Trifolirhizin on the SNU-5 cells. The experiments were performed in triplicate.

triggered the formation of autophagosomes in the SNU-5 gastric cancer cells, indicative of autophagy (Figure 2). In addition, Trifolirhizin also caused shrinkage of the nuclei of the SNU-5 cells, suggestive of apoptosis. For the confirmation of autophagy, the expression of autophagy-associated proteins was examined and it was found that Trifolirhizin caused upsurge of Beclin-1 and LC3-II expression. However, no effects were observed on the expression of LC3-I and Vps34 (Figure 3). The apoptosis was confirmed by AO/EB staining which showed considerable changes in the nuclear morphology (Figure 4). Annexin V/PI staining showed that the percentage of the apoptotic SNU-5 cells increased with increase in the concentration of Trifolirhizin (Figure 5). The apoptosis was further confirmed by the increased expression of Bax and decreased expression of the Bcl-2 in SNU-5 cells (Figure 6).

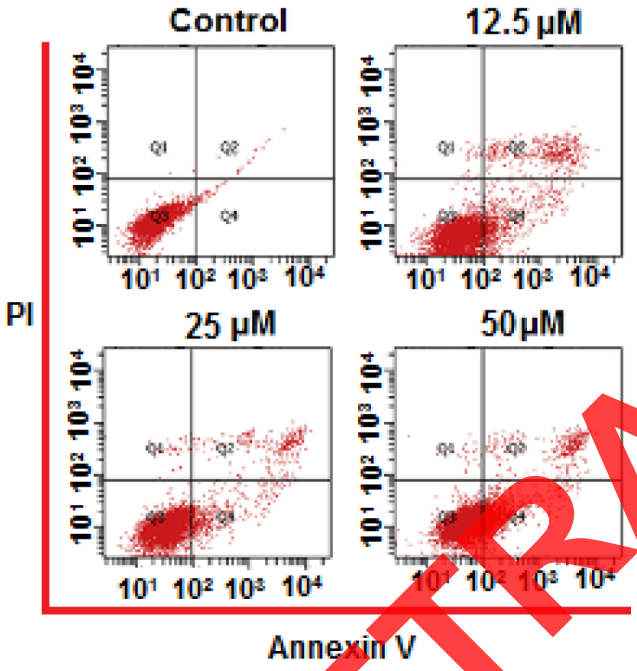

Figure 5. Estimation of the apoptotic cell populations in Trifolirhizin treated SNU-5 cells as depicted by annexin V/PI staining. The Figure shows that the percentage of apoptotic SNU-5 cells increased with the increase in the concentration of Trifolirhizin. The experiments were performed in triplicate.

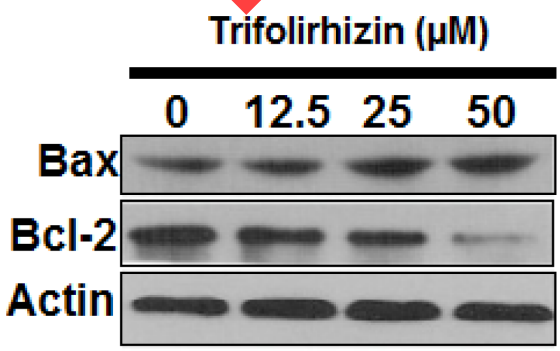

Figure 6. Effect of Trifolirhizin on the expression of Bax and Bcl-2 proteins as depicted by western blot analysis. The image shows that Trifolirhizin increased Bax and decreased Bcl-2 expression. The experiments were repeated in triplicate.

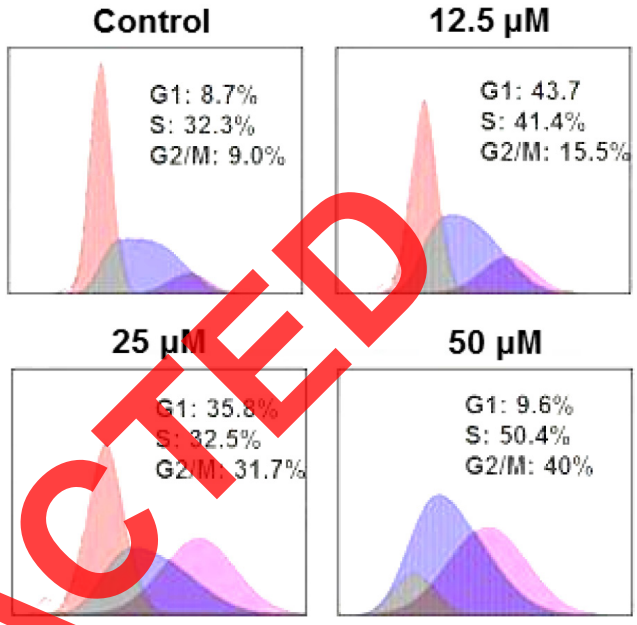

Figure 7. Effect of Trifolirhizin on cell cycle as revealed by flow cytometry. The Figure shows that Trifolirhizin triggers arrest of SNU-2 cells at the G2/M phase of the cell cycle in a dose-dependent manner. The experiments were performed in triplicate.

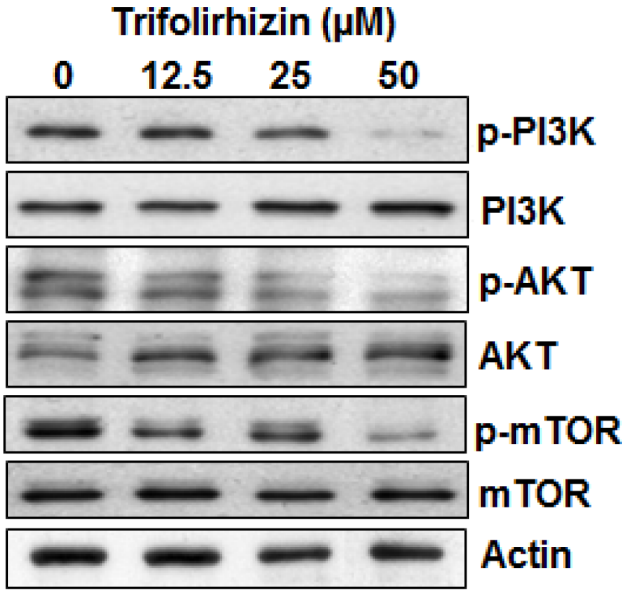

Figure 8. Effect of Trifolirhizin on the mTOR/PI3K/KT signalling pathway as depicted by western blot analysis. The Figure shows that Trifolirhizin blocked the PI3K/AKT/mTOR signaling pathway dose-dependently. The experiments were performed in triplicate.

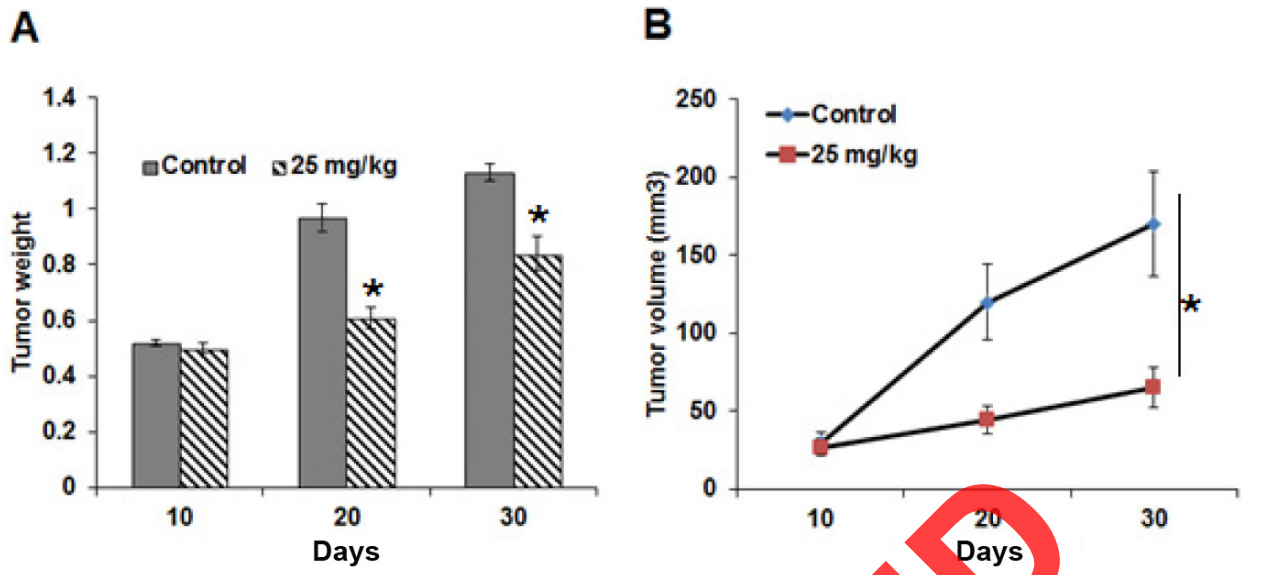

Figure 9. Effect of Trifolirhizin on tumor growth of xenografted tumor growth. **(A)** Tumor volume, **(B)** Tumor weight. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01).

Trifolirhizin causes the G_2/M arrest of gastric cancer cells

The effects of Trifolirhizin on the distribution of SNU-5vcells in various cell cycle phases was assessed by flow cytometry. It was found that Trifolirhizin caused remarkable increase in the percentage of the SNU-5 cells in the G_2 phase of the cell cycle. The percentage of SNU-5 cells in the G_2 phase increased from 9 to 40% upon treatment with Trifolirhizin (Figure 7). These results clearly indicate that Trifolirhizin induces G_2/M cell cycle arrest of the gastric cancer cells.

Trifolirhizin inhibits the PI3K/AKT signalling pathway

The effects of Trifolirhizin were also examined on the PI3K/AKT signalling pathway of SNU-5 gastric cancer cells. It was found that Trifolirhizin caused concentration-dependent decline in the expression of p-AKT, p-PI3K and p-mTOR, while no visible effect was observed on the expression of AKT, PI3K and AKT (Figure 8). Taken together, the results indicate that Trifolirhizin inhibits the mTOR/PI3K/AKT signal transduction pathway in SNU-5 cells.

Trifolirhizin inhibits tumor growth in vivo

Since Trifolirhizin showed potent anticancer effects on gastric cancer cell lines *in vitro*, we sought to examine the anticancer effects of Trifolirhizin *in vivo* in xenografted mice models. It was observed that Trifolirhizin at the dosage of 25 mg/kg significantly inhibited the tumor weight and volume concentration-dependently (Figure 9A and B).

Discussion

Gastric cancer is one of the frequently detected cancers worldwide and is responsible for significant morbidity and mortality across the globe. It has been reported that the major cause of gastric cancer-related mortality is due to late diagnosis which results to local invasion and distant metastasis [17]. The chemotherapeutic agents used for the management of gastric cancer are generally inefficient and exhibit severe adverse effects on the overall health of the patients [18]. Plant-derived anticancer agents have attracted remarkable attention in the recent past due to their minimal toxic effects. As such, an ever-increasing number of plant-derived natural products are being evaluated against cancer cells for their anticancer activity [19]. Herein, the anticancer effects of Trifolirhizin were examined against human gastric cancer cell lines and it was found that Trifolirhizin could cause considerable decline in the viability of these cells. In a previously carried out study, Trifolirhizin has also been reported to inhibit the growth of several types of cancer cells [10]. Autophagy is a vital process that triggers death of the harmful cells and protects the survival of normal cells [20]. Similarly, apoptosis eliminates the harmful cells from the body [21]. In this study investigation of the mechanism of action of Trifolirhizin revealed that Trifolirhizin prompts both autophagy and apoptotic cell death of the SNU-5 gastric cancer cells. This was also associated with changes in the expression of autophagy as well in the apoptosis-related protein expression. In addition, Trifolirhizin caused arrest of the SNU-5 at the G_2/M check point and thereby halted their growth.

Previous studies have indicated that several of the anticancer molecules induce autophagy, apoptosis and cell cycle arrest of cancer cells [22]. It has been found that mTOR/PI3K/AKT pathway is activated in many cancer types and this promotes their proliferation [19]. In this study we found that Trifolirhizin could inhibit the mTOR/PI3K/AKT signal transduction pathway in SNU-5 cells concentration-dependently. Because of the potential anticancer activity of Trifolirhizin *in vitro*, we sought to know the antiproliferative effects of Trifolirhizin *in vivo* and found that Trifolirhizin could inhibit the growth of xenografted tumors, indicative of the potential of Trifolirhizin in the treatment of gastric cancer.

Conclusion

Taken together, it is concluded that Trifolirhizin inhibits the proliferation of gastric cancer cells by autophagy and apoptotic cell death. In addition it could also induce cell cycle arrest and inhibited the tumor growth in vivo. As such Trifolirhizin could prove to be an important therapeutic agent and warrants further investigation.

Conflict of interests

The authors declare no conflict of interests.

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