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ORIGINAL ARTICLE

Totarol, a natural diterpenoid, induces selective antitumor activity in SGC-7901 human gastric carcinoma cells by triggering apoptosis, cell cycle disruption and suppression of cancer cell migration

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Summary

Purpose: *Totarol is a plant-derived natural product and has been reported to exhibit important pharmacological activities. However, the anticancer activity of totarol has not been evaluated yet. Therefore, the present research work was designed to evaluate the antitumor effects of totarol in SGC-7901 human gastric cancer cells and human gastric epithelial mucosa cell line GES-1 (used as normal cell line model) together with examining its effects on induction of apoptosis, cell cycle phase distribution and cell migration.*

Methods: *The effect of totarol on cell cytotoxicity was evaluated by MTT cell viability assay. Inverted phase contrast microscopy was used to identify the effects on cell morphology, while transmission electron microscopy indicated the apoptosis-driven morphological changes in cancer cells. The effects on cell apoptosis were also evaluated by annexin V/PI staining, while cell cycle analysis was done by flow cytometry. In vitro wound healing assay estimated the effects of totarol on cell migration.*

Results: *The results indicated that totarol induced selective cytotoxic effects in SGC-7901 human gastric cancer cells concentration-dependently and exhibited lower toxicity in GES-1 normal cells. The totarol-treated cells showed significant alterations in cell morphology including rounding and cellular shrinkage. Untreated SGC-7901 cells exhibited normal cellular morphology with undamaged plasma membrane. However, treating cells with totarol led to damaged plasma membrane along with appearance of rounded protrusions (apoptotic bodies) containing damaged and broken chromatin material. Treatment with different doses of totarol led to profound suppression of wound healing. Totarol treatment also led to G2/M phase cell cycle arrest in these cells in a concentration-dependent manner.*

Conclusions: *The present study indicated that totarol diterpene has the tendency to show selective anticancer effects in SGC-7901 human gastric cancer cells along with inducing apoptosis, cell cycle arrest and inhibition of cell migration.*

Key words: *apoptosis, cell migration, flow cytometry, gastric cancer, totarol*

Introduction

Gastric cancer is one of the deadly malignancies that has become a serious global health issue. Gastric cancer is the second main cause of cancer-related death and the fourth most prevalent kind of malignant neoplasm in the world. This cancer still has a poor prognosis despite advances in treatment

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strategies, better prevention and improved diagnostic tools [1,2]. There is a significant geographical variation in the worldwide incidence of gastric carcinoma cases which indicates wide-ranging factors responsible for this cancer. Most of the gastric cancer cases are reported from Asian countries while Europe and America together constitute less than a quarter of the global disease burden. The populations with lower socioeconomic status are usually more affected than the affluent class of people [3,4]. In China, gastric cancer is one of the most prevalent types of malignancy with an estimated 380,000 new cases reported every year. This figure accounts for about 40% of the global annual cancer incidence. In China, it has been reported that rural areas (particularly in Henan, Hebei, Gansu, Shanxi and Shaanxi Provinces) are high-risk zones for gastric cancer than urban areas. The gastric cancer-related deaths both for men and women in China are the highest globally [5,6]. Gastric carcinoma is directly associated with an infectious microbe namely *Helicobacter pylori* (*H. pylori*). This microbe colonizes the stomach of about half of the global population and has been linked to peptic ulcer, gastric lymphomas, chronic gastritis etc. *H. pylori infection* is the well-known known risk factor for the development of gastric cancer [7].

Treatment strategies for gastric cancer depend on the location and size of the primary tumor. The most useful treatment approach involves surgical resection with subtotal or total gastrectomy followed by adjuvant or neoadjuvant chemotherapy. Chemotherapy kills cancer cells by inducing toxicity and apoptosis in cancer cells, however, it also affects normal cells leading to serious side-effects [8]. Therefore, the focus of the research studies carried out throughout the globe is to screen, identify and develop anticancer agents which target cancer cells without causing too much damage to normal cells. Various naturally occurring compounds have been identified to have such a potential [9-12]. The phenolic diterpenoid totarol is a major constituent isolated from the sap of *Podocarpus-totara* and exhibits antiplasmodial, antifungal and antimicrobial activity against several organisms, including *Propionibacterium acne* [13]. Besides, this compound inhibits bacterial respiratory transport, disrupt phospholipid membranes, or function as an efflux-pump inhibitor [13]. However, the anticancer activity of totatrol has not been explored as yet.

Therefore, the aim of the current research work was to evaluate the cytotoxic effects of totarol against SGC-7901 human gastric cancer cells. Its effects on mitochondrial-mediated apoptosis, cell cycle phase distribution and cancer cell migration were also demonstrated in the present study.

Methods

Chemicals and other reagents

Totarol (≥95% purity as determined by HPLC), MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), dimethyl sulfoxide, 5% heat-inactivated fetal calf serum, penicillin, streptomycin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Propidium iodide (PI), and acridine orange/ethidium bromide (AO/EB) were purchased from Wuhan Boster Biological Technology Ltd. (Wuhan, China).

Cell lines, cell culture conditions and MTT cell viability assay

Human gastric epithelial mucosa cell line GES-1 and gastric cancer cell line SGC-7901 were procured from the cell bank of the Basic Medical College of Huazhong University of Science and Technology (HUST). The cells were cultured in RPMI 1640 medium containing 15% fetal bovine serum (FBS) along with 100 U/ml penicillin and 100 µg/ml streptomycin in an incubator at 37°C in a humidified atmosphere with 5% CO_2. The cytotoxic effect of totarol was evaluated by MTT assay which is a colorimetric assay based on the reduction of yellow colored MTT by succinate dehydrogenase which is present in mitochondria.

Both the cell lines (SGC-7901 and GES-1) at a density of 2×10^5 cells/well were seeded in a 96-well plate, incubated for 24 hrs and then treated with increasing doses (0, 10, 20, 40, 100, 200, 400 µM) of totarol for 24 hrs. The untreated cells were considered as control group. After incubation, the cells were washed with phosphate buffered saline (PBS) twice and then 100 µl

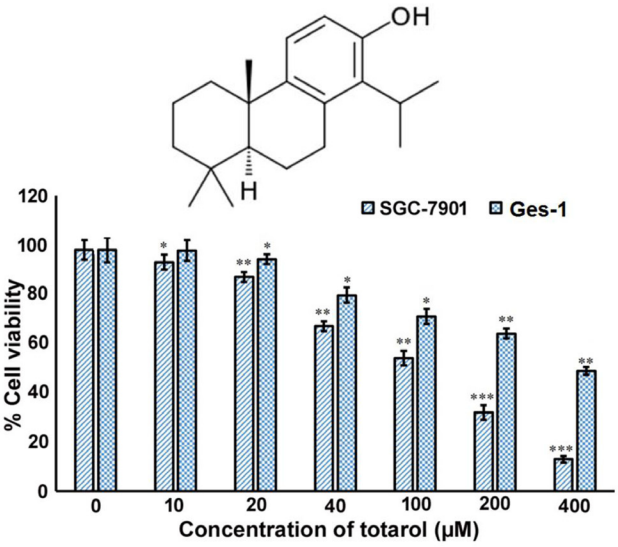

Figure 1. Chemical structure of totarol. Totarol exhibits cytotoxicity in SGC-7901 human gastric cancer cells. The cell cytotoxicity was evaluated by MTT assay at various concentrations. All experiments were carried out in triplicate and expressed as mean±SE. Significance of difference between SGC-7901 and Ges-1 cells was indicated as *p<0.05, **p<0.01 and ***p<0.001.

of MTT solution was added and the whole cell culture was again incubated for 50 min. Finally, the absorbance was measured at 490 nm using ELISA plate reader (ELX 800; Bio-Tek Instruments, USA).

Inverted phase contrast microscopy

SGC-7901 cells were grown in 6-well plates at 2×10^6 cells/ml and then maintained at favorable conditions for 24 hrs. Then, the cells were treated with increasing doses of totarol (0, 40, 100 and 400µM) for 48 hrs. Inverted light microscope (Nikon Corp., Tokyo, Japan) was used to examine culture plates following drug treatment and images were taken. Dimethyl sulfoxide (DMSO) served as a control.

Transmission electron microscopy (TEM) study

The fact that totarol induces apoptosis in SGC-7901 cells was demonstrated by using transmission electron microscope. In brief, the cells were seeded in a flask and then treated with increasing concentrations (0, 40, 100, 400 µM) of totarol for 48 hrs. The cells were collected and washed with PBS twice before adding 2.5% glutaraldehyde for microtome sectioning using ultramicrotome (JEOL Co: Japan). TEM analysis was done by a Transmission Electron Microscope (JEOL Co; Japan).

Estimation of apoptotic populations by annexin V/PI staining

The gastric cancer cells at a density of 2×10^5 cells/well were seeded in 6-well plates and were administered 0, 40, 100 and 400 µM totarol for 48 hrs. The cells were then subjected to annexin V/IP staining. Afterwards, the cell sample was examined by flow cytometry.

In vitro wound healing assay

The effect of totarol on the migration of SGC-7901 cells was evaluated by *in vitro* wound healing assay. SGC-7901 cells at a cell density of 2×10^5 cells per ml were seeded in a 6-well plate and incubated for 24 hrs to acquire a 100% confluence. The cells were starved for another 12 hrs, after which a straight cell-free wound was made using a 100 ml pipette. Each well containing cells was washed with PBS twice and then treated with 0, 40, 100, 400 µM of totarol for 48 hrs, after fixing the totarol-treated cells and staining with 2.5% ethanol comprising 3.5% crystal violet for 30 min. The cells were then examined under light microscope and the percentage of cells that migrated to the scratched area was determined.

Cell cycle analysis

For this assay, SGC-7901 human gastric cancer cells were seeded in 60-mm plates and treated with 0, 40, 100, 400 µM of totarol for 48 hrs. Following drug administration, cells were subjected to trypsinization and rinsed at least twice with PBS. Afterwards, these cells were fixed with 70% ethanol and administered 30 µg/mL RNase A, followed by staining with 10 µg/mL of PI. Lastly, DNA content and cell distribution was studied using flow cytometer FACS Calibur instrument (BD Biosciences, San Jose, CA, USA).

Statistics

All experiments were carried out in triplicate and expressed as mean±SE. The statistical analyses were carried out by Student's t-test (for comparisons between two samples) and one way ANOVA followed by Tukey's test (for comparisons between more than two samples) using GraphPad prism 7 software. The values were considered significant at *p<0.05, **p<0.01 and ***p<0.001.

Results

Totarol exhibited potent antitumor activity in SGC-7901 cells

In order to evaluate the antitumor effects of totarol in SGC-7901 cells we used MTT cell viability assay which is a colorimetric assay based on the decrease of yellow colored MTT by succinate dehydrogenase which is present in mitochondria. The results are shown in Figure 1 and indicate that totarol led to dose-dependent cytotoxicity in SGC-7901 cells as compared to the normal GES-1 cells. Even at a high dose of 400 µM, at which only 13.2% SGC-7901 cells showed viability, GES-1 cells were not affected too much. This indicates that totarol targets cancer cells without causing too much cytotoxicity to normal cells (GES-1), suggesting totarol may prove a safer and useful lead molecule.

Totarol induced morphological changes in SGC-7901 cells

The morphological changes induced by different doses of totarol in SGC-7901 cells were ob-

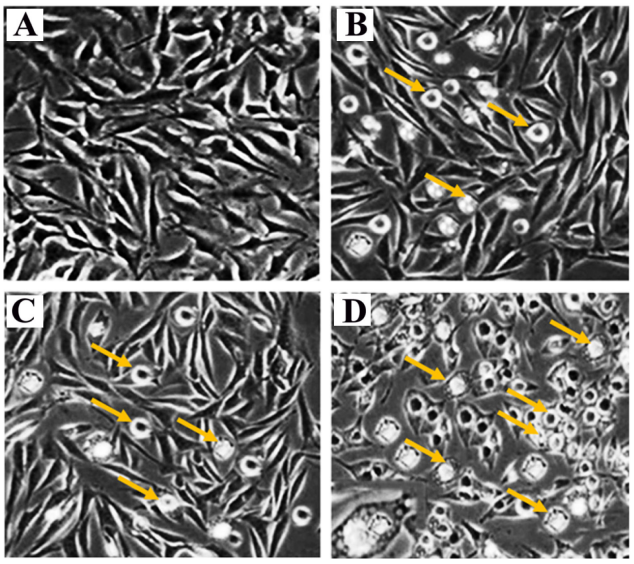

Figure 2. Morphological changes induced by totarol in SGC-7901 human gastric cancer cells. The cells were treated with 0 **(A)**, 40 **(B)**, 100 **(C)** and 400 **(D)** µM dose of totarol for 48 hrs and then examined under phase contrast microscope at 100x magnification. With increasing dose of the drug, significant cellular shrinkage could be seen (yellow arrows).

served using inverted phase contrast microscopy. It was observed that in comparison to control cells which exhibited normal cell morphology, the totarol-treated cells showed significant alterations in cell morphology including rounding and cellular shrinkage. There was a significant decrease in the attachment of the cells to the substratum as well as to one another, forming small clusters of cells (Figure 2).

Apoptotic changes induced by totarol in SGC-7901 cells

To fully establish whether totarol induces apoptosis in SGC-7901 cells, we used transmission electron microscopy after treating cells with totarol. The results are shown in Figure 3 and reveal that untreated SGC-7901 cells exhibited normal cellular morphology with normal chromatin material and undamaged plasma membrane (Figure 3 A). However, treating cells with increasing doses of totarol led to damaged plasma membrane along with appearance of rounded protrusions containing damaged and broken chromatin material. These protrusions are known as apoptotic bodies and it was observed that their number increased with increasing dose of totarol (Figure 3 B-D). Apoptotic body formation is a key step involved in the apoptotic process, indicating that anticancer effects of totarol are mediated via apoptotic induction in these cells. The apoptosis inducing potential of totarol was further confirmed by annexin V staining (Figure 4A-D) and it was observed that totarol induced apoptosis in a dose-dependent manner.

Totarol suppressed cell migration in SGC-7901 cells

The effect of totarol on the migration of SGC-7901 cells was demonstrated by *in vitro* wound healing assay. The outcomes are depicted in Figure 5 and show that wound scratch in vehicle-treated control cells was virtually fully closed after 48 hrs of incubation. However, treatment with 0, 40, 100 and 400 µM dose of totarol (Figure 5) led to suppression of wound healing dose-dependently. Thus, these results reveal that totarol might show anticancer activity by inhibiting cancer cell migration. Chemotherapeutic agents which suppress cancer cell migration are believed to be promising antitumor drugs because cancer cell migration has a direct relationship with cancer metastases.

Totarol induced G2/M phase cell cycle arrest in SGC-7901 human gastric cancer cells

Flow cytometry was used in evaluating the effects of totarol on the cell cycle phase distribution in SGC-7901 cells. The findings are shown in Figure 6 and reveal that totarol had a profound effect on the cell cycle phase distribution in these cells. In the untreated control group, the G2/M phase cells percentage was only 1.2% which increased to 6.7% ($p< 0.05$), 26.2% ($p< 0.01$) and 57.9% ($p< 0.001$) in

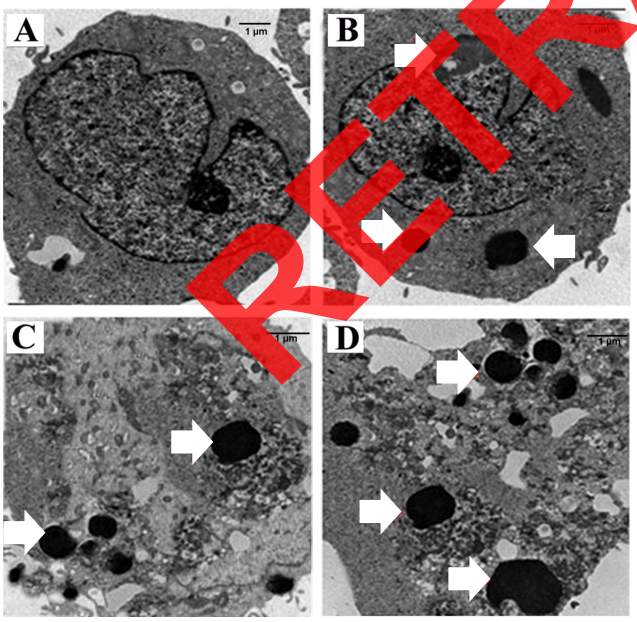

Figure 3. Totarol induced apoptosis-related ultrastructural changes in SGC-7901 human gastric cancer cells. The cells were treated with 0 **(A)**, 40 **(B)**, 100 **(C)** and 400 **(D)** µM dose of totarol for 48 hrs and then examined by transmission electron microscope at 8000x magnification. As compared to the untreated control cells, totarol-treated cells showed obvious apoptotic bodies which appeared as rounded protrusions. These apoptotic bodies increased in number with increasing dose of totarol. Arrows depict apoptotic bodies.

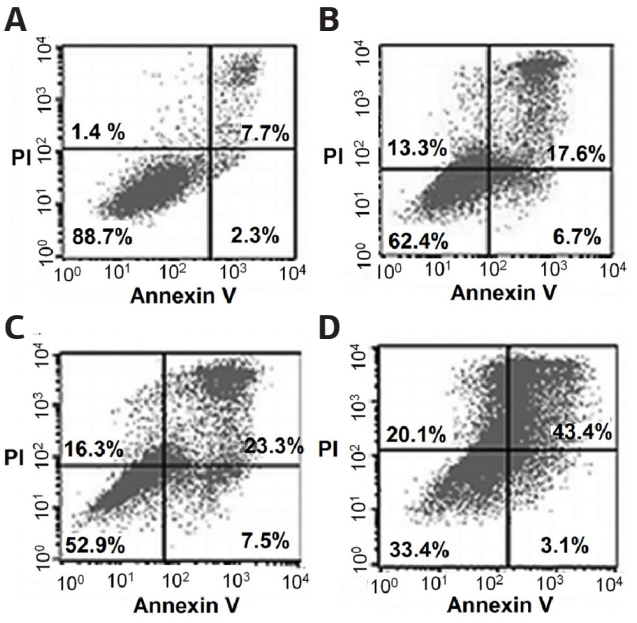

Figure 4. Totarol triggered apoptosis in SGC-7901 human gastric cancer cells as indicated by annexin V/PI staining and different concentrations of totarol **(A)** 0 **(B)**, 40 **(C)**, 100 **(D)** µM.

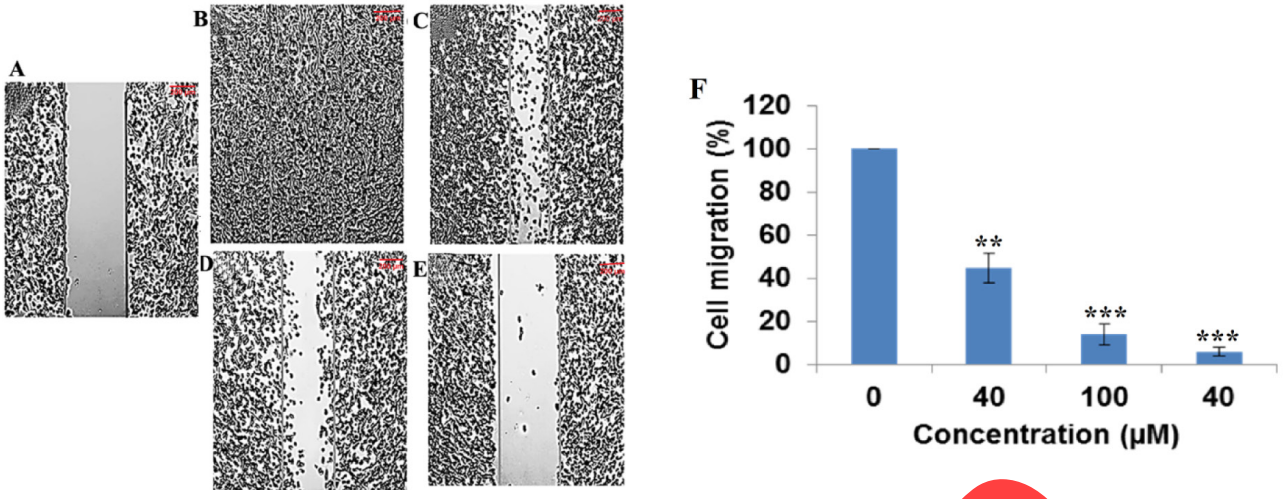

Figure 5. Totarol led to inhibition of migration in SGC-7901 human gastric cancer cells. Cells were treated with 0 **(B)**, 40 **(C)**, 100 **(D)** and 400 **(E)** μM dose of totarol. A shows a simulated wound prepared by scratching with a micropipette tip. **(F)** Quantification of cell migration. All experiments were carried out in triplicate. Significance of difference was indicated as **p<0.01 and ***p<0.001.

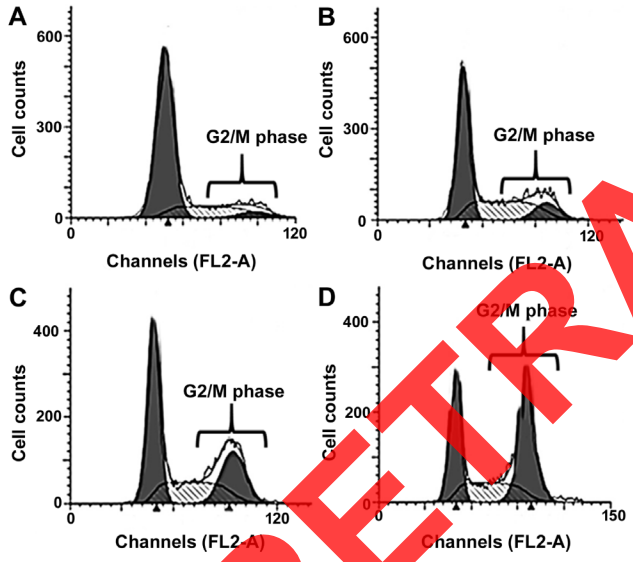

Figure 6. Totarol induced G2/M phase cell cycle arrest in SGC-7901 human gastric cancer cells. After treating the cells with 0 (A), 40 (B), 100 (C) and 400 (D) μM dose of totarol for 48 hrs, flow cytometry was used for analysis. The percentage of G2/M phase cells increased from 1.2% in control to 6.7%, 26.2% and 57.9% in groups treated with 40, 100 and 400 μM dose of totarol, respectively.

groups treated with 40, 100 and 400 μM dose of totarol, respectively. Thus, this experiment reveals that anticancer effect of totarol in SGC-7901 cells is mediated via G2/M cell cycle arrest.

Discussion

Gastric cancer is one the main causes of cancer related deaths across the globe and the treatment options are limited with lot of side effects. There is a pressing need to explore efficient and more effective drugs for the treatment of gastric cancer. Consistently, plant-based compounds include a wide spectrum of naturally occurring molecules belonging to diverse classes including flavonoids, sesquiterpenes, alkaloids, diterpenoids, and polyphenolics and most of these compounds display anticancer effects against a wide variety of cancer cells. Herbal medicinal agents have been shown to exert a wide range of pharmaceutic activities and various herbal agents have been used especially to treat different forms of human malignancies [14]. Totarol is a plant natural product that has been reported to exhibit several bioactivities, for instance, totarol has been shown to prevent the proliferation of several pathogenic Gram-positive bacteria including *Mycobacterium tuberculosis* [15]. In the present study, we reported for the first time the anticancer activity of totarol against gastric cancer cells. It was observed that totarol caused a concentration-dependent inhibition of SGC-7901 cells as compared to SEG-1 control cells. Additionally, totarol induced significant alterations in cell morphology such as rounding and cellular shrinkage. TEM showed that treating cells with increasing doses of totarol led to appearance of rounded protuberances (apoptotic bodies) containing damaged and broken chromatin material. Apoptosis is a programmed cell death and an extremely well-planned physiological mechanism to put an end to injured or dysfunctional cells. Apoptotic cells show characteristic morphological features and distinctive molecular expression [16]. In normal individuals, apoptosis plays crucial role in embryogenesis and helps maintaining homeostasis. Apoptosis is easily detectable from the changes in cellular

morphology including cell shrinkage, chromatin condensation, membrane blebbing, along with disorientation of cell organelles [17]. It is also characterized by certain biochemical features including activation of caspases and modulation of Bcl-2 and Bax proteins [16-18]. These proteins are closely related with the mitochondrial function. Bcl-2 is an anti-apoptotic protein and prevents the release of cytochrome c from mitochondria. On the contrary, Bax is a pro-apoptotic protein which promotes the release of cytochrome c from the mitochondria [19,20]. Our results are in agreement with a previous study wherein many anticancer drugs, such as 5-fluorouracil, exert their anticancer effects by inducing apoptosis in cancer cells [21].

We also evaluated the effect of totarol on the migration of SGC-7901 cells and observed that this molecule inhibited the cell migration dose-dependently. Invasion and migration of tumor cells into neighboring tissues and the vasculature is a preliminary stage in tumor metastasis. This involves chemotactic migration of tumor cells which is navigated by swelling of the cell membrane and its adherence to the extracellular matrix. This tendency of cancer cells to migrate and invade surrounding tissues permits them to change position within the tissue. As such, migration and invasion are responsible for the invasion of cancer cells into the lymphatic and blood circulation and eventually achieve metastatic growth in distant tissues [22-24].

Analysis of cell cycle phase distribution of totarol-treated gastric cancer cells showed that totarol triggered G2/M phase cell cycle arrest. In the untreated control group, the G2/M phase cells percentage was only 1.2% which increased to 6.7%, 26.2% and 57.9% in the groups treated with 40, 100 and 400 μM dose of totarol respectively. The link between cancer cell growth and cell cycle is very evident, while as cancer involves uncontrolled cell proliferation, cell cycle machinery controls cell proliferation. Cancers involve the presence of too many cells and this excess of cell number is "trapped" within a vicious circle. Any failure within the cell cycle leads to abnormal cell proliferation due to loss of cell cycle checkpoints, eventually leading to cancer [14,25,26].Our results are in concordance with a previous study wherein several natural products such as kaempferol have been reported to induce cell cycle arrest [27].

Taken together, we conclude that totarol exerts potent anticancer effects on gastric cancer cells, mainly due to induction of apoptosis and cell cycle arrest. Additionally, totarol also exhibits significant inhibitory effects on cell migration, indicating that it may prove a lead molecule for development of new gastric cancer therapy and deserves further research endeavors.

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Conflict of interests

The authors declare no conflict of interests.

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