Naturally Occurring Sesquiterpene Lactone-Santonin, Exerts Anticancer Effects in Multi-Drug Resistant Breast Cancer Cells by Inducing Mitochondrial Mediated Apoptosis, Caspase Activation, Cell Cycle Arrest, and by Targeting Ras/Raf/MEK/ERK Signaling Pathway

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Background: Sesquiterpene lactones have gained tremendous attention owing to their potent anticancer properties. The main focus of this study was to evaluate the anticancer effects of a naturally occurring sesquiterpene lactone, santonin, against human breast cancer SK-BR-3 cells.

Material/Methods: Cell counting kit 8 assay was used for the determination of cell viability. Apoptosis was detected by DAPI (4',6-diamidino-2-phenylindole) and annexin V/propidium iodide (PI) staining. Flow cytometry was used for cell cycle analysis and western blotting was used for the estimation of protein expression.

Results: Results showed that santonin exerts significant anti-proliferative effects on the SK-BR-3 breast cancer cells in a concentration dependent manner. Santonin showed an IC_{50} of 16 µM against SK-BR-3 cells. DAPI staining showed that santonin caused DNA fragmentation in the SK-BR-3 cells, which is indicative of apoptosis. Annexin V/PI staining showed that apoptotic cell percentage increased up to 34.32% at 32 µM concentration of santonin. Santonin also caused an increase in the expression of Bax, caspase-3, and caspase-9, and a decrease in the expression of Bcl-2. Santonin also caused the arrest of the SK-BR-3 cells at the G2/M phase of the cell cycle and suppressed the expression of cyclin A and B1. Finally, santonin could also block the Raf/MEK/ERK pathway in breast cancer cells.

Conclusions: The findings of this study suggest the potential for the naturally occurring sesquiterpene lactone santonin in breast cancer treatment and also suggest that it could be developed as a promising anticancer agent.

MeSH Keywords: Apoptosis • Cell Cycle • Flow Cytometry • Inflammatory Breast Neoplasms

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Background

Accounting for significant mortalities, breast cancer is the most prevalent type of cancer in women in USA [1]. Moreover, it is also one of the leading causes of cancer related mortality in women across the world. Although the incidence of breast cancer has been reported to be lower in several Asian countries, such as Japan, over last few years breast cancer incidence has shown a rapid increase in these countries [2]. The risk of developing breast cancer increases with age. Ovarian hormones which prompt cell division in breasts have also been shown to have a role in the development of breast cancer [3]. Moreover, the emergence of drug resistance among the breast cancers makes it even more difficult to treat [2,3]. In order to curb the increasing incidence of breast cancer, the identification of new and effective anticancer drugs is urgently needed. One of the major concerns nowadays is to find novel and efficient chemotherapeutic agents that have comparatively lower adverse effect. Additionally, there is a pressing need to explore anticancer agents that target cancer cells selectively, and decrease their malignancy without affecting normal cells [4]. Higher species plants have been shown to be exceptional sources of chemical entities with enormous medicinal potential [5]. Of all the plant-derived natural products, sesquiterpene lactones have shown potent and diverse biological activities [6]. Sesquiterpene lactones include a diverse and large group of natural metabolites found across the plant kingdom. Approximately 5 hundred sesquiterpene lactones have been isolated and characterized so far from the plant kingdom [7]. Over the last couple of decades, sesquiterpene lactones have gained considerable attention owing to their potent bioactivities such as anticancer and anti-microbial activities [8,9]. A number of sesquiterpene lactones have been reported to exhibit significant anticancer activity and many of these are currently undergoing clinical trials [10]. Santonin is an important sesquiterpene lactones found in several plant species [11]. However, the anticancer activity of santonin has not been explored so far. This study was therefore undertaken to investigate the anticancer effects of santonin against the human SK-BR-3 breast cancer cells. The results showed that santonin inhibited the growth of the breast cancer cells concentration dependently via induction of apoptosis and G2/M cell cycle arrest. The Ras/Raf/MEK/ERK signaling pathway has been reported to be implicated in the progression and development of several cancers [12], and herein it was found that santonin could deactivate this pathway. To sum up, santonin may prove beneficial in breast cancer treatment.

Material and Methods

Cell culture conditions

The drug resistant ovarian SK-BR-3 cells were obtained from the Cancer Research Institute of Beijing (Beijing, China) and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen Life Technologies, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Massachusetts, USA), 100 µg/mL streptomycin and 100 U/mL penicillin G (HiMedia, PA, USA) in an incubator at 37°C with 5% CO₂.

Cell counting kit-8 (CCK-8) assay

The SK-BR-3 cells were inoculated in a 96-well plate at the density of 1×10⁶ cells/well, and subjected to treatment with santonin at various concentrations (0 µM to 320 µM) and the number of SK-BR-3 cells was measured at each concentration. The procedures were as follows: the culture Dulbecco’s modified Eagle’s medium (Invitrogen Life Technologies, MA, USA) was discarded and we added 100 µL CCK-8 reagent. The 60-well plate was incubated in a CO₂ incubator for 2 hours. The OD values were measured by a microplate reader at the wavelength of 450 nm.

4’,6-diamidino-2-phenylindole (DAPI) and annexin V/propidium iodide (PI) staining

The SK-BR-3 cells at the density of 0.6×10⁶ were cultured in 6-well plates. Following an incubation period of around 12 hours, the SK-BR-3 cells were subjected to santonin treatment (0, 8, 16, and 32 µM) for 24 hours at 37°C. As the cells sloughed off, 25 µL cell cultures were put onto a glass slides and subjected to staining with DAPI. The slides were covered with a coverslip and examined with a fluorescent microscope (BD Biosciences, San Jose, CA, USA). Annexin V/PI staining was performed as described previously [13].

Cell cycle analysis

The SK-BR-3 cells were treated with varied concentrations of santonin and incubated for 24 hours at 37°C. The cells were subjected to washing with phosphate buffered saline (PBS). Afterwards, the santonin (0, 8, 16, and 32 µM) treated SK-BR-3 cells were stained with propidium iodide (PI) and subjected to staining with DAPI. The procedures were as follows: the culture Dulbecco’s modified Eagle’s medium (Invitrogen Life Technologies, MA, USA) was discarded and we added 100 µL CCK-8 reagent. The 60-well plate was incubated in a CO₂ incubator for 2 hours. The OD values were measured by a microplate reader at the wavelength of 450 nm.

Western blot analysis

Protein expression estimation was carried out by western blotting. The santonin treated (0, 8, 16, and 32 µM) SK-BR-3 cells were harvested with centrifugation. The SK-BR-3 cells were then lysed in lysis buffer containing the protease inhibitor. Around 45 µg of proteins from each sample were subjected to separation 10% and followed by transferring it to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 hour. Afterwards, the membranes were treated with primary antibodies at 4°C.
overnight. Subsequently, the membranes were subjected to incubation with secondary antibodies. Finally, the signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalization.

**Statistical analysis**

The experiments were carried out in triplicate and data are presented as mean ± standard deviation (SD). Statistical analysis was performed using Student's t-test. Values of $P<0.05$ were taken as statistically significant difference.
Results

Santonin inhibited the proliferation of the SK-BR-3 cells

The growth inhibitory effects of santonin (Figure 1A) were investigated by CCK-8 assay against the SK-BR-3 cells. Results showed that santonin exerts antiproliferative effects on the breast cancer SK-BR-3 cells. The IC\textsubscript{50} of 16 µM was observed for santonin against the SK-BR-3 cell line as calculated by CCK-8 assay. In addition, it was revealed that the anticancer effects of santonin on the breast cancer cells were concentration dependent (Figure 1B).

Santonin induced apoptosis in breast cancer cells

To decipher if santonin exerted anti-proliferative effects on the SK-BR-3 cells are because of the induction of apoptotic cell death, DAPI, and annexin V/PI staining assays were performed. It was observed that the percentage of the DAPI positive cells increased suggestive of the apoptosis in the SK-BR-3 breast cancer cells (Figure 2). The annexin V/PI staining showed that breast cancer cell percentage increase in a concentration dependent manner. The apoptosis percentage increased to 34.32% at 32 µM concentration as compared to the 0.01% in the controls (Figure 3). For the validation of apoptosis, the expression of apoptosis associated proteins was examined, and it was found that santonin caused an upsurge of Bax and downregulation of Bcl-2 in SK-BR-3 cells (Figure 4).

Santonin caused the activation of caspase-3 and caspase-9 in SK-BR-3 cells

The effects of santonin were also investigated on the expression of caspase-3 and caspase-9 at 0, 8, 16, and 32 µM.
Figure 4. Effect of santonin on the expression of Bax and Bcl-2 as depicted by western blot analysis. The figure shows that santonin increases apoptosis percentage of SK-BR-3 cells concentration dependently. The experiments were performed in triplicate.

Figure 5. Effect of santonin on the expression of caspase-3 and caspase-9 as depicted by western blot analysis. The figure shows that santonin increases caspase-3 and caspase-9 expression in SK-BR-3 cells concentration dependently. The experiments were performed in triplicate.

Figure 6. Effect of santonin on cell cycle distribution of Sk-BR-3 cells in different phases. The experiments were performed in triplicate.
concentrations by western blotting. The results showed that santonin caused the downregulation of both caspase-3 and caspase-9 in the SK-BR-3 breast cancer cells (Figure 5). These effects of santonin on the breast cancer cells were found to be concentration dependent.

Santonin caused G2/M arrest in SK-BR-3 cells

The effects of santonin were also investigated on the cell cycle distribution of the SK-BR-3 cells by flow cytometry. It was found that santonin caused significant increase in the percentage of the G2/M phase SK-BR-3 cells. The percentage of the G2/M phase cells increased to 36% at 32 µM as compared to 15% in control (Figure 6). The induction of the G2/M phase arrest by santonin in the SK-BR-3 cells was also concomitant with concentration dependent decrease in the expression of cyclin A and B1 (Figure 7).

Santonin inhibited the Raf/MEK/ERK signaling pathway in SK-BR-3 cells

We also sought to know the effects of santonin on the Raf/EK/ERK signaling pathway by western blot analysis at 0, 8, 16, and 32 µM concentrations. The results showed that santonin caused decrease in the expression of p-c-Raf, p-MRK1/2, and p-ERK1/2 in a concentration dependent manner (Figure 8).

Discussion

Breast cancer is the prevalently detected cancer among woman and causes significant mortality throughout the world [14]. The need of the hour is to find out efficient treatment regimens for the treatment of breast cancer. Against this backdrop, there has been increasing pursuit to identify plant metabolites that can selectively eliminate the cancer cells without any harm to the normal cells of the body [15]. Thus, this study examined the anticancer effects of santonin against the SK-BR-3 breast cancer cells. It was found that santonin exerts significant anticancer effects on the SK-BR-3 ovarian cancer cells by inhibiting the proliferation of the SK-BR-3 cancer cells concentration dependently. These observations confirmed previous investigations wherein sesquiterpene lactone was shown to halt the growth of the cancer cells; for example, alantolactone which is a plant derived sesquiterpene has been reported to suppress the growth of the breast cancer cells [16]. Similarly, calein C, which is another sesquiterpene lactone has been reported to suppress the growth of cancer cells [17]. Moreover, santonin synthesized by organic chemistry efforts has also been shown to suppress the growth of the cancer cells [18,19]. The DAPI and annexin V/PI staining showed that santonin induced apoptotic cell death of SK-BR-3 breast cancer cells which was allied with increased expression level of Bax and decreased expression level of Bcl-2. Moreover, santonin also caused a significant increase in the expression of caspase-3 and caspase-9. Previously, several studies have shown that sesquiterpene lactones induced the apoptotic cell death of the SK-BR-3 cells. In a recent study, the sesquiterpene lactone arglabin was found to cause apoptosis of oral cancer cells both in vitro and in vivo [20]. In yet another study, bigelovin, which is a plant derived sesquiterpene lactone, was also reported to induce apoptosis of the cancer cells [21]. Sesquiterpenes have also been shown to cause inhibition of cancer cell progression by triggering of cell cycle arrest. For example, lactucopicrin has been shown to cause the arrest of the skin cancer cells at the G2/M check point [22]. In the present study, we also observed that santonin caused the arrest of the SK-BR-3 cancer cells at the G2/M phase of the cell cycle which was also concomitant with the suppression of cyclin A and B1 expression. The Raf/MEK/ERK pathway has been shown to be activated in many cancer types and is considered to be the therapeutic target for cancer treatment [11]. In this study, it was found that santonin could block this pathway suggesting the potent anticancer effects of santonin.
The findings of this study suggest that santonin may prove beneficial in the treatment of ovarian cancer as it induces apoptosis and cell cycle arrest in breast cancer cells by deactivation of the Raf/MEK/ERK signaling pathway. However, more research will be required for the further evaluation of santonin.

**Conclusions**

**Conflict of interest**

None.

**References:**

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