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ORIGINAL ARTICLE

# Imperatorin shows selective antitumor effects in SGC-7901 human gastric adenocarcinoma cells by inducing apoptosis, cell cycle arrest and targeting PI3K/Akt/m-TOR signalling pathway

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## Summary

***Purpose:*** *The main purpose of the present study was to determine the anticancer properties of imperatorin - a naturally occurring coumarin compound - against SGC-7901 human gastric adenocarcinoma cells and the mouse fibroblast cell line 3T3 (normal cell line).*

***Methods:*** *Imperatorin effects on apoptosis induction, cell cycle phase distribution and PI3K/Akt/m-TOR signalling pathways were studied. MTT cell viability assay examined the compound's cytotoxic potential, while inverted phase contrast microscopy and fluorescence microscopy techniques were used to study morphological changes induced in SGC-7901 cells by imperatorin. Flow cytometry examined its effects on cell cycle progression while Western blot assay was used to study changes in protein expressions of PI3K/Akt/m-TOR pathway.*

***Results:*** *Imperatorin induced a dose-dependent growth inhibition of the SGC-7901 gastric cancer cells with an $IC_{50}$ value 62.6 μM, while in case of normal 3T3 mouse fibroblast cells, the drug did not show significant toxicity ($IC_{50}$ value 195.8 μM), indicating that the drug selectively induced cytotoxicity in gastric cancer cells. The cells became rounded up, shrunken in size and got detached from the monolayer attached to well surface. Cells treated with 10, 75 and 175 μM imperatorin indicated that they began to emit yellow or red fluorescence which is an indication of early or late apoptosis respectively. Imperatorin also induced significant DNA fragmentation along with increasing the fraction of sub-G1 cells, indicating a sub-G1 cell cycle arrest.*

***Conclusion:*** *Imperatorin could prove an important lead molecule for the treatment of gastric cancer and deserves further research in vivo against more cell lines.*

***Key words:*** *apoptosis, cell cycle arrest, fluorescence, gastric cancer, imperatorin*

## Introduction

Gastric carcinoma is one of the most lethal kinds of human malignancies making it an important health problem. This cancer is one of the principal causes of cancer-related deaths not only in developing countries but also in developed countries. China is one of the few countries with a very high incidence of gastric cancer and accounts for more than 45% of all the new cases diagnosed worldwide. In China, gastric cancer is the third leading cause of cancer related mortality [1,2]. This cancer is characterized by high recurrence rate and low survival rate. The majority of gastric cancer patients have already advanced disease stage at the time of diagnosis making treatment options problematic [3]. A lot of research has been conducted worldwide to understand the underlying biochemical and genetic factors leading to gastric cancer, still only a limited number of chemothera-

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peutic and chemopreventive agents have been designed and developed so far [4]. The treatment options for gastric cancer include surgical resection and chemotherapy. But these treatment options are only successful only if the disease is diagnosed at an early stage. It has been reported that the 5-year survival of gastric cancer patients in about 30%, which is quite low. Therefore, it is imperative that novel and more effective treatment strategies are designed to combat this growing health problem globally [5,6]. It has been reported that compounds which induce apoptosis (programmed cell death) in cancer cells have the tendency to curb tumor cell proliferation which can be a promising and effective treatment option to inhibit the development of cancer [7,8]. Additionally, these compounds should have the tendency to selectively target cancer cells without causing much damage to normal cells. However, most of the tested compounds as well as those drugs which have been clinically approved for cancer treatment cause serious side effects due to their non-selectivity as they kill cancer cells as well as normal cells.

In this study, we report on the anticancer activity of imperatorin, which is a naturally occurring coumarin natural product isolated from various plant species, particularly from Angelica species. It was shown that imperatorin selectively targets SGC-7901 gastric cancer cells without causing much damage to normal cells. The effects of imperatorin on apoptosis induction as well as on PI3K/Akt/m-TOR signalling pathway were also evaluated.

## Methods

*Chemicals and other reagents*

Imperatorin (>95% purity as checked by HPLC), Annexin V-FITC, propidium iodide, acridine orange and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] (MTT) were obtained from Sigma Aldrich, St. Louis, MO, USA. RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco BRL, Carlsbad, CA, USA. The various antibodies were purchased from Cell Signaling Technology, USA. Fetal bovine serum (FBS), trypsin, penicillin, streptomycin, DMSO, RIPA Buffer, SDS, were obtained from Hangzhou Sijiqing Biological Products Co. Ltd, China. Bichinconinic acid protein assay kit was obtained from Pierce Biotechnology, Rockford, IL, USA.

*Cell line and cell culture conditions*

SGC-7901 human gastric adenocarcinoma cell line was procured from Institute of Cell Biology, Chinese Academy of Science, Shanghai, China. The mouse fibroblast cell line 3T3 was obtained from American Type Culture Collection (Manassas, VA). Mouse fibroblast cell line 3T3 was grown in DMEM while SGC-7901 cells were grown in DMEM supplemented with 10 % FCS, 1% penicillin and 0.01% streptomycin. Cells were cultured in $CO_2$ incubator (Thermo Scientific, Waltham, USA) at 37°C, 95% humidity and 5% $CO_2$.

*MTT assay for cell viability*

The effect of imperatorin on cell viability was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. In brief, SGC-7901 gastric cancer cells and 3T3 normal cells were seeded in a 96-well plate at a density of $2x10^6$ cells/well. The cells were incubated for 48 hrs and then treated with various concentrations (0, 10, 50, 75, 100, 125 and 175 µM) of imperatorin and incubated for 24, 48 and 72 hrs. In the control group, the cells were treated with the solvent only (DMSO) and not the drug. Following incubation, the cells were washed with PBS three times and a 200 µl solution of MTT dye was added and the whole cell sample was again incubated for 40 min. After this, the absorbance was measured at 490 nm using ELISA plate reader (ELX 800; Bio-Tek Instruments, USA).

*Inverted phase contrast microscopy*

The morphological changes induced in SGC-7901 gastric cancer cells by imperatorin were measured by inverted phase contrast microscopy. SGC-7901 cancer cells were seeded in 6-well plates at a density of $2x10^6$ cells/well. The cells were treated with varying doses of imperatorin including 0, 10, 75 and 175 µM. The cells without drug treatment served as vehicle control. Following treatment, the cells were incubated for 48 hrs before being analyzed under an inverted phase contrast microscope (Olympus IX71, Japan).

*Fluorescence microscopy using acridine orange/propidium iodide double staining*

Fluorescence microscopy using acridine orange/propidium iodide gives a good idea about the morphological changes which occur during apoptosis. In brief, SGC-7901 human gastric carcinoma cells were seeded in a 6-well plate at a cell density of $2x10^6$ cells/well and incubated for 24 hrs. After incubation, the cells were treated with 0, 10, 75 and 175 µM doses of imperatorin and again incubated for 48 hrs. Both the treated and untreated cells were incubated with acridine orange and propidium iodide (10 µg/ml each) and then the cells were assessed under a fluorescent microscope (Olympus IX71, Japan) at 200x magnification.

*DNA fragmentation analysis*

In brief, SGC-7901 cells were seeded in a 60-mm cell culture plate and then incubated for 48 hrs before being treated with 0, 10, 75 and 175 µM of imperatorin for 48 hrs. After harvesting the cells were washed three times with PBS and then the pellets were lysed with a DNA lysis buffer for 1 hr. The sample containing the cells was centrifuged at 12,000 rpm and the supernatant was prepared in an equivalent amount of 2.0 % sodium-dodecyl sulphate, and then incubated with 10 mg/ml RNase A for 2 hrs. Eventually, DNA was dissolved in

gel loading buffer and separated using electrophoresis in 2.5% agarose gel, stained with ethidium bromide and then examined under ultraviolet light.

*Measurement of cell cycle phase distribution using flow cytometry*

Further experiments using flow cytometry were carried out in order to evaluate the effect of imperatorin on the cell cycle phase distribution in SGC-7901 human gastric carcinoma cells. For this purpose, the cells were seeded at a cell density of $2x10^6$ cells/ml and then incubated for 12 hrs. The overnight cell culture medium was replaced by a fresh DMEM and then different doses (0, 10, 75 and 175 µM) of imperatorin drug were added. The cells were further incubated for 48 hrs before being trypsinized and washed twice with ice-cold PBS. The cells were then fixed using 70% methanol for 50 min and were washed again with PBS. The cells were then stained with 10 µg/mL propidium iodide and then 10 µg/mL RNase A was added for 30 min. Eventually, the cells were analyzed using a flow cytometer (FACS Calibur; BD Biosciences, Singapore) and the data were processed using cell cycle analysis software (Modifit 2.0).

*Western blot assay*

In order to evaluate the effect of imperatorin on the expression levels of various key proteins like PI3K/Akt/m-TOR, Western blot assay was used. SGC-7901 human gastric cancer cells were seeded in a plate (20 cm) and incubated for 24 hrs. Subsequently, the old medium was substituted by a fresh DMEM. The cells were treated with varying doses (0, 10, 75 and 175 µM) of imperatorin and further incubated for 48 hrs. The medium was removed and the cells were washed with ice-cold PBS three times. The cells were then detached and lysed using RIPA buffer and protease inhibitor for 20 min. The contents were then centrifuged and the protein content was estimated by bichinconinic acid protein assay kit. About 100 µg of cellular protein from each sample was applied to 10% SDS-polyacrylamide gels and probed with antibodies against p-Akt, Akt, m-TOR and glyceraldehyde phosphate dehydrogenase (GAPDH) at 4°C overnight, followed by exposure to horseradish peroxidase-conjugated goat anti-mouse antibodies. Blots were then developed using the West Pico Chemiluminescent substrate (Pierce; Woburn, MA, USA).

*Statistics*

All results were expressed as mean±standard error (SE) from at least three independent experiments. The differences between groups were analyzed by one-way ANOVA, and the significance of difference was designated as *p<0.05, and **p<0.01.

## Results

*GSelective antitumor activity of imperatorin against SGC-7901 human gastric cancer cells*

The cytotoxic effects of imperatorin in SGC-7901 human gastric cancer cells and the mouse fibroblast cell line 3T3 (normal cells) were evaluated by MTT cell viability assay (Figure 1). The results which are shown in Figure 2 indicate that imperatorin induced a dose-dependent growth inhibition of the SGC-7901 gastric cancer cells with an $IC_{50}$ value of 62.6 µM. $IC_{50}$ value which gives an indication of the potency of the compound revealed that imperatorin induced significant cytotoxic effects in these cancer cells. However, as compared to the SGC-7901 cancer cells, the 3T3 mouse fibroblast cells were not affected so much, even at an imperatorin dose of 175 µM, indicating that the drug selectively induced cytotoxicity in gastric cancer cells sparing the normal mouse fibroblast cells.

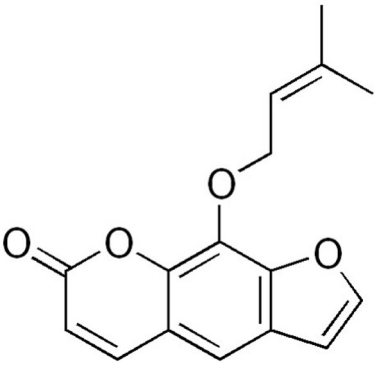

**Figure 1.** Chemical structure of imperatorin.

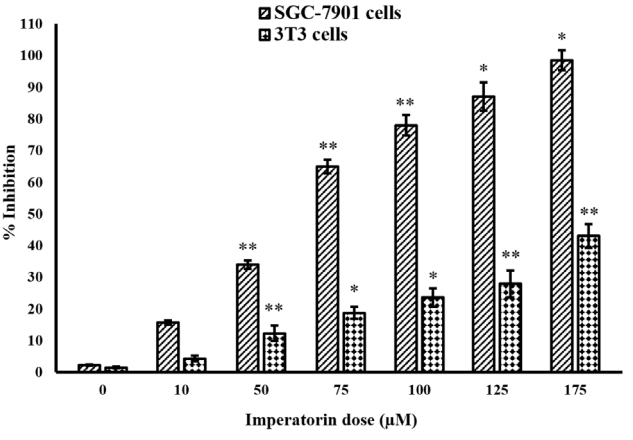


**Figure 2.** Selective cytotoxicity induced by imperatorin in SGC-7901 human gastric carcinoma cells and the mouse fibroblast cell line 3T3. Data are shown as mean±SD of three independent experiments. *p<0.05, **p<0.01, vs 0 µM (control).

*Imperatorin induced morphological changes in SGC-7901 cells*

The morphological alterations induced by imperatorin in SGC-7901 human gastric cancer cells are shown in Figure 3. The results indicate that the untreated control cells exhibited normal cellular morphology with uniform cell growth and no signs of cellular shrinkage. However, on treating cells with 10, 75 and 175 µM dose of imperatorin, cell shrinkage along with distortion of normal cel-

lular structures was observed. These effects were seen to increase with increasing doses of the imperatorin (Figure 3, B-D). The cells became rounded up, shrunken in size and got detached from the monolayer. It was also observed that the number of cells also reduced in the treated cells in comparison to the untreated control cells.

*Imperatorin induced apoptosis in SGC-7901 cells as evaluated by fluorescence microscopy*

The morphological changes characteristic of apoptosis induced by imperatorin in SGC-7901 human gastric cancer cells were demonstrated by fluorescence microscopy. As shown in Fig.4 A-D, the control cells exhibited normal cell morphology emitting only green fluorescence which is a sign of viable cells. However, cells treated with 10, 75 and 175 µM imperatorin indicated that cells began to emit yellow or red fluorescence which is an indication of early or late apoptosis, respectively. The percentage of red fluorescence-emitting cells increased with increasing doses of the drug. Further, the treated cells showed cell shrinkage, blebbing of the plasma membrane along with chromatin condensation.

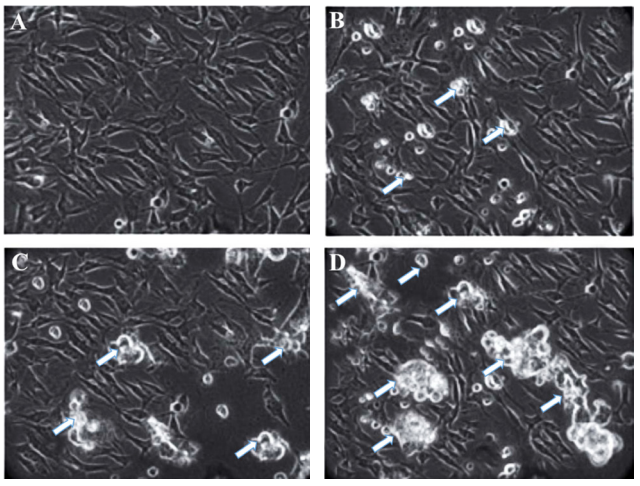


**Figure 3.** Morphological evaluation of the SGC-7901 human gastric cancer cells following treatment with 0 **(A)**, 10 **(B)**, 75 **(C)** and 175 µM **(D)** of imperatorin for 48 hrs using inverted phase contrast microscopy. The white arrows indicate the changes in cell morphology. As compared to the control cells, imperatorin-treated cells showed altered cell morphology.

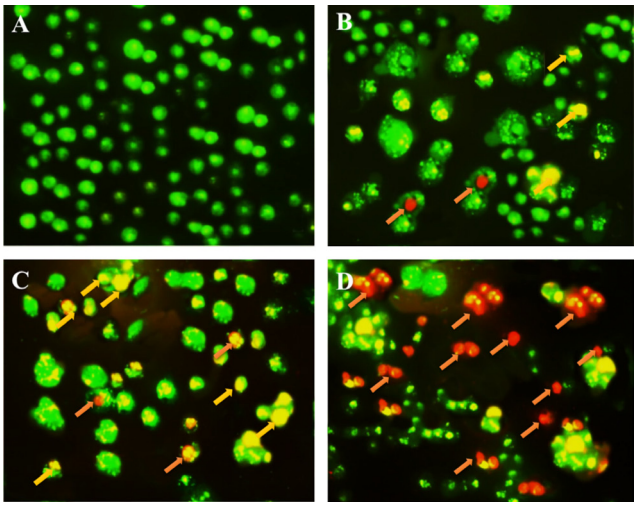


**Figure 4.** Fluorescence microscopy evaluation of the apoptosis induction in SGC-7901 human gastric cancer cells using acridine orange/propidium iodide double staining. The cells were treated with increasing doses (0 **(A)**, 10 **(B)**, 75 **(C)** and 175 (D) µM) of imperatorin for 48 hrs, stained and then analyzed by the fluorescence microscope at 200 x magnification. Yellow arrows indicate early apoptotic cells, red arrows indicate late apoptotic cells.

*Imperatorin induced significant DNA damage in SGC-7901 gastric cancer cells*

A further experiment using agarose gel electrophoresis was designed to study effects of imperatorin on the DNA ladder formation in SGC-7901 cells. Results are shown in Figure 5 and reveal that as compared to untreated control cells, imperatorin-treated cells showed considerable DNA laddering formation indicating DNA damage induced by imperatorin in these cells. The DNA marker (M) indicates the size of the fragments of the DNA ladder.

*Imperatorin induced sub-G1 cell cycle arrest*

Using flow cytometry coupled with propidium iodide, it was shown that imperatorin induced significant effects on cell cycle phase distribution. As shown in Figure 6, increasing doses of imperatorin led to increase in the percentage of

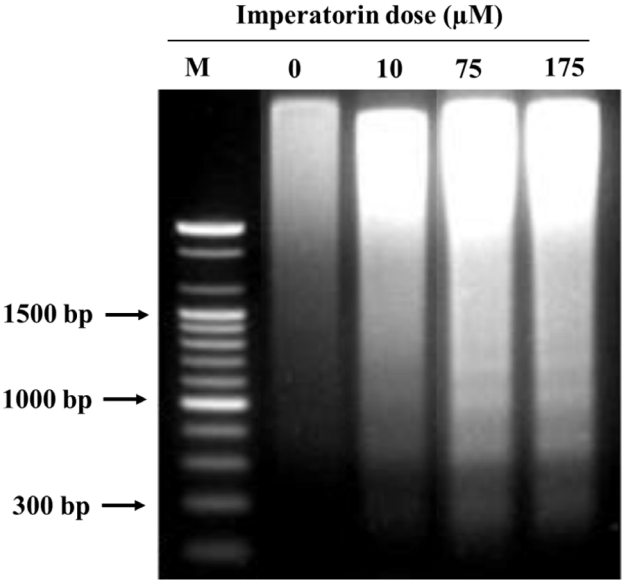


**Figure 5.** DNA fragmentation analysis of SGC-7901 human gastric cancer cells after the cells were treated with 0, 10, 75 and 175 µM dose of imperatorin and incubated for 48 hrs. The bands were analyzed by agarose gel electrophoresis. The gel images show that imperatorin induces DNA damage in a concentration-dependent manner.

the sub-G1 cells. As the concentration of imperatorin increased from 0 to 10, 75 and 175 μM, the percentage of sub-G1 cells (apoptotic cells) also increased from 2.3% to 8.6%, 23.5% and 76.3%, respectively. In addition, a decrease in the G0/G1 cells was also seen as the dose of imperatorin increased.

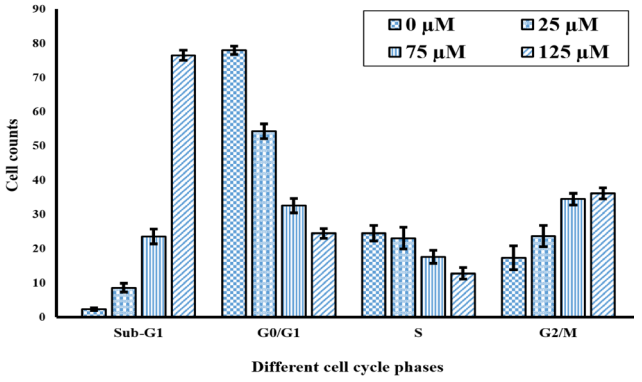


**Figure 6.** Imperatorin induced sub-G1 cell cycle arrest in SGC-7901 human gastric cancer cells. The cells were treated with 0, 10, 75 and 175 μM dose of imperatorin and incubated for 48 hrs, after which cells were analyzed using flow cytometry. It was observed that imperatorin induced sub-G1 cell cycles arrest in SGC-7901 cells ($p<0.01$ vs control).

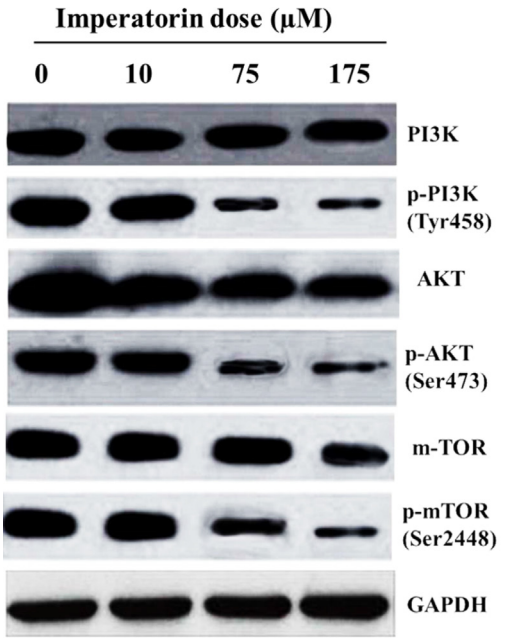


**Figure 7.** Effect of increasing doses of imperatorin on the expression levels of PI3K/Akt/m-TOR pathway-related proteins. The cells were treated with 0, 10, 75 and 175 μM dose of imperatorin and incubated for 48 hrs and then subjected to Western blot assay. The results indicate that imperatorin downregulated the expression of the important proteins P13K/Akt/m-TOR pathway.

*Imperatorin led to downregulation of phosphatidylinositol 3-kinase (PI3K)/Akt/m-TOR pathway-related protein expressions*

Using Western blot assay, the effects of imperatorin on the expressions of PI3K/Akt/m-TOR signalling proteins were evaluated. It has been reported that the phosphatidylinositol 3-kinase (PI3K) Akt/m-TOR pathway is mostly disturbed in many human malignancies. Imperatorin led to downregulation of PI3K/Akt/m-TOR signalling proteins in a dose-dependent manner (Figure 7). The expression levels of p-PI3K, p-Akt and pm-TOR were much higher in treated cells as compared to the untreated control cells. Thus it can be concluded that imperatorin induced anticancer effects in SGC-7901 gastric cancer cells via downregulation of PI3K/Akt/m-TOR pathway.

## Discussion

Imperatorin is a furanocoumarin isolated from many plant species including *Angelica dahurica*. It has been reported to exhibit many pharmacological activities including anticancer and anticonvulsant effects and has been used in many herbal formulations for the treatment of hypertension and cardiovascular disorders. Reports have revealed that imperatorin isolated from *Angelica dahurica* induced apoptosis in Hep-G2 cells through Death-Receptor and Mitochondria-Mediated Pathways [9]. Imperatorin has also been shown to inhibit cell growth of SNU-449 liver cancer cells as well as colon cancer cells (HCT-15) in a dose-dependent manner. Different doses of imperatorin were shown to induce morphological alterations in these cancer cells without damaging normal dermal fibroblast cells. It also induced cell death via cell cycle arrest and downregulation of Bcl-2 protein expression [10]. Imperatorin has also been reported to inhibit HT-29 colon cancer cell growth via apoptosis induction and upregulation of p53 and caspases. It has also been reported to induce cell cycle arrest in the G1 phase [11]. It also induces cytochrome c-dependent apoptosis in human promyelocytic leukemia (HL-60) cells [12]. Furthermore, imperatorin has been reported to inhibit cell proliferation of melanoma cells and cell cycle arrest at G2/M phase under ultraviolet radiation [13]. Imperatorin has also been shown to affect scopolamine-induced cognitive impairment and oxidative stress in mice [14]. There are no reports on the anticancer effects of imperatorin in SGC-7901 human gastric cancer cells or apoptosis induction, cell cycle arrest and PI3K/Akt/m-TOR signalling pathways. Therefore, in the current study, our main objective was to study the anticancer effects of imperatorin along with effects on apoptosis induction, cell cycle phase distribu-

tion and PI3K/Akt/m-TOR signalling cascade in SGC-7901 human gastric adenocarcinoma cells. The results indicated that imperatorin induced dose-dependent and selective antitumor effects in SGC-7901 gastric cancer cells. Our findings also indicate that imperatorin induced significant morphological changes including cell shrinkage and rounding up of the cells. The number of cells was also shown to decrease with increasing imperatorin dose. Cells treated with 10, 75 and 175 μM of imperatorin began to emit yellow or red fluorescence which is an indication of early or late apoptosis, respectively. The percentage of red fluorescence-emitting cells increased with increasing doses of the drug. Imperatorin was also shown to induce DNA fragmentation which also increased with the concentration of the drug. It also led to sub-G1 cell cycle arrest as the percentage of sub-G1 cells (apoptotic cells) increased from 2.3% to 8.6%, 23.5% and 76.3% respectively as the dose of imperatorin increased from 0 to 10, 75 and 175 μM, respectively. Imperatorin also led to downregulation of PI3K/Akt/m-TOR signalling proteins in a dose-dependent manner.

In conclusion, it can be summed up that imperatorin induced selective antitumor effects in SGC-7901 human gastric cancer cells without causing too much cytotoxicity to the normal mouse fibroblast cells (3T3 cells). It also induced apoptosis, sub-G1 cell cycle arrest, DNA fragmentation and downregulation of PI3K/Akt/m-TOR signalling pathway.

## Conflict of interests

The authors declare no conflict of interests.

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