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**Zerumbone inhibits prostate cancer cell viability and induces cell death by non-apoptotic pathway**

## Zerumbone inhibits prostate cancer cell viability and induces cell death by non-apoptotic pathway

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### Abstract

The aim of the present study was to investigate the role of zerumbone on the proliferation, cell cycle arrest and cell death in DU-145 prostate cancer cell lines. The MTT assay revealed that zerumbone (20  $\mu$ M) reduced proliferation of DU-145 cells to 39.0% at 48 hours. It also increased the proportion of propidium iodide stained cells to 53.4% compared 1.0% in control. However, the population of annexin V-stained cells remained unaffected indicating induction of non-apoptotic cell death by zerumbone. Treatment of DU-145 cells with zerumbone (20  $\mu$ M) caused 8-fold enhancement in the level of reactive oxygen species (ROS). On the other hand, exposure of the zerumbone treated DU-145 cells to glutathione inhibited the generation of ROS. Flow cytometry using propidium iodide staining revealed that zerumbone treatment increased proportion of cells in G1 phase to 71.3% on compared to 34.7% in the control. The results from Western blot analysis revealed a significant increase in the expression of cyclin D1 protein in DU-145 cells on treatment with 20  $\mu$ M concentration of zerumbone. Thus, zerumbone treatment inhibits prostate cancer cell viability and can be used for its treatment.

### Introduction

Prostate carcinoma at present is one among the common causes of cancer related deaths in males throughout the world. Among newly diagnosed cancer cases, around 30% include prostate cancer patients.

The cancer can be inhibited at the initial stage by use of hormonal therapy, radiation therapy or combination of two (Jemal et al., 2010). However, it has been reported that use of hormonal therapy leads to the development of a disorder in which hormones become non-responsive.

Natural products have undergone millions of years of modifications in the living organisms which led to loss of any harmful effect and are used for the treatment of various diseases (Gupta et al., 2010). Taking into account very less or no toxic effects of natural products,

they are preferred for the treatment of various types of diseases.

Zerumbone a natural isolate from the *Zingiber zerumbet* Smith has traditional Chinese medicinal importance with no side effects in the living organisms (Yob et al., 2011). Zerumbone inhibits inflammation and cancer growth in various types of carcinoma cells (Murakami et al., 2002; Sung et al., 2008; Yodkeeree et al., 2009; Kirana et al., 2003; Kim et al., 2009; Xian et al., 2007; Sung et al., 2009). Furthermore, zerumbone treatment has been shown to induce expression of tumor necrosis factor- $\alpha$ , enhance the secretion of oxygen free radicals in cancer cells (Murakami et al., 2002; Sung et al., 2008; Takada et al., 2005).

In the present study effect of zerumbone on cell viability, cell death and arrest of cell cycle in prostate cancer cells was investigated. It was observed that

zerumbone treatment inhibited cell viability, caused arrest of cell cycle and led to death of cells by non-apoptotic mechanism.

## Materials and Methods

### Cell lines and reagents

Prostate DU-145 carcinoma cell line was purchased from the American Type Culture Collection (USA). The cell culture was performed in RPMI-1640 medium containing 10% fetal bovine serum (FBS). In addition, the medium contained antibiotics. Cell culture was performed in incubator at 37°C with 5% CO<sub>2</sub> and 95% air. Zerumbone and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (USA).

### MTT assay

Effect of zerumbone on the proliferation of DU-145 prostate carcinoma cells was analyzed using MTT assay. The cells were seeded at  $3 \times 10^6$  concentration into the 96-well plates per well. Culture of cells was carried out for 24 hours duration in humid atmosphere at 37°C containing 5% CO<sub>2</sub> in incubator. Following 24 hours, the cells were treated with various concentrations of zerumbone for various time periods. Following incubation, a solution of 20 µL of MTT was added to the well of the plates and incubation was continued for 4 hours more. The supernatant was then decanted and to each of the well 150 µL DMSO was added. For each of the well absorbance was recorded at 490 nm in triplicates using an ELISA reader.

### Cell cycle analysis

For the purpose of cell cycle analysis, the cells after treatment with zerumbone for 48 hours were trypsinized and fixed in 70% ethyl alcohol after 24 hours. Distribution of cells in various phases of the cell cycle was examined by FACScan flow cytometer using stain propidium iodide.

### Cell death analysis

Following treatment with zerumbone for 48 hours cells were trypsinized and then subjected to washing with phosphate buffered saline. The cells at a density of  $2 \times 10^6$  cells in each milliliter were resuspended in binding buffer. Into the suspension of cells, a solution of annexin-V-fluorescein isothiocyanate and propidium iodide each 5 µL were added followed by 10 min incubation under dark conditions. Death of cells on treatment with zerumbone was analyzed by flow cytometry using annexin V apoptosis detection kit (Beckman Coulter Inc.). Cytomics FC500 Flow Cytometer CXP and CXP analysis software (Beckman Coulter Inc.) were used for the analysis of the cell staining and determination of the proportion of non-viable cells.

### Analysis of production of reactive oxygen species (ROS)

The cells after treatment with zerumbone were analyzed for ROS generation using non-fluorescent probe. The cells were treated with a 5 µM solution of 2',7'-dichlorofluorescein diacetate (DCFH-DA). The mean fluorescence intensity for DCFH formed by the oxidation of DCFH by ROS was measured by flow cytometry (FC500).

### Western blot assay (Video Clip)

The cells after washing thrice with PBS were treated with lysis and subsequently lysates were centrifuged to remove the debris. The expression level of proteins was determined using BCA method and the proteins were isolated by electrophoresis on 10% SDS-PAGE. Equal protein samples were loaded on to the nitrocellulose membranes which were prior treated with 5% non-fat-milk solution. The membranes were incubated overnight with primary antibodies against cyclin D1 protein followed by washing again with PBS. Incubation of the membranes for a period of 1 hour was performed with mouse peroxidase-labeled secondary antibody. The band visualization was carried out by enhanced chemi-luminescence (ECL) detection technique (Amersham Corporation).

### Statistical analysis

Statistical Package for Social Sciences (SPSS for Windows, version 17.0; SPSS, Inc., Chicago, IL, USA) was used for the data processing. Data analysis was performed by using the monofactorial analysis of variance. All the data presented are the mean of three experiments performed independently. P values were considered significant statistically at  $p < 0.05$ .

## Results

### Rate of proliferation of DU-145 cells

Zerumbone treatment led to reduction in proliferation of prostate carcinoma cells in concentration- and time-based manner (Figure 1). The reduction in proliferation rate was maximum at 20 µM concentration of zerumbone after 48 hours treatment. Treatment with 5, 10, 15 and 20 µM zerumbone for 48 hours reduced proliferation rate of DU-145 cells to 92, 76, 51 and 39%, respectively compared to 100% in the control cells.

### Cell death in non-apoptotic manner

Treatment of DU-145 carcinoma cells with different doses of zerumbone for 48 hours revealed no significant increase in the proportion of annexin V-stained cells (Figure 2). On the other hand, proportion of propidium iodide stained cells increased significantly at 20 µM

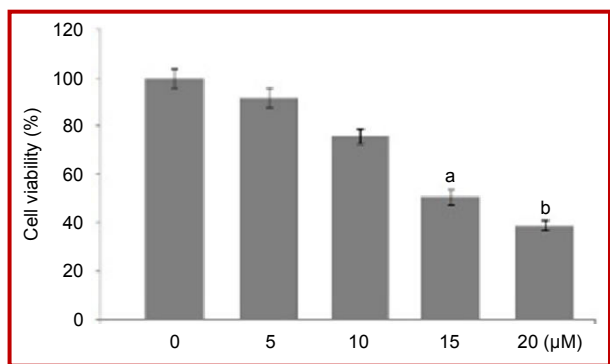


Figure 1: Zerumbone treatment caused proliferation inhibition in DU-145 carcinoma cells. The cells were incubated in presence of 5, 10, 15 and 20  $\mu\text{M}$  zerumbone for 48 hours or left untreated as control and then analyzed by MTT assay. The values expressed are the mean  $\pm$  SEM in relation to DMSO treated cells.  $a_p < 0.05$  compared to control

concentration of zerumbone. The population of propidium iodide stained cells in cultures treated with 20  $\mu\text{M}$  concentration of zerumbone increased to 53.4% compared 1.0% in control cultures.

#### Generation of ROS in prostate carcinoma cells

Incubation of DU-145 cells with zerumbone for 48 hours led to increased production ROS (Figure 3). In DU-145 cells incubation with 20  $\mu\text{M}$  concentration of zerumbone the level of ROS was 8-fold higher than those of DMSO treated cells. For the confirmation of the effect of

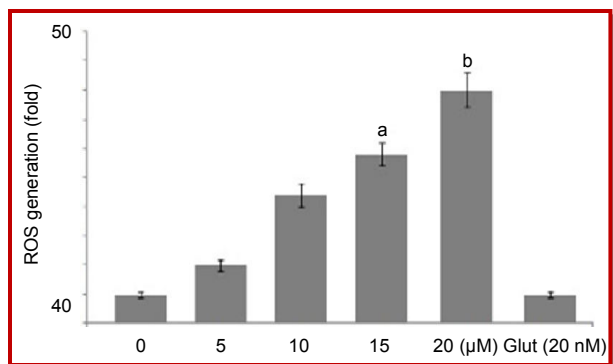


Figure 3: Zerumbone treatment induced production of ROS in prostate carcinoma cells. The cells were treated with different doses of zerumbone for 48 hours and then then analyzed for ROS generation using DCFH-DA dye

zerumbone on production of ROS, the cells were treated with glutathione. Exposure of the zerumbone treated cells to glutathione inhibited the zerumbone induced generation of ROS in DU-145 cells.

#### Arrest of cell cycle in DU-145 cells

The results from flow cytometry using propidium iodide staining revealed that zerumbone treatment promoted the population of cells in the G1 phase (Figure 4). Zerumbone treatment at 20  $\mu\text{M}$  concen-

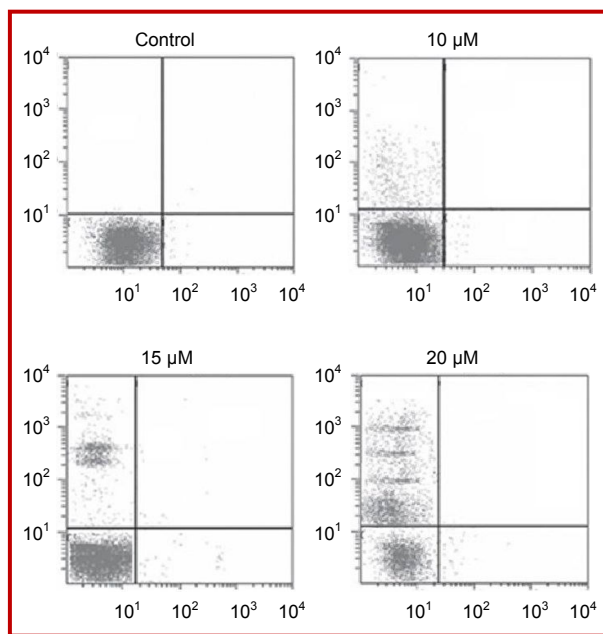


Figure 2: Zerumbone induced cell death in DU-145 prostate carcinoma cells. The cells were incubated with various doses of zerumbone for 48 hours and then subjected to annexin V or PI binding staining followed by flow cytometry

tration promoted the percentage of cells significantly in G1 phase with subsequent reduction in the S and G2/M phases. The proportion of cells in G1 phase was enhanced to 71.3% on treatment with zerumbone (20  $\mu\text{M}$ ) compared to 34.7% in the control.

#### Expression of G1 phase marker

Analysis of Western blots showed a significant enhancement in the level of cyclin D1 protein in DU-145 cells on treatment with 20  $\mu\text{M}$  concentration of zerumbone (Figure 5).

## Discussion

The current study demonstrates the effect of zerumbone on proliferation, apoptosis induction and cell cycle arrest in prostate carcinoma cells. Zerumbone treatment is reported to induce the production of nitric oxide synthase, cyclooxygenase-2 and promote the expression of tumor necrosis factor- $\alpha$  (Murakami et al., 2002; Sung et al., 2008; Takada et al., 2005). In the present study treatment of DU-145 prostate carcinoma cells with zerumbone led to reduction of cell viability in dose- and time-dependent manner. Analysis of mechanism of zerumbone induced cell proliferation inhibition revealed cell death in a non-apoptotic way. This was evident from the finding that zerumbone treatment resulted no alteration in proportion of annexin V-stained DU-145 prostate carcinoma cells compared to control cells.

The results from the flow cytometry showed a significant increase in the population of cells in G1

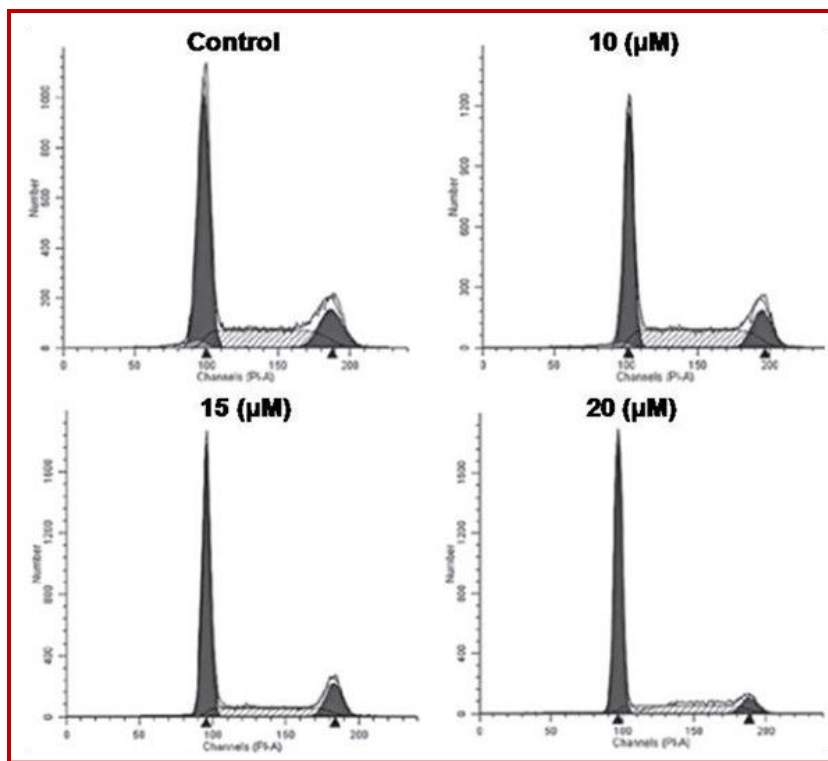


Figure 4: Zerumbone treatment caused arrest of cell cycle in DU-145 carcinoma cells. The cells were incubated with zerumbone, stained with PI and then examined by flow cytometry

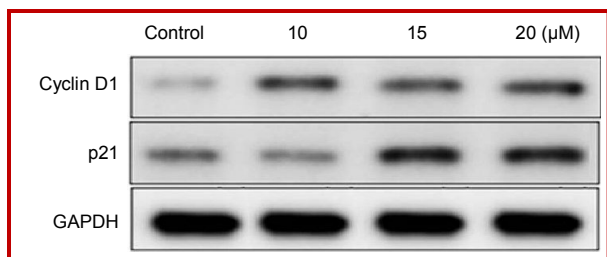


Figure 5: Zerumbone treatment induced expression of cyclin D1 protein in DU-145 cells. The cells were treated with zerumbone for 48 hours and then subjected to Western blot analysis

phase of cell cycle with subsequent decrease in S and G2/M on treatment with zerumbone. Thus, zerumbone treatment caused arrest of cell cycle in the G1 phase in DU-145 cells. Analysis of the expression of marker of G1 phase, cyclin D1 showed higher expression in zerumbone treated cells compared to the control cells. Because of the role of cyclin D1 in inducing p21 (Abbas and Dutta, 2009), it appears that zerumbone treatment exhibits its anti-proliferative effect through p21 dependent pathway.

Because of the role of zerumbone in inducing nitric oxide synthase production the reduction in prostate cell viability may be due to generation of ROS (Murakami et al., 2002; Sung et al., 2008). Production of ROS plays an important role in the induction of carcinoma cell

death (Di Stefano et al., 2006). Further it has been reported that production of ROS causes oxidative stress which then results cell necrosis (Choi et al., 2009). Results from the present study showed that zerumbone treatment for 48 hours caused a marked increase in the production of ROS in DU-145 cells. GSH is known to quench the produced ROS in the cells and therefore prevent the harmful effects of ROS (Hood et al., 2010). Results from the present study showed that treatment of DU-145 cells with GSH prevented zerumbone induced generation of ROS.

The proliferation of prostate cancer cell can be inhibited by tangeretin which might be due to induction of apoptosis via inhibiting critical pathways in cancer development- AR signalling and PI3/Akt/mTOR - Notch signalling pathways (Guo et al., 2015). Naringenin modulates the metastasis of human prostate cancer cells by down-regulating the matrix metalloproteinases -2/-9 via ROS/ERK1/2 pathways (Lin et al., 2014).

## Conclusion

The present study demonstrates that zerumbone treatment induces cell death in prostate cells through non-apoptotic pathway. Thus zerumbone can be used for the treatment of prostate cancer.



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