IMPACT: International Journal of Research in Applied, Natural and Social Sciences (IMPACT: IJRANSS)
ISSN(P): 2347-4580; ISSN(E): 2321-8851
Vol. 5, Issue 4, Apr 2017, 49-54

ZERUMBONE INHIBITS PROLIFERATION AND INDUCES APOPTOSIS, CELL CYCLE ARRESTIN HUMAN COLON CANCER CELLLINE, HCT15

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ABSTRACT

Zerumbone is a natural cyclic sesquiterpene derived from the rhizome of *Zingiber zerumbet* Smith. Zerumbone has shown topossess anticancer properties in various types of carcinoma cells. The present study investigates the *in vitro* effect of zerumbone on colon cancer cell line HCT15. Cisplatin, a conventional chemotherapeutic drug was taken as a positive control. The cytotoxicity of zerumbone and cisplatin were determined using MTT tetrazolium salt assay. Zerumbone and cisplatin exhibited growth inhibition of HCT15 cells in a dose (0-100µg/ml) and time (24h, 48h, 72h) dependentmanner. Morphological analysis showed changes in the treated HCT15 cells. Fluorescent microscopic studies showed typical apoptotic features in treated cells. In addition, flow cytometry studies showed cancer cells arrest at G_2/M phase by zerumbone. Overall, these results suggest that zerumbone can be a potent chemotherapeutic compound for the treatment of colon cancer.

KEYWORDS: Proliferation, Apoptosis, Cell Cycle, Zerumbone, HCT15, MTT

INTRODUCTION

Zerumbone, a sesquiterpene is a major constituent of Zingiber zerumbet Smith. The volatile oil of the plant contains large amount of zerumbone [1]. Previous studies have showed that Zerumbone inhibits cell proliferation in different cell lines such as HT-29, CaCo2, MCF-2, GBM 8401, HepG2 and HCT116 with extra investigations on apoptotic pathway [2-5]. Treatment with zerumbone induces cell cycle arrest in leukemia cells, laryngeal carcinoma cells, ovarian and cervical cancer cells [6-8]. Studies haveexplored the role of zerumbone in controlling tumor angiogenesis in pancreatic and gastric cancer [9, 10].

Colorectal cancer is an epithelial malignant tumor which is a major public health concern in the developed countries. It is the third most commonly diagnosed cancer in males and the second in females, with an estimated 1.4 million cases and 693,900 deaths occurring in 2012. Rates are higher in men than in women in most parts of the world [11]. Despite the advances in the treatment and prevention of colorectal cancer; it remains a major cause of death with poor five-year survival rates. The survival rate of colorectal cancer is higher when it is detected at an early pathological stage. Many advanced screening techniques such as fecal occult blood test [FOBT], sigmoidoscopy, stool DNA test, colonoscopy and Computed tomographic colonography(CTC) are used for detection of colorectal cancer[12]. Researchers have targeted Chemoprevention and screening programs for the prevention of colorectal cancer. Chemoprevention is defined as the use of specific pharmacologic or nutrient agents to prevent, reverse, or inhibit the process of carcinogenesis [13, 14]. In the present study, chemopreventive activity of zerumbone on colorectal cancer cell line HCT15 was studied.

The phytochemical has shown to inhibit cell proliferation, induce apoptosis and arrest cell cycle progression. Cisplatin, a conventional cancer drug is used as a positive control.

MATERIALS AND METHODS

Chemicals

Zerumbone, cisplatin, MTT, propidium iodide were purchased from Sigma Aldrich Ltd, USA. Fetal bovine serum, RPMI 1640 and trypsin were purchased from Himedia. All other chemicals were of analytical grade and were obtained from Sigma Aldrich Ltd, USA or Himedia, India.

Cell Culture and Maintenance

Human colon cancer cell line, HCT15 was obtained from National Centre for Cell Science, Pune (India). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100µg/ml) at 37ºC in a humidified incubator with 5% CO_2 atmosphere and passaged twice weekly to maintain a sub-confluent state.

MTT Assay

The antiproliferative effect of zerumbone was assessed by MTT (3, 4, 5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide) assay [15], which is based on the reduction of MTT by mitochondrial dehydrogenase of intact cells to a purple formazan crystal. Cells were seeded at a density of 2 x 10^4cells/well in 12 well plates. Cells were treated against zerumbone and cisplatin at 0-100µg/ml for 24h, at 0-50µg/ml for 48h and at 0-25µg/ml for 72h. The untreated cells served as control. After incubation, cells were washed with phosphate buffer saline (PBS) and incubated with MTT (100µg/ml) at 37ºC in dark for 5h. Dimethyl sulfoxide was used to dissolve the formazan crystals and O.D. was taken at 540nm. The absorbance from untreated cells was considered 100% viable cells. The percent viable cells were plotted on Y-axis against concentration of zerumbone and cisplatin on X-axis. IC_{50} values of zerumbone and cisplatin on HCT15 were interpolated from the graph.

Morphological Study

Phase contrast microscopy was used to observe morphological changes in treated and untreated cells

Propidium Iodide Staining

Propidium iodide fluorescence staining method was used to observe the apoptotic morphological changes in the treated cells. Cells were seeded in 6 well plates at a density of 4 x 10^5 cells/well. Cells were treated with IC_{50} values of zerumbone for 24h, 48h and 72h. The untreated cells served as control. After incubation, cells were washed with PBS, fixed in absolute alcohol for 30min at 4ºC, rehydrate with PBS and incubated with 100µl propidium iodide(25µM) at 37ºC for 5min. Photomicrographs were taken under a fluorescent microscope.

Determination of Cell Cycle Arrest by Flow Cytometry

The cells were seeded at the density of 4 x 10^5 cells/well in 6 well plates. Cells were exposed to IC_{50} concentration of zerumbone for 24h. The untreated cells served as control. Both treated and untreated cells were harvested and washed with PBS. Cells were then fixed with cold 70% ethanol. After fixation cells were washed with PBS and incubated with

100µg/ml RNase and then stained with propidium iodide (50µg/ml)[16]. Those cells that were less intensely stained than G_0/G_1 cells (sub-G_1) in flow cytometric histograms were considered apoptotic. The red fluorescence of individual cells was measured on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Ten thousand events were analyzed per sample. The percentage of DNA content in each cell cycle phase was analyzed using Cell Quest Pro software Version 5.2.1 (Becton Dickinson).

RESULTS AND DISCUSSIONS

Chemotherapy and chemoprevention by using natural product is gaining importance due to the toxic side effects of most of the conventional therapeutic cancer drugs. Zerumbone is one such product which is used for different human ailments since time immemorial [17]. zerumbone has shown to exert anticancer activity on various carcinomas in vivo and in vitro[18]. The current study demonstrates the effect of zerumbone on proliferation, apoptosis and cell cycle progression in colorectal adenocarcinoma cells, HCT15. Zerumbone and cisplatin treatment on HCT15 cells led to the reduction of cell viability in dose dependent (0-100µg/ml) and time dependent (24h, 48h 72h) manner. Treated cells have undergone morphological changes associated with apoptosis such as loss of adherence, nuclear condensation, membrane blebbing, cell shrinkage and nuclear fragmentation. (Figure 1&3). The percentage of cell viability was calculated by ratio of O.D. of treated cells and O.D. of untreated cells multiplied by 100. The IC_{50} values of zerumbone and cisplatin on HCT15 were calculated (Table1). The IC_{50} values indicate that zerumbone is more cytotoxic than cisplatin on HCT15 (Figure 2). The cytotoxic effect of zerumbone appears to be attributed to the α,β-unsaturated carbonyl group in its structure, which plays an important role in the interaction of the zerumbone with the most biologically active molecules[19]. Zerumbone has shown to arrest HCT15 cell cycle in G_2/M phase (Figure 4) with evidenced by increase in DNA content. This is in concurrence with induction of G_2/M cell cycle arrest and apoptosis in human leukemia cells and human laryngeal carcinoma cells by zerumbone [6, 7].

CONCLUSIONS

The present findings exhibited the cytotoxic effect of zerumbone on colorectal cancer cell line HCT15 in a dose and time dependent manner. Although zerumbone has shown to be a promising molecule in cancer chemoprevention and chemotherapy, further efforts to explore its therapeutic strategy is needed. Therefore, there is a necessity to conduct animal model studies and clinical trials to ascertain its efficacy and safety as an intended pharmaceutical drug.

ACKNOWLEDGEMENTS

Rima Thiyam acknowledges the financial support from DST INSPIRE Division, DST in the form of DST INSPIRE Fellowship.

CONFLICT OF INTERESTS

The authors have no conflicts of interest in this work.

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APPENDICES

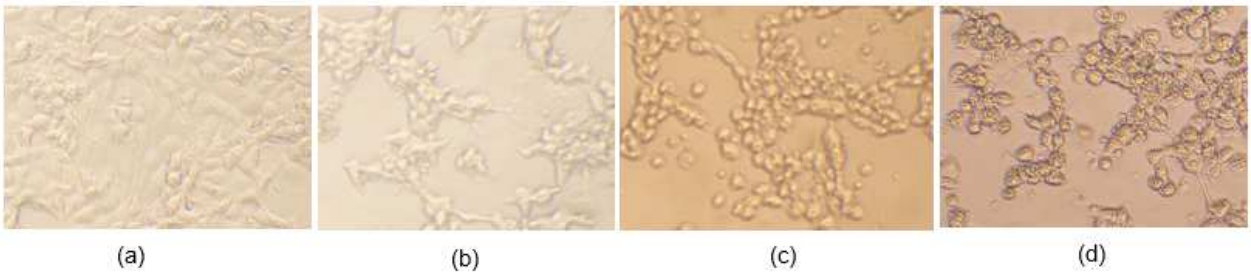

Figure 1: Photomicrographs of HCT15 Cells Showing Morphological Changes

(a) Untreated Control Cells after 24h, (b) Cells Treated with 18µg/ml of Zerumbone for 24h, (c) Cells Treated with 9µg/ml of Zerumbone for 48h and (d) Cells Treated with 4µg/ml of Zerumbone for 72h. Cells were visualized Under a Phase Contrast Microscope (Magnification 100 xs)

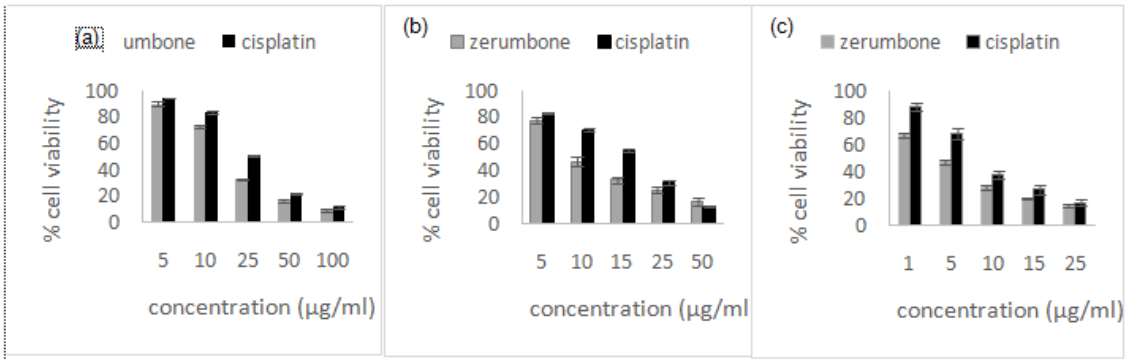

Figure 2: Cytotoxic Activity of Zerumbone and Cisplatin on HCT15 Cells as Determined by MTT assay. HCT15 Cells were Treated with Zerumbone and Cisplatin for (a) 24h, (b) 48h and (c) 72h. Values are Expressed as Mean ± SD (N=5) Percentage Cell Viability

Table 1: IC_{50} Values of Zerumbone and Cisplatin on HCT15 for 24h, 48h and 72h

Compound	IC_{50} (µg/ml)		
	24h	48h	72h
Zerumbone	18	9	4
Cisplatin	25	16	8

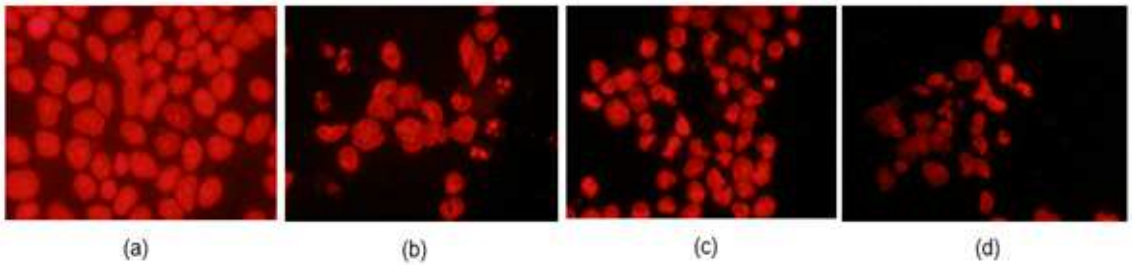

Figure 3: Photomicrographs of HCT15 cells Showing Apoptotic Morphological Changes(a) Untreated Control Cells after 24h, (b) Cells Treated with 18µg/ml of Zerumbone for 24h, (c) cells Treated with 9µg/ml of Zerumbone for 48h and (d) Cells Treated with 4µg/ml of Zerumbone for 72h. Cells were visualized Under a Fluorescent Microscope (Magnification 200x)

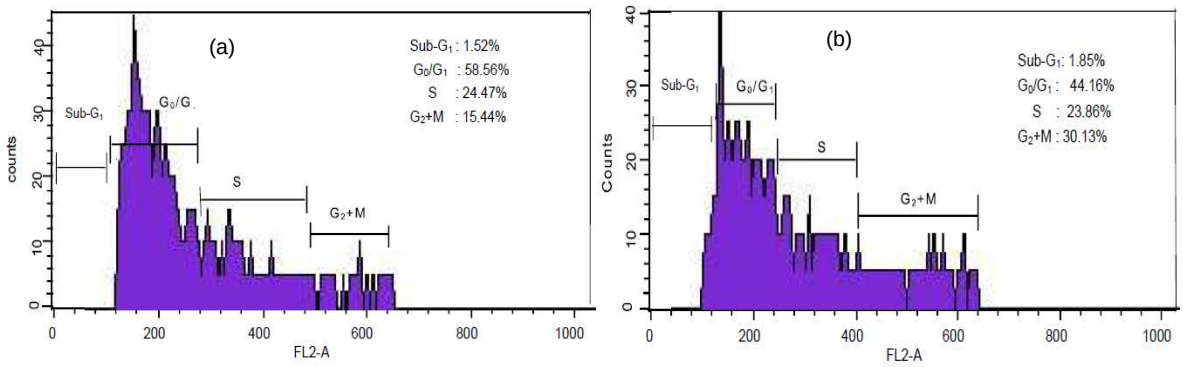

Figure 4: Cell cycle Analysis of HCT15 cells. The DNA Content was analyzed by Means of Flow Cytometry. (a) Untreated Control Cells after 24h and (b) Cells Treated with 18µg/ml Zerumbone for 24h. G_0/G_1, S and G_2+M Indicate the Cell Phases, and Sub-G_1 DNA Content refer to the Portion of Apoptotic Cells