Research Article

Casticin treatment inhibits squamous cell carcinoma cell proliferation and arrests cell cycle in S phase

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Abstract

The present study demonstrates the effect of casticin on esophageal squamous cell carcinoma cell lines, TE-1 and TE-15. The cells were treated with various concentrations (10-50 μM) of casticin for different time periods. The results revealed that casticin treatment significantly inhibited the rate of cell proliferation in both TE-1 and TE-15 cell lines after 48 hours. Casticin treatment induced cell cycle arrest in S phase, enhanced the expression of proapoptotic gene, Bax and activation of caspase-3. Moreover, the morphological features of the cells were altered resulting in apoptosis. Casticin also inhibited the migration potential of TE-1 cells. Thus, casticin exhibits inhibitory effect on the esophageal squamous cell carcinoma cell lines by inhibiting cell proliferation, arresting cell cycle, inducing apoptosis and inhibiting migration. Therefore, casticin can be of therapeutic importance for the treatment of esophageal squamous cell carcinoma.

Introduction

Squamous cell carcinoma is one among the two sub-types of esophageal cancer and is frequently detected in Asia (Jemal et al., 2011; Cook et al., 2009). From the last three decades the prevalence of squamous cell carcinoma has rapidly increased in the developing countries (Szumiło, 2009; Rubenstein and Chen, 2014). Some of the causes include intake of excessive alcohol, consumption of hot beverages and red meat (Rubenstein and Chen, 2014). The absence of characteristic clinical symptoms in the early stage hinders the detection and treatment (Gaur et al., 2014). Therefore, in most of the cases squamous cell carcinoma is detected at metastasis stage making the treatment difficult.

The commonly used treatment strategies at present are surgical extraction, radiotherapy and chemotherapy (Smith, 1996; Tepper et al., 2008). Use of radio-therapy worsens the life quality of the patients by inducing chronic toxicities (Blazeby et al., 2000; Blazeby et al., 1995). The five-year survival rate of the squamous cell carcinoma patients is estimated to be <13% (Kim et al., 2011). Therefore, discovery and screening of the novel molecules for the treatment of squamous cell carcinoma is required.

Natural products either as such or after chemical modification have been the source of a large number of anticancer drugs. The fruit of the plant *Vitex trifolia L.*, is the source of a medicine *Fructus viticis*. The medicine has a long traditional importance of use against various types of cancers in the China (Pharmacopoeia Commission, 2010). *Fructus viticis* is also used as a potent candidate against inflammatory reactions (Pharmacopoeia Commission, 2010).

The phytochemical analysis of *Fructus viticis* led to the isolation of casticin as the major component. Casticin (3',5-dihydroxy-3,4',6,7-tetrame-thoxyflavone), exhibits inhibitory effect on the secretion of prolactin and induces apoptosis in leukemic carcinoma cells through mitotic catastrophe (Ye et al., 2010). It also interacts with phosphatidylinositol-3-kinase in synergistic manner to

inhibit cancer cell growth (Shen et al., 2009). Casticin has been reported to induce apoptosis and inhibits cell viability in glioma stem-like cells (Yang et al., 2011; Feng et al., 2012). However, the role of casticin in inhibiting cell viability and preventing cell migration in squamous cell carcinoma cell lines is not investigated. Therefore, the present study was performed to investigate the effect of casticin on TE-1 and TE-15, squamous cell carcinoma cell lines.

Materials and Methods

Reagents

Casticin was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China) and dissolved in dimethyl sulfoxide to prepare stock solution. MTT, Hoechst 33258 and DMSO were purchased from Sigma-Aldrich (USA).

Cell culture

TE-1 and TE-15, squamous cell carcinoma cell lines were obtained from American Type Culture Collection (USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing with 10% fetal bovine serum and antibiotics (Gibco Life Technologies, USA).

Cell viability assay

TE-1 and TE-15 cells were treated with different concentrations of casticin for various periods of time. CCK-8 kit (Dojindo Molecular Technologies Inc) was used for the analysis of cell viability. For this purpose, the cells were distributed at a density of 2×10^6 onto 96-well plates and incubated with various concentrations of casticin for indicated periods of time. After incubation, 10 µL of CCK-8 solution was put into each well followed by incubation for 4 hours at 37°C. Absorbance for each well was measured at 455 nm using a microplate reader (Bio-Rad, Richmond, USA). All the measurements were performed at least three times.

Wound healing assays

For the analysis of migration in squamous cell carcinoma cell lines wound healing assays were performed. TE-1 and TE-15 cells were distributed onto 6-well plates and allowed to attain 70% confluency. The plastic tips were used to make small holes in the cell monolayers. The cells were incubated in medium supplemented with 2% serum and containing casticin for 24 hours. After incubation with casticin, images were captured and the distance of migration was measured using an Olympus-CX31 microscope (Olympus Corp.).

Morphological observation

Squamous cell carcinoma cell lines were distributed at a density of 2×10^5 cells per well onto 96-well microtiter plates. The cells were allowed to grow exponentially in 5% CO_2 atmosphere at 37°C. Castacin was added to each of the well and the plates were incubated for 24 hours. Following incubation, Leica DM IRB (Leica Microsystems, Germany) inverted microscope was used to examine the alterations in morphology of the cells.

Western blotting

The cells after attaining confluence were cultured in media with or without (control) casticin. The cells were incubated for 48 hours, washed with PBS and lysed in 1X RIPA lysis buffer. Protein assay was used for determination of the quantity of proteins in cell lysates. The protein samples were resolved in NuPAGE® Novex Bis-Tris Mini Gels (Invitrogen Life Technologies) and then transferred to the polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes blocked using 5% skimmed milk was incubated with primary antibodies overnight. Primary antibodies were used against cleaved caspase-3, Bax and β-actin (Cell Signalling Technology, Inc., USA). After PBS washing the membranes were incubated with secondary antibodies for anti-mouse or anti-rabbit horse-radish peroxidase-conjugated IgG (Promega Corporation). The expressed proteins were visualized using western Lightning® Plus enhanced chemiluminescence (ECL) substrate (Perkin-Elmer) and autoradiography. Alpha Imager System (ProteinSimple, USA) using Alpha View software was used for the densitometric analysis of X-ray films.

Cell-cycle analysis by flow cytometry

TE-1 and TE-15 cells after incubation with casticin for 48 hours were harvested and fixed in ethyl alcohol overnight. The cells were then rinsed twice in PBS followed by PI staining under dark conditions for 1 hour at 37°C. Flow cytometry (Beckman Coulter Cell, USA) was used for the assessment of the stained cells and FlowJo 7.6.5 software (FlowJo, LLC., USA) for analysis of the cells.

Statistical analysis

The data presented are the mean ± standard deviation. SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses of the data. Comparisons were made using Student's t-test, and statistical differences were determined by one-way or two-way analysis of variance. $p < 0.05$ was considered to represent a statistically significant difference.

Results

Inhibition of TE-1 and TE-15 cell viability

Casticin treatment caused a significant reduction in the viability of TE-1 and TE-15 cells in dose- and time-dependent manner (Figure 1). With the increase in concentration of casticin from 10 to 50 µM, the viability of TE-1 cells decreased from 98 to 24%. In TE-15 cells,
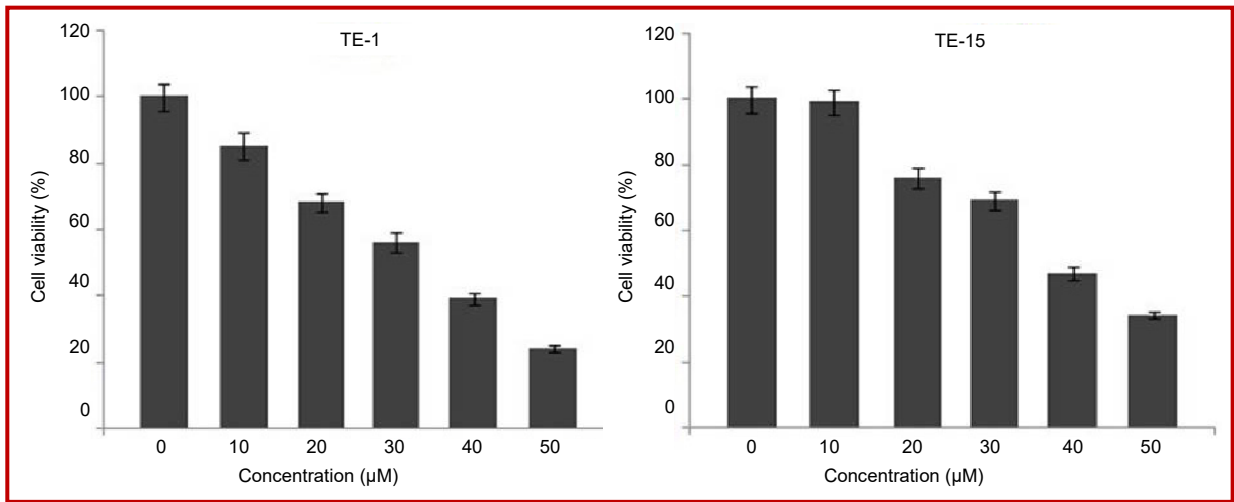
Figure 1: Casticin inhibited cell viability of TE-1 and TE-15 squamous carcinoma cell lines. The cells were treated with various concentrations of casticin or left untreated (as control) for 48 hours. Absorbance was measured at 565 nm to determine the cell viability (p<0.05)

viability decreased from 99 to 34% with increase in concentration of casticin from 10 to 50 µM. The inhibition was significant after 48 hours of casticin treatment in both the cell lines.

Morphological alterations in TE-1 cells

Treatment of TE-1 cells with 50 µM concentration of casticin for 48 hours induced various morphological changes including decrease in cell size, vacuolar cytoplasm, granular and detached cells (Figure 2). Fluorescent microscopy revealed the presence of condensed and fragmented chromatin material along with apoptotic cells in the casticin-treated cultures after 48 hours (Figure 2).

Inhibition of TE-1 cell migration

The results from wound healing assay revealed a significant decrease in the migration of TE-1 cells on treatment with casticin (50 µM) after 48 hours. Examination of the migratory capacity revealed that casticin treatment decreased the migratory rate in TE-1 cells to 8.6% compared to 64.7% in the control cells following 48 hours (Figure 3)

Effect on cell cycle distribution in TE-1 cells

Casticin treatment at 50 µM concentration induced accumulation of TE-1 cells in S phase with a subsequent reduction in G_1 phase. The population of TE-1 cells in the S phase increased to 28.4% on treatment with casticin for 48 hours compared to 11.8% in the control (Figure 4). However, the population in G_1 phase decreased from 35.6 to 17.3%. Therefore, casticin treatment for 48 hours inhibits cell viability by arresting cell cycle in the S phase.

Effect on expression of proapoptotic proteins

Western blot analysis was performed to analyze the effect of casticin on expression of proapoptotic proteins, Bax and cleaved caspase-3. The results revealed that casticin treatment in TE-1 cells significantly enhanced the expression of Bax and cleaved caspase-3 after 48 hours compared to the untreated cells (Figure 5).

Discussion

Casticin, treatment in leukemic carcinoma cells induces apoptosis through mitotic catastrophe and inhibits secretion of prolactin (Ye et al., 2010). It inhibits viability of glioma stem-like cells by inducing apoptosis (Yang et al., 2011; Feng et al., 2012). However, the effect of casticin on cell viability, migration, cell cycle arrest and induction of apoptosis in squamous cell carcinoma is not yet studied. The present study was performed to investigate the effect of casticin on the rate of cell viability, migration capacity and induction of apoptosis in TE-1, squamous cell carcinoma cells.

Since one of the most important characteristic of carcinoma cells is the capacity to proliferate at infinite rate, the effect of casticin on rate of proliferation in TE-1 and TE-15 cells was investigated. It was observed that casticin treatment inhibited the viability of both the tested carcinoma cell lines significantly. It is reported that enhanced rate of cell proliferation in carcinoma cells is associated with the cell cycle disorder (Kastan and Bartek, 2004). The mechanism of action of several antitumor drugs including, cisplatin involves arrest of cell cycle (Chu, 1994). The rate of proliferation in carcinoma cells is related with the progression of cell cycle (Nurse, 2000; Nurse et al., 1998), therefore, the effect of casticin on cell cycle progression and distribution was also analyzed. The results revealed that casticin enhanced the population of cells in S phase with subsequent reduction in G1 phase. Therefore,
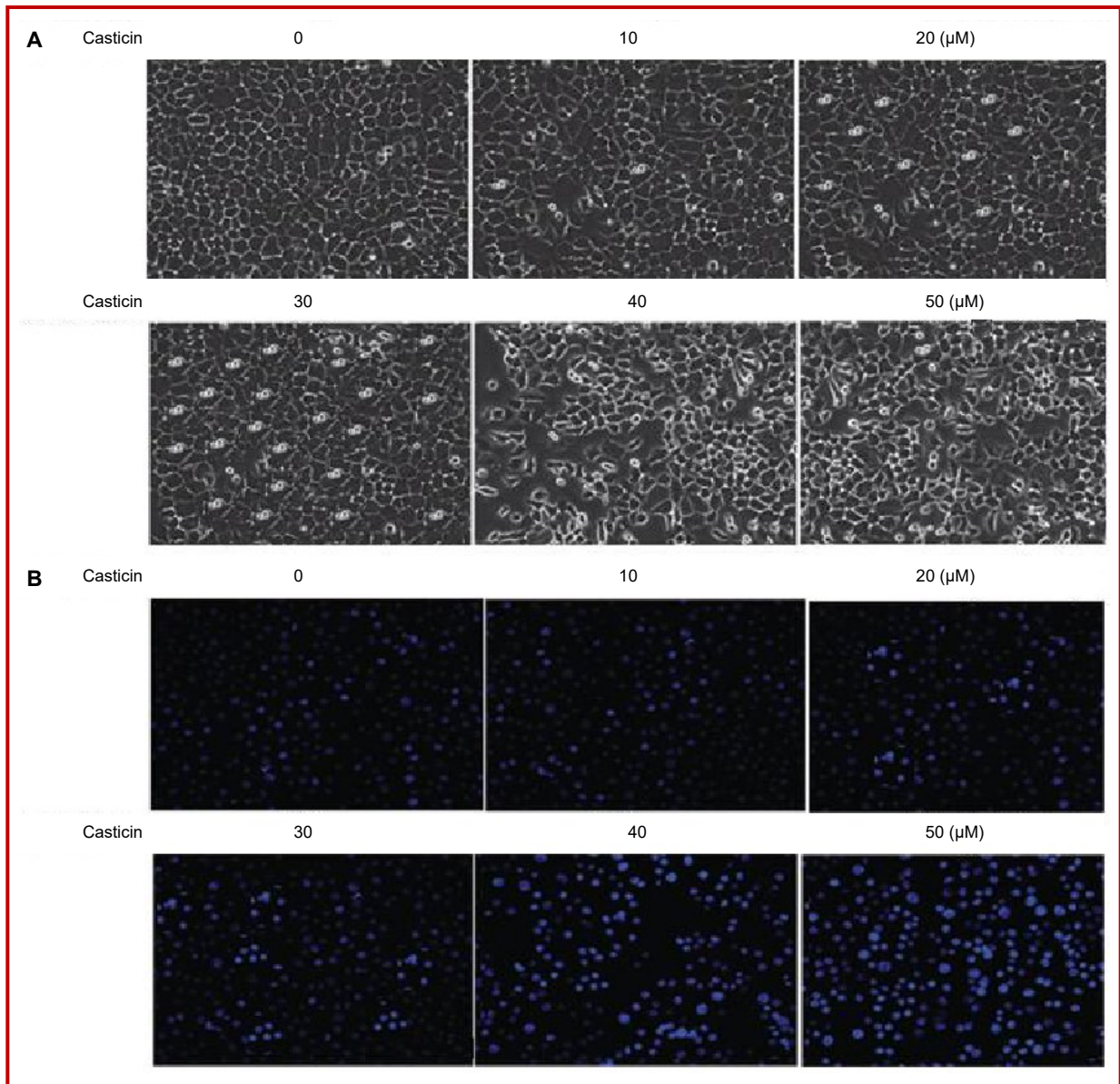
Figure 2: Casticin induced morphological alterations in TE-1 cells. The cells were treated with 10, 20, 30, 40 and 50 µM/L doses of casticin or left untreated as control for 48 hours (A) and subjected to Hoechst 33258 staining (B). The cells were then observed under light microscope to examine the apoptosis

casticin treatment in TE-1 squamous carcinoma cells caused cell cycle arrest in S phase.

Another characteristic feature of carcinoma cells is their capacity to evade the process of apoptosis (Warner, 1972). Clinicians are developing various drugs to induce apoptosis in carcinoma cells in order to inhibit growth and proliferation of cancer (Wyllie, 1985). Results from the present study revealed that casticin induced apoptosis in squamous carcinoma cells which was evident by the formation of vacuolar cytoplasm, shrinkage of cells and condensation and fragmentation of chromatin material. Nuclear translocation of Bax increases mitochondrial membrane permeability and

induces cytochrome c release which then activates caspase-3 (Li et al., 2004; Luo et al., 1998). Activation of caspase-3 has a vital role in inducing the carcinoma cell apoptosis (Porter and Jänicke, 1999). The present study demonstrated that casticin treatment enhanced the expression of proapoptotic proteins, Bax and caspase-3.

The capacity of esophageal carcinoma cells to penetrate the adjacent tissues, blood and lymph vessels is responsible for the resistance to treatment strategies (Napier et al., 2014). The present study demonstrated that casticin treatment caused a marked reduction in the migration capacity of squamous cell carcinoma cell lines.
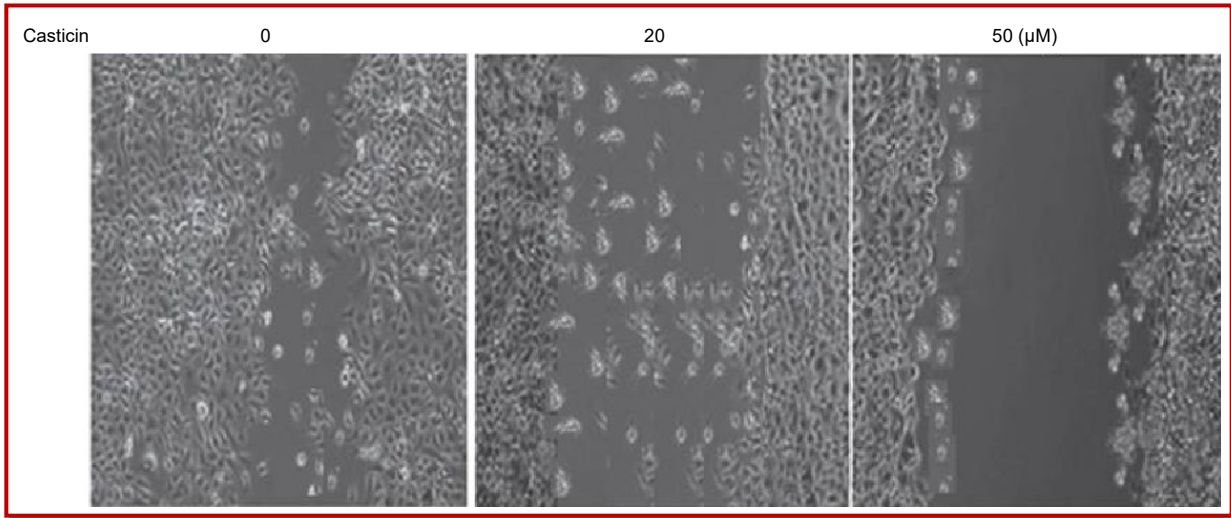
Figure 3: Casticin treatment inhibited the migration of TE-1 cells. Migration capacity of TE-1 cells was analyzed after 24 and 48 hours of casticin treatment
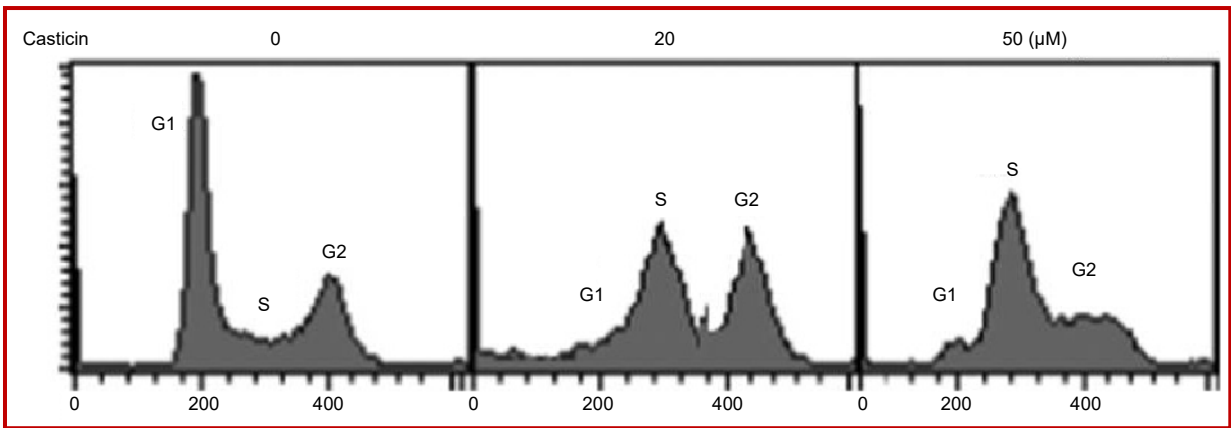

Figure 4: Casticin increased the population of cells in the S phase of cell cycle with subsequent reduction in G1 phase. The cells were analyzed by flow cytometry after treatment with casticin for 48 hours
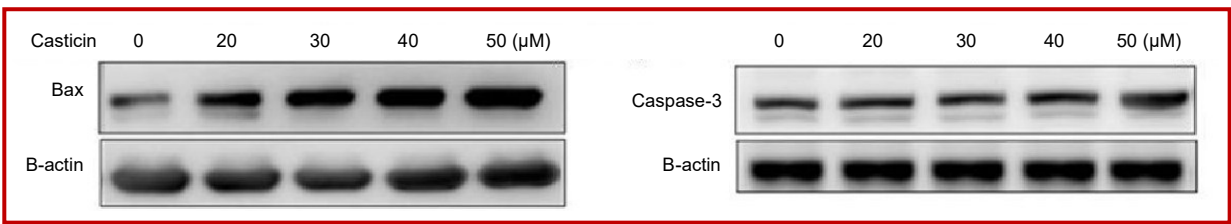

Figure 5: Casticin promoted the expression of proapoptotic protein, Bax and activation of cleaved caspase-3 in TE-1 cells

Conclusion

Thus, casticin inhibited squamous cell carcinoma through inhibition of cell viability, cell cycle arrest in S phase, enhanced expression of Bax, activation of caspase-3 and induction of apoptosis and suppression of cell migration. Therefore, casticin can be of therapeutic importance for the treatment of squamous cell carcinoma.

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Conflict of Interest

Authors declare no conflict of interest

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