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Full Length Research Paper

Casticin induces apoptosis and mitotic arrest in pancreatic carcinoma PANC-1 cells

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Pancreatic cancer is one of the most malignant tumors that responded poorly to currently available chemotherapy. Casticin, a flavonoid compound, has been reported to induce apoptosis in various cancer cell lines. However, there is no report on the anti-pancreatic cancer potential of casticin. In the present study, we showed for the first time that casticin strongly inhibits the growth of PANC-1 pancreatic carcinoma cells. The anti-proliferative effect assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay demonstrated that casticin inhibited the growth of PANC-1 cells in a dose-dependent manner. Further analysis using flow cytometry and immunoblot showed that the growth inhibitory effect of casticin was associated with cell cycle arrest at G2/M phase and induction of apoptosis. Mechanistic studies indicated that the induction of apoptosis was associated with up-regulation of pro-apoptotic protein Bax, down-regulation of anti-apoptotice protein Bcl-2 and activation of caspase-3. These findings suggest that casticin may be a promising candidate for treating human pancreatic cancer.

Key words: Casticin, PANC-1, apoptosis, G2/M phase arrest, caspase-3.

INTRODUCTION

Pancreatic cancer is one of the most malignant tumors worldwide. It is difficult to diagnose at early stage, and cannot be removed surgically at the time of diagnosis. Due to its inaccessible location, proximity to other vital organs and inherently aggressive pattern of growth, it has extremely poor survival rate (Awasthi et al., 2011; Warshaw and Fernandez-del Castillo, 1992; Duffy et al., 2003). At present it is the fourth leading cause of cancer-related deaths in United States with nearly identical rates of incidents and mortality. In the United States, pancreatic cancer accounted for more than 28,000 deaths per year (Jemal et al., 2009).

Despite advancements in diagnosis, surgery, radiotherapy and chemotherapy, the overall survival rate remains less than 5 years, while the median survival rate is about 6 months (Yip-Schneider et al., 2005). Surgical operation remains the only choice for long term survival of patients. However, difficulty in achieving early diagnosis and aggressive nature of this type of cancer limited the surgical operation to only about 10% patients. Therefore, majority of pancreatic cancer patients are treated with chemotherapy (Wilkowski et al., 2006). Currently, gemcitabine is the best chemotherapeutic drug for the treatment of pancreatic cancer. However, the objective tumor response remains considerably low (Arlt et al., 2003; Li et al., 2004). Several other cytotoxic and chemotherapy agents, such as cisplatin, fluorouracil, docetaxel, erlotinib, oxaliplatin and irinotecan have been tested as single agent or in combination with gemcitabine for the treatment of advanced pancreatic cancer. However, most of these studies failed to ameliorate overall patient survival compared to gemcitabine (Awasthi et al., 2011). Therefore, there is an urgent need to identify new compounds that can effectively kill pancreatic cancer cells.

Flavonoids are a group of phenolic compounds which occur widely in plants and have multiple biological, pharmacological and medicinal properties, including anti-inflammatory, antiviral, antiallergic, antithrombotic and

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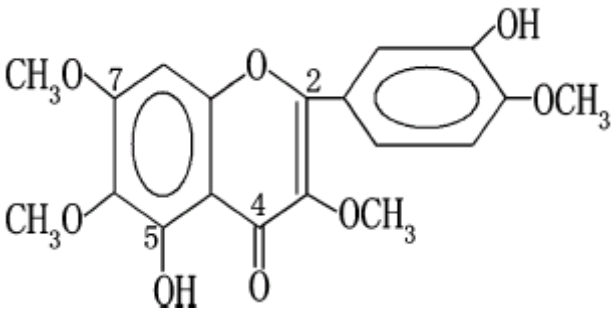

Figure 1. Structure of casticin.

anticancer effect (Kim et al., 2008; Khaki et al., 2011). Casticin is a flavonoid compound that has been shown to induce apoptosis and cell cycle arrest in breast cancer, epidermoid cancer and leukemia (Kobayakawa et al., 2004; Haidara et al., 2006; Shen et al., 2009). However, no report is at present available on anticancer activity of casticin against human pancreatic carcinoma. In the present study, we have evaluated the anticancer activity of casticin on human pancreatic carcinoma cells PANC-1 for the first time. The results support that casticin possesses anti-pancreatic cancer potential by arresting the cell cycle and induction of apoptosis through intrinsic pathway.

MATERIALS AND METHODS

Chemicals and reagents

Casticin (Figure 1) was obtained from Tauto Biotech Co Ltd. (Shanghai, China) and purity (> 98%) was determined by high performance liquid chromatography (HPLC). Dulbecco's Modified Eagle's Medium (DMEM), fatal bovine serum (FBS), propidium iodide (PI), RNase A, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT), calcein acetoxymethylester (calcein AM) and dimethyl sulfoxide (DMSO) were purchased from Sigma (Beijing, China). Annexin V-FITC apoptosis detection kit was purchased from Beyotime (Shanghai, China). Antibodies specific to Bax, Bcl-2 and caspase-3 were purchased from Beyotime. β-actin antibody and secondary antibodies were purchased from Santa Cruz (USA).

Cell culture and treatments

Pancreatic carcinoma cell line PANC-1 was obtained from Shanghai Cell Bank and maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin in 5% CO_2 at 37°C. Cells were treated with various concentrations of casticin dissolved in DMSO with a final DMSO concentration of 1% for 24 h. DMSO treated cells were used as control.

Determination of cell viability

The antiproliferative effect of casticin on PANC-1 cells was measured by MTT assay as describe previously (Al Shawi et al., 2011). Briefly, PANC-1 cells were seeded into 96-well plate and incubated at 37°C. After 24 h, cells were treated with DMSO (1 µl) or with various concentrations of casticin (5 to 100 µM) in 1 µl DMSO for 24 h. 10 µl (5 mg/ml) MTT reagent was then added to each well and the cells were further incubated for 4 h. Subsequently, 150 µl DMSO was added to dissolve farmazan crystals and absorbance was measured at 570 nm in a microplate reader. Results were expressed as the percentage of MTT reduction, assuming that the absorbance of control cells was 100%.

Morphological changes of PANC-1 cells by light microscopy

PANC-1 cells were treated with DMSO or with 20 and 40 µM casticin for 24 h. After incubation, morphological changes were observed by phase contrast microscopy (Olympus 1 × 71).

Quantification of live and dead cells

Live and dead cells were quantified using the fluorescent probes calcein AM and PI as described previously (Khan et al., 2012). Calcein AM is cell membrane permeable. In viable cells, it is converted into calcein by esterases and emits strong green fluorescence. PI, a nuclei staining dye, is cell membrane impermeable. Cells with impaired plasma membrane integrity are stained red due to entry of PI and failure to retain calcein. Since, both calcein and PI can be excited with 490 nm, simultaneous monitoring of viable and dead cells is possible with a fluorescence microscope. PANC-1 cells were incubated with DMSO or with 20 and 40 µM casticin for 24 h. After incubation, floating and adherent cells were collected, rinsed with PBS and incubated with PBS solution containing 2 µM calcein AM and 4 µM PI in the dark for 20 min at room temperature. At the end, 100 cells were counted microscopically for the percentage of live and dead cells.

Flow cytometric analysis of apoptosis

PANC-1 cells were treated with DMSO or with 20 and 40 µM casticin for 24 h. Following incubation, the cells were harvested, rinsed twice with PBS and resuspended in 200 µl of binding buffer containing 5 µl FITC-conjugated annexin V according to the manufacturer's instructions (Beyotime, Shanghai, China). After incubation in dark for 10 min and then labeled with PI, the samples were immediately analyzed on a flow cytometer (Beckman Coulter, Epics XL).

Flow cytometric analysis of cell cycle

PANC-1 cells were treated with DMSO or with 20 and 40µM casticin for 24 h. The cells were then collected, washed with PBS and fixed with 70% ice cold ethanol at 4°C for overnight. After washing twice with PBS, cells were stained with a solution containing 50 µg/ml of PI and 100 µg/ml RNase A for 30 min in the dark at room temperature. The stained cells were analyzed for DNA contents and cell cycle phase distribution by flow cytometry (Beckman Coulter, Epics XL).

Western blot

PANC-1 cells were treated with DMSO or with 20 and 40 µM casticin for 24 h, and were rinsed twice with PBS and lysed on ice with WIP cell lysis reagent (BIOSS, Beijing Biosynthesis Biotechnology Co LTD) supplemented with 1% phenylmethylsulphonyl fluoride (PMSF) for 30 min. The insoluble protein lysate was removed by centrifugation at 12000 rpm for 15

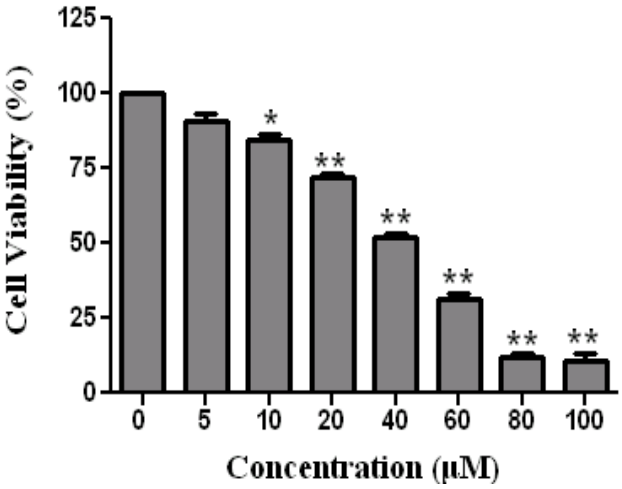

Figure 2. Determination of PANC-1 cells viability using MTT assay. PANC-1 cells were treated with various concentrations of casticin for 24 h. Data are expressed as Mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01 compared with control.

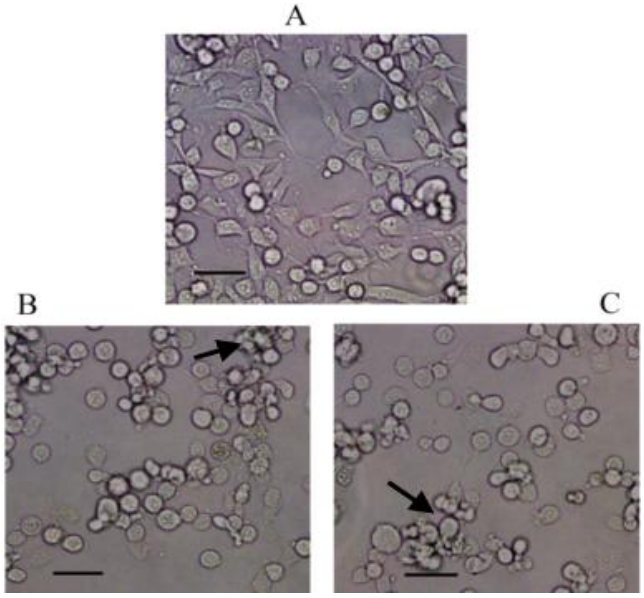

Figure 3. Morphological changes of PANC-1 cells by phase contrast microscopy. (A) Control, (B) Cells treated with 20 µM casticin for 24 h, (C) Cells treated with 40 µM for 24 h. Arrow indicates the floating cells in medium. Scale bar = 50 µm.

min at 4°C. The protein concentrations were determined using NanoDrop 1000 (Thermo Scientific) spectrophotometer. 40 µg of proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% (w/v) non-fat milk and washing with tris-buffered saline-tween solution (TBST), membranes were incubated with respective primary antibodies at 4°C overnight. After washing three times with TBST, the blots were then incubated with anti-rabbit or anti-mouse horse raddish peroxidase conjugated secondary antibodies for 1 h. After washing with TBST three times, signals were detected using ECL plus chemiluminescence kit on X-ray film (Milipore, Corporation).

Statistical analysis

The results are expressed as the mean ± SEM from three independent experiments and statistically compared with the control group using student t-test and *P-value < 0.05 was considered statistically significant.

RESULTS

Effect of casticin on PANC-1 cells proliferation

To detect the growth inhibitory effect of casticin on PANC-1 cells, we performed MTT assay. Our results showed that casticin reduced the cell viability in a dose-dependent manner (Figure 2). At low concentration, only a minor growth inhibition was observed. However, at 10 µM, a statistically significant growth inhibition was observed after 24 h as compared to the control group. The maximum growth inhibition was observed at 80 µM, which was nearly 90%.

Microscopic study of PANC-1 cells

Morphological changes were observed with phase contrast microscopy. Cells were treated with DMSO or with 20 and 40 µM casticin for 24 h. Cells treated with casticin displayed drastic morphological changes, including a reduction in total number of cells and an increase in floating cells in culture medium in a dose-dependent manner (Figure 3).

Furthermore, live and dead cells were quantified using fluorescent probes calcein AM and PI and fluorescence microscopy. Figure 4 shows the staining of cells with calcein and PI. DMSO treated PANC-1 cells (Figure 4A) took up calcein AM, de-esterified and retained the green calcein dye while cells treated with casticin (Figure 4B and C) were unable to retain the intracellular calcein or to exclude PI. Live (green) and dead cells (red) were counted microscopically. The data showed that the viability of PANC-1 cells treated with 20 and 40 µM casticin for 24 h was significantly lower (68.4 and 49.6% versus 100% in control group, P < 0.05).

Casticin induces apoptosis in PANC-1 cells

Apoptosis and cell cycle arrest are two main causes of cell growth inhibition. To further evaluate casticin induced inhibitory effect, PANC-1 cells were treated with casticin and the percentages of cells undergoing apoptosis/ necrosis were determined by flow cytometric analysis after staining with annexin V-FITC and PI. The results

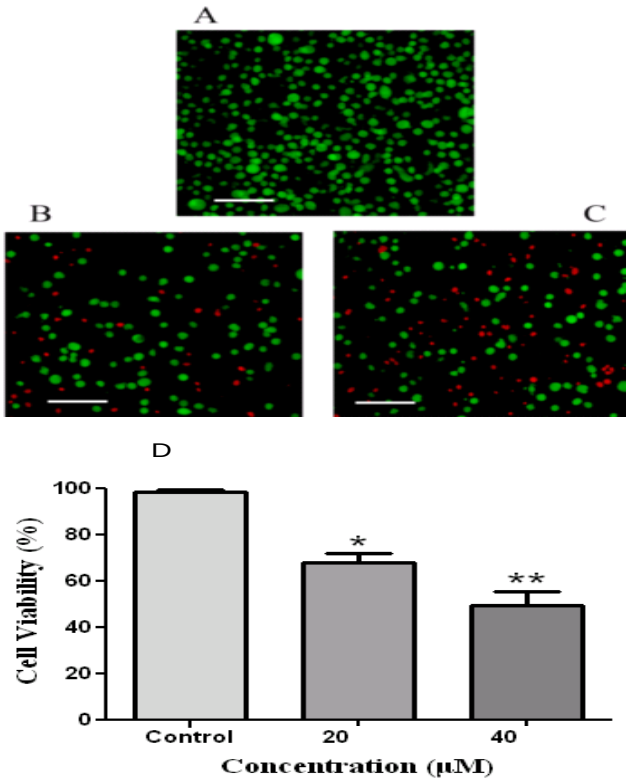

Figure 4. Quantification of live and dead cells using fluorescent probes calcein AM and PI. (A)Control cells, (B anc C) Cells were treated with 20 and40 μM casticin for 24 h, respectively. Dead cells stained red while viable cells stained green. Scale bar = 200 μm. (D) Data are expressed as Mean ±SEM of three independent experiments. *P < 0.05, **P<0.01 compared with control.

showed that casticin induced apoptosis in PANC-1 cells in a dose-dependent manner as shown in Figure 5.

Casticin induces G2/M phase arrest in PANC-1 cells

To further investigate whether cell cycle arrest is involved in PANC-1 cells' growth inhibition, we analyzed cell cycle profile using PI staining and flow cytometry. The data showed that casticin arrested the cell cycle of PANC-1 cells at G2/M phase with a corresponding decrease in S phase and G0/G1 phase in a dose-dependent manner (Figure 6).

Casticin induces apoptosis in PANC-1cells through intrinsic pathway

To further characterize in detail the mechanism of casticin-induced apoptosis in PANC-1 cells, we performed western blot analysis of some major proteins involved in cell apoptosis. The changes in protein expression in the cells treated with casticin are as shown in Figure 7. The expression of pro-apoptotic protein Bax gradually increased in a dose-dependent manner in cells treated with casticin for 24 h. Meanwhile, the expression of anti-apoptotic protein Bcl-2 markedly decreased in cells of the treatment group in a dose-dependent manner. Next, we observed the possible changes in the expression level of caspase-3. Figure 7 clearly indicates the cleavage of caspase-3 in cells of treatment group. The data suggest that casticin induces apoptosis in PANC-1 cells through intrinsic pathway.

DISCUSSION

In this study, we investigated the inhibitory effect of casticin on PANC-1 pancreatic carcinoma cells. Although, casticin has been reported to induce growth inhibition in various cell lines of breast cancer, epidermoid cancer and leukemia (Kobayakawa et al., 2004; Haidara et al., 2006; Shen et al., 2009), yet this is the first report describing the effect of casticin on growth inhibition of pancreatic cancer and its molecular mechanism. Many anticancer compounds exert their growth inhibitory effect either by arresting the cell cycle at a particular checkpoint of cell cycle or by induction of apoptosis or a combined effect of both cyclic block and apoptosis (Chan et al., 2010; Shen et al., 2009). To characterize in detail the inhibitory effect of casticin on PANC-1 cells, we performed apoptosis and cell cycle analysis, and the results showed that casticin arrested the cell cycle at G2/M phase in a dose-dependent manner. The results concur with those of Kobayakawa et al. (2004), Haidra et al. (2006), Shen et al. (2009) and Li et al. (2005) who demonstrated a similar effect of casticin on human epidermoid carcinoma KB cell line, human myeloid leukemia, K562 cell line and breast cancer MN1 and MDD2 cell lines.

Apoptosis, a form of programmed cell death, is highly organized and evolutionary conserved process characterized by membrane blebbing, DNA fragmentation, loss of plasma membrane integrity, activation of proteases and endonucleases, enzymatic cleavage of DNA into oligonucleosomal fragments and segmentation of cells into membrane-bound apoptotic bodies (Lee et al., 2000; Lin 2001; Gong et al., 2004). Apoptosis has been recognized to play a key role in maintenance of tissue homeostasis by selective elimination of damaged or unwanted cells (Lin, 2001). Many anticancer agents exert growth suppressive effect on malignant cells by inducing apoptosis (Gong et al., 2004). In the present study, apoptosis inducing effect of casticin on PANC-1 cells was indicated by morphological changes and verified by flow cytometry analysis by staining the cells with annexin V and PI. The data showed that casticin induced apoptosis in PANC-1 cells in a dose-dependent manner. Our results are in line with those reported previously (Kobayakawa et al., 2004; Haidara et al., 2006; Shen et al., 2009; Li et al.,

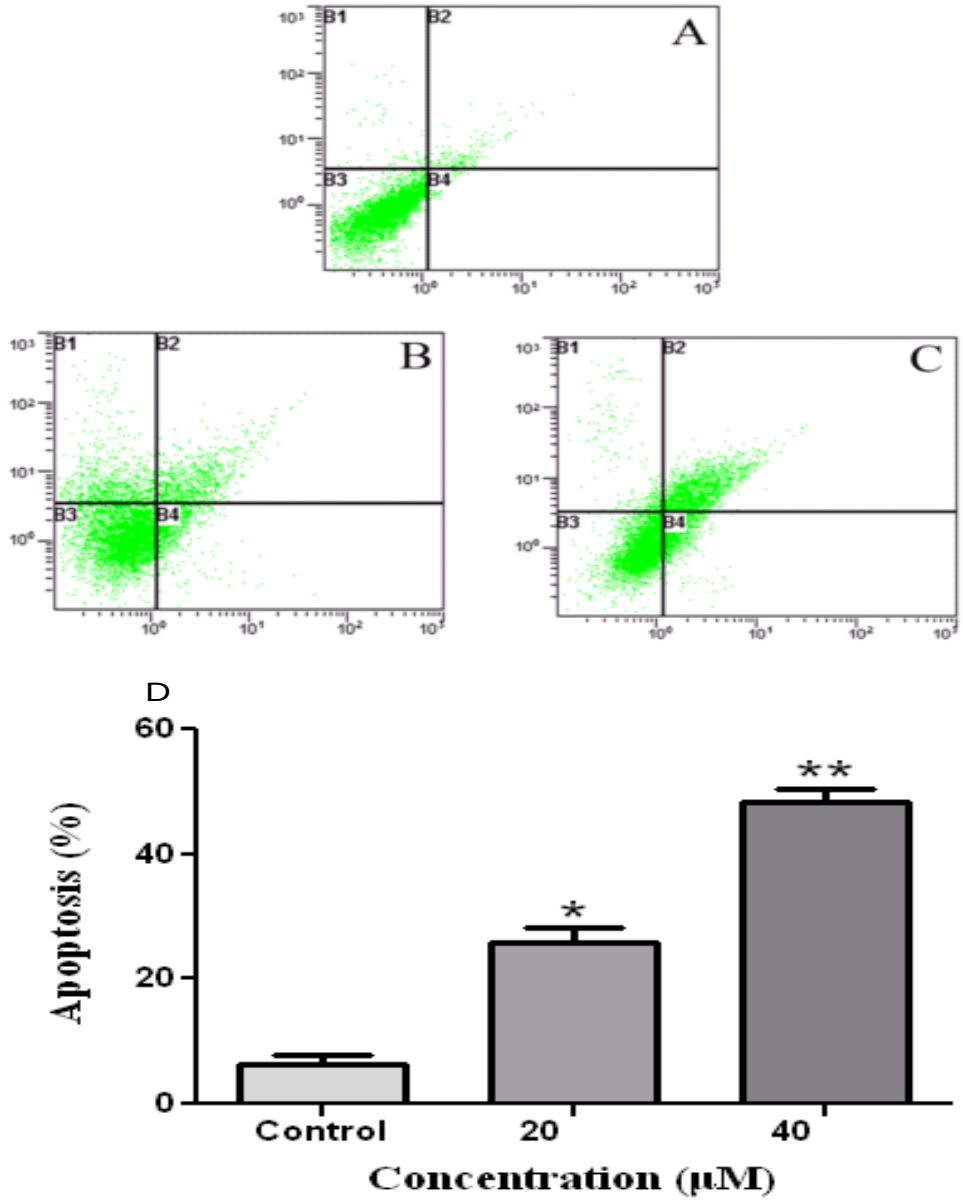

Figure 5. Flow cytometric analysis of apoptosis in PANC-1 cells. (A) Control, (B & C) PANC-1 cells treated with 20 and 40 µM casticin for 24 h, respectively. (D) Data are expressed as Mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01 compared with control.

2005). Bcl-2 family proteins play a key role in cell survival or cell death. The members of Bcl-2 family can function either as pro-apoptotic or anti-apoptotic molecules. Under normal circumstances, Bax, a pro- apoptotic protein resides in the cytosol, and is negatively regulated by anti-apoptotic protein Bcl-2. Thus, Bcl-2/Bax ratio determines the fate of cell. In the presence of apoptotic stimulus, Bcl-2/Bax ratio decreases and cell undergoes apoptosis (Ji et al., 2011; Antonsson et al., 2001; Ji et al., 2008; Wong et al., 2011). In the present study, we observed a remarkable increase in Bax expression and a dramatic decrease in Bcl-2 expression in casticin-treated PANC-1 cells. These results are consistent with previous study in which casticin induced apoptosis in K562 cell via mitochondrial-controlled apoptosis (Hai-yan et al., 2005).

Caspase-3 has been identified as the main executioner ofapoptotic response inside the cells. Cleavage of caspase-3 is a hallmark in the process of apoptosis. Activated caspase-3 induces the release of death substrate and lyses DNA in nucleus resulting in DNA fragmentation (Ji et al., 2011). Our western blot study clearly indicates the cleavage of caspase-3 in casticin-treated PANC-1 cells.

Conclusively, our data suggested that casticin inhibited

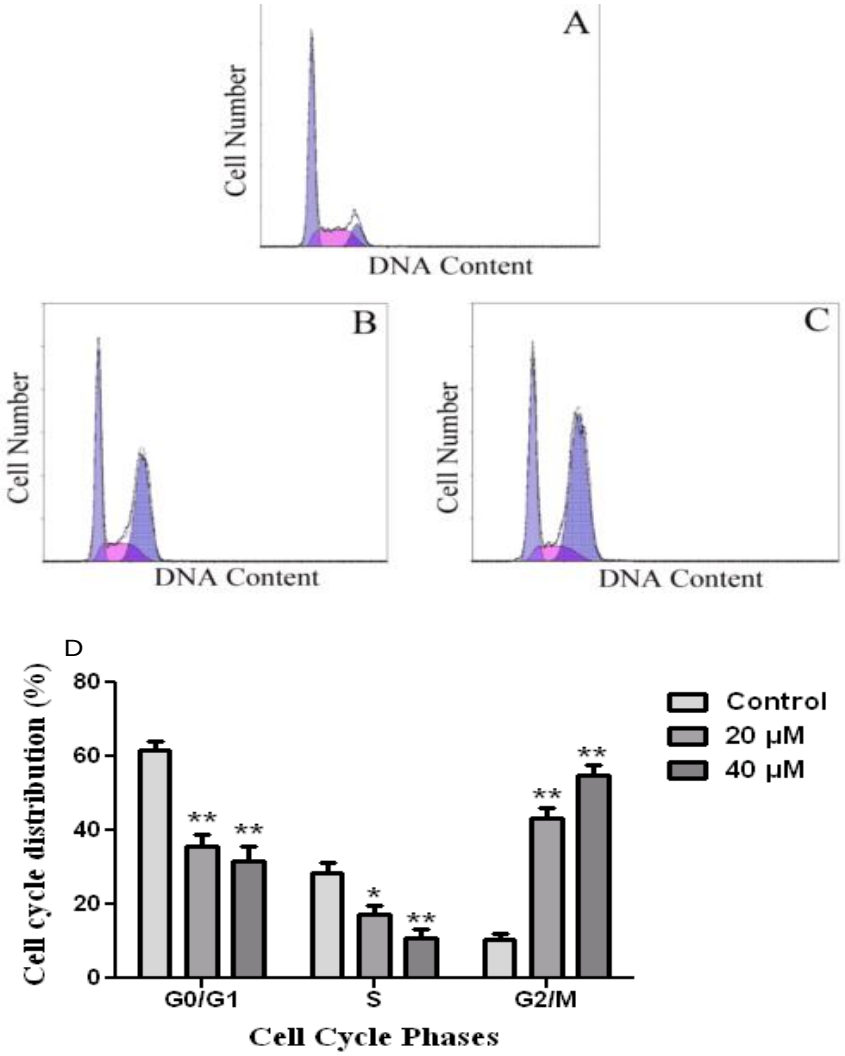

Figure 6. Flow cytometric analysis of the cell cycle phase distribution in PANC-1 cells. (A) Control cells, (B & C) PANC-1 cells were treated with 20 and 40 μM casticin for 24 h, respectively. (D) Data are expressed as Mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01 compared with control.

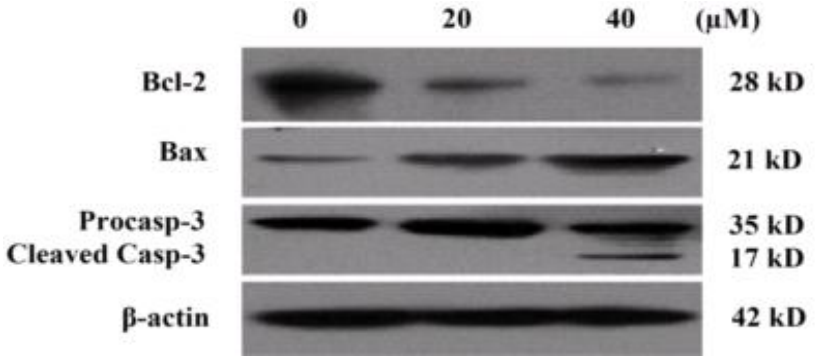

Figure 7. Effect of casticin on the expression of apoptosis regulators. PANC-1 cells were treated with DMSO or with 20 and 40 μM vitexicarpin for 24 h. The changes in expression level of Bax, Bcl-2 and Caspase-3 (procaspase-3 and cleaved caspase-3) were determined by western blot analysis.

the growth of PANC-1 cells by arresting the cell cycle at G2/M phase and inducing apoptosis through up-regulation of Bax protein expression, down-regulation of Bcl-2 protein expression and cleavage of caspase-3. Thus, casticin may be a promising candidate for treating human pancreatic cancer.

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