

EVALUATION OF CYTOTOXIC AND GENOTOXIC EFFECTS OF ZERUMBONE ON COLON ADENOCARCINOMA COLO205 CELLS AND HUMAN LYMPHOCYTES

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ABSTRACT

Objective: The objective of the present study was to investigate the growth inhibitory effect, apoptosis initiation and genotoxicity of zerumbone, a phytochemical and cisplatin, a chemotherapeutic drug on human colorectal cancer cell line COLO205 and normal human lymphocytes.

Methods: The antiproliferative activity of zerumbone and cisplatin (positive control) on COLO205 cells and lymphocytes was analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Morphological analysis of the cells was studied by using an inverted phase contrast microscope. Propidium iodide staining method was used to observe the apoptotic morphological changes in the treated cells. Finally, comet assay was conducted to observe the extent of DNA damage induced by zerumbone and cisplatin on COLO205 and lymphocytes.

Results: Zerumbone and cisplatin exhibited growth inhibition in a dose and time-dependent manner against COLO205 with not much considerable effect on lymphocytes. The IC₅₀ values of zerumbone and cisplatin on COLO205 for 24h, 48h and 72h were 19 µg/ml, 10 µg/ml, 5 µg/ml and 38 µg/ml, 24 µg/ml, 15 µg/ml. Morphological changes such as cell shrinkage, membrane blebbing and nuclear condensation were observed in COLO205 while a more prominent change was not observed in lymphocytes. Fluorescence imaging studies confirmed apoptotic cell death in treated COLO205 cells while cell death was not clearly observed in treated lymphocytes. Comet assay revealed a significant DNA damage in treated COLO205 cells.

Conclusion: The present study demonstrated the cytotoxic and genotoxic effects of zerumbone on colon cancer cells with minimal effect on normal lymphocytes. The results of the study have revealed that zerumbone can be used as an effective therapeutic drug in treating colon cancer.

Keywords: Zerumbone, Cisplatin, COLO205, Lymphocytes, MTT assay, Comet assay

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INTRODUCTION

Plant-derived compounds are most commonly used as medicines in the world today. Every culture on Earth, through written or oral tradition, has relied on the huge variety of natural products found in healing plants for their curative properties. One-fourth of modern drugs prescribed worldwide are plant-derived, where 121 of these bioactive compounds are currently used in the treatment of various ailments [1]. Natural phytochemicals hold a great promise in clinical therapy as they possess no side effects that are usually associated with chemotherapy or radiotherapy. They are also comparatively inexpensive as they are ubiquitous and thus significantly reduce health care cost. Phytochemicals are the plant compounds with specific biological activities that support human health [2].

Zerumbone is a phytochemical, present in the rhizomes of *Zingiber zerumbet* Smith and *Zingiber aromaticum* [3]. Its molecular formula is C₁₅H₂₂O. The IUPAC name of this phytochemical is (2E, 6E, 10E)-2, 6, 9, 9-tetramethyl cycloundeca-2, 6, 10-trien-1-one [4]. It was found to possess many biomedical properties such as antioxidant, anti-inflammatory and antimicrobial activities [5,6]. It has been proven to possess anti-cancer, immunomodulatory and apoptosis-inducing properties against various human tumour cells, but with least effect on normal cells [7-11].

A variety of *in vitro* genotoxicity test systems have been developed in the cultured mammalian cell systems such as human peripheral blood lymphocytes for the screening of potentially cytotoxic, mutagenic and carcinogenic agents [12-14]. According to the literature survey, there exists a lack of good basic information about the genotoxic potential of zerumbone on colon cancer cells. In this context, the present work has been carried out to study the genotoxic potential of zerumbone on human colorectal cancer cell line, COLO205 and human peripheral blood lymphocytes. The current work can be seen as the comparative analysis of zerumbone genotoxic activity against the COLO205 cells and

lymphocytes, as well the cell growth inhibition and apoptosis induction by zerumbone in COLO205 cells and lymphocytes were studied.

MATERIALS AND METHODS

Chemicals

Zerumbone, cisplatin, lymphocyte isolation medium (Histopaque 1077), MTT and propidium iodide were purchased from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum, RPMI 1640, trypsin and dimethyl sulfoxide (DMSO) were procured from Hi media (Mumbai, India). All other chemicals and reagents used in this study were of analytical or HPLC grade and obtained from Sigma Aldrich (St. Louis, MO, USA) and Himedia (Mumbai, India).

Cell culture and maintenance

Human colorectal cancer cell line, COLO205 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 (100 µg/ml) at 37 °C in a humidified incubator with 5% CO₂ atmosphere. Once the cells reached ~90% confluency, they were trypsinized, washed thoroughly with media and subcultured into a new 25 cm² culture flask for expansion.

Isolation of peripheral blood lymphocytes

Peripheral blood was aseptically collected from healthy, non-smoking donors (below 30 y) by venepuncture into preservative-free EDTA tubes. The blood was diluted by adding phosphate buffered saline (PBS), pH 7.4 and carefully layered onto Histopaque 1077, centrifuged at 400 x g for 30 min, at interface lymphocytes were suspended. Trypan blue exclusion was used to assess the viability of isolated lymphocytes and the viability of these cells was found to be above 90%. The final concentration of lymphocytes was adjusted to about 2x10⁵ cells/ml.

Anti-proliferative activity of zerumbone and cisplatin

The MTT assay was performed to study the antiproliferative effect of zerumbone and cisplatin on COLO205 and lymphocytes. Cells were treated with varying concentrations (0-100 µg/ml) of zerumbone and cisplatin for different time intervals (24h, 48h, 72h) at 37 °C, 5% CO₂ along with the untreated control samples (which contained an equivalent concentration of DMSO). After every incubation period, cells were washed with PBS, treated with MTT and again incubated for 4h after which MTT was removed, then DMSO was added and finally, the plate was read at 540 nm in an ELISA plate reader. The data were represented as the percent post-treatment recovery (% live cells), whereas the absorbance from untreated control cells was defined as 100 % live cells.

Morphological studies using normal inverted microscope

Treated cells were observed under a normal inverted microscope following 24h, 48h and 72 h of incubation. The changes in cellular morphology due to death were examined and compared with untreated control. Photomicrographs were taken under the inverted microscope at 100× magnification.

Propidium iodide staining

Propidium iodide fluorescence staining method was used to observe the apoptotic morphological changes in the treated cells. Both COLO205 cells and lymphocytes were treated with IC₅₀ concentrations of zerumbone and cisplatin for 24h. The untreated cells served as control. After incubation, cells were washed with PBS, fixed in absolute alcohol for 30min at 4 °C, rehydrated with PBS and incubated with 100µl propidium iodide (25µM) at 37 °C for 5min. Photomicrographs were taken under a fluorescent microscope at 200× magnification.

Comet assay

DNA damage was assessed using the comet assay according to the method of Tice *et al.* [15]. 10 µl of the treated and untreated cells were suspended in 70 µl of 1% low melting agarose in PBS and were layered onto the slides pre-coated with 1% normal melting agarose. The slides were incubated at 4 °C for 30 min. A third layer was coated with 1% normal melting agarose and the slides were kept to dry. After solidification, the slides were kept in cold lysis buffer (10mmol Tris, 2.5 M NaCl, 100mmol EDTA, 1% N-lauroylsarcosine; pH 10) with fresh 1% Triton X-100 and 10% DMSO overnight at 4 °C. For unwinding of the DNA, the slides were incubated in freshly prepared alkaline electrophoresis buffer (300 mmol NaOH, 1 mmol EDTA; pH 13) for 20 min. The DNA was electrophoresed at 25 V and 300 mA for 20 min at 4 °C. After electrophoresis, the slides were washed thrice with a neutralizing buffer (0.4 M Tris-HCl; pH 7.5), left to dry for 30 min and fixed in 75% ethanol for 10 min. After staining with 25 µM propidium iodide, comets from individual cells were examined using a fluorescence microscope. OPEN COMET software was used to score 50 comets for each experiment.

Statistical analysis

Values were expressed as the mean ± standard deviation (SD), where n=5 for the MTT assay and n=3 for the comet assay. The values were subjected to analysis of variance (ANOVA) using GraphPad Prism software, Version 6.01 (California, USA). A statistically significant difference was considered to be present at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of zerumbone and cisplatin on cell growth

The antiproliferative effect of zerumbone and cisplatin on colorectal cancer cell line, COLO205 and normal human lymphocytes was determined by MTT assay. The growth of the cancer cells was inhibited in a dose (0-100µg/ml) and time (24h, 48h, 72h) dependent manner after exposure to zerumbone and cisplatin whereas lymphocytes were not affected after the exposure (fig.1). Previous studies reported the growth inhibition property of zerumbone on human colonic adenocarcinoma cell lines in a dose-dependent manner, while the growth of normal human dermal and colon fibroblast was minimally affected [16]. Our studies exhibited that zerumbone can induce a cytotoxic effect on colon cancer cells in a dose as well as time-dependent manner.

The IC₅₀ values of zerumbone and cisplatin on COLO205 cells for different incubation periods (24h, 48h and 72h) were found to be 19 µg/ml, 10 µg/ml, 5 µg/ml and 38 µg/ml, 24 µg/ml, 15 µg/ml respectively (table 1). The IC₅₀ concentrations were considered for further studies. Zerumbone, a natural dietary compound found in the rhizome of *Zingiber zerumbet*, exerts anticancer activity against various cancers, both *in vivo* and *in vitro* [17]. Literature stated that zerumbone hits a redox-regulated mechanism to selectively suppress the proliferation of cancer cells [18]. The cytotoxic activity of zerumbone on cancer cells is said to be attributed by the presence of a α, β -unsaturated carbonyl group in its structure, which plays a major role in interaction with the most biologically active molecules. According to Murakami *et al.*, zerumbone displayed a selective cytotoxic characteristic towards cancer cell lines and normal cell lines [3]. The results of our cytotoxic studies have also proved that zerumbone can induce a cytotoxic effect selectively on colon cancer cells but not on the normal lymphocytes.

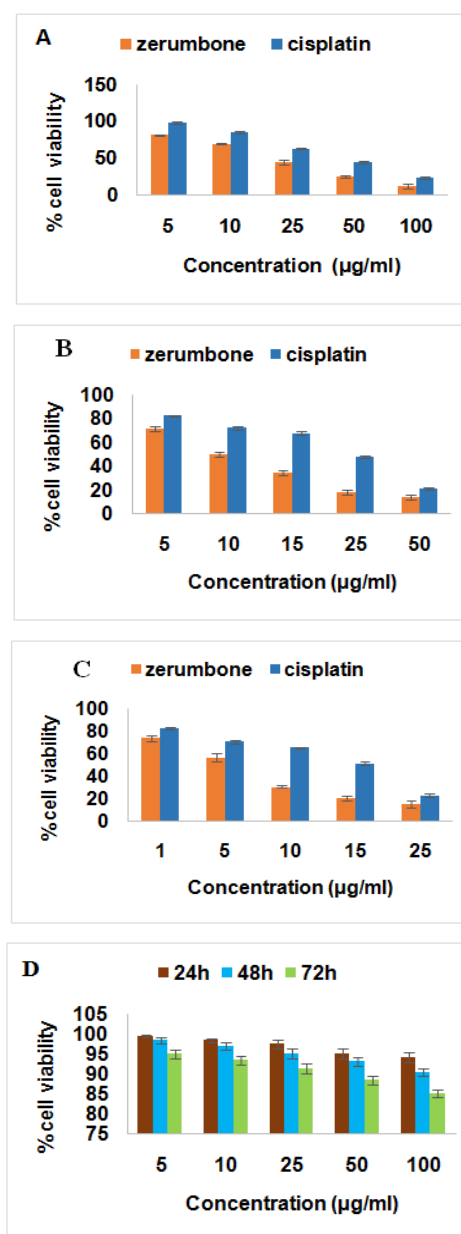


Fig. 1: Cytotoxic activity of zerumbone and cisplatin on COLO205 and lymphocytes as determined by MTT assay, COLO205 cells were treated with zerumbone and cisplatin for (A) 24h, (B) 48h and (C) 72h. Lymphocytes treated with zerumbone for 24h, 48h and 72h is shown in (D). Values are expressed as mean ± SD (N=5) percentage cell viability

Table 1: IC₅₀ values of zerumbone and cisplatin on COLO205 cells for 24 h, 48 h and 72 h

Compound	IC ₅₀ (µg/ml)		
	24 h	48 h	72 h
Zerumbone	19	10	5
Cisplatin	38	24	15

Effect of zerumbone and cisplatin on apoptotic cell death

COLO205 cells and lymphocytes were exposed to IC₅₀ concentrations of zerumbone and cisplatin. The treated cancer cells demonstrated early signs of apoptosis such as membrane blebbing and cytoplasmic shrinkage (fig.2). The untreated COLO205 cells and treated lymphocytes showed a negligible change in the membrane morphology (fig. 2,3). In the present study, it is proposed that zerumbone inhibits the cell proliferation by subsequent induction of apoptotic cell death of the colon cancer cells. This is clearly evident by the morphological changes in zerumbone-treated COLO205 cells that include distinctive nuclear morphology, chromatin condensation and fragmentation, loss of cell volume and production of apoptotic bodies (fig.4), which are all features of apoptosis [19]. Several studies have exposed the apoptotic nature of zerumbone against various cancer cells, but it has a negligible effect on normal cells [3].

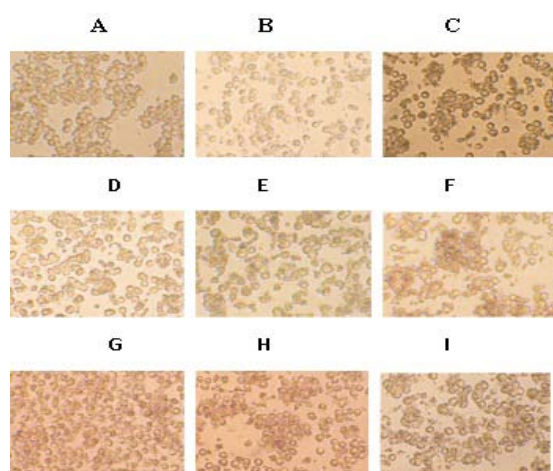


Fig.2: Effect of zerumbone and cisplatin on COLO205. Cells were treated for 24h: (A) control, (B) 19 µg/ml zerumbone, (C) 38 µg/ml cisplatin; for 48h: (D) control, (E) 10 µg/ml zerumbone, (F) 24 µg/ml cisplatin and for 72h: (G) control, (H) 5 µg/ml zerumbone, (I) 15 µg/ml cisplatin

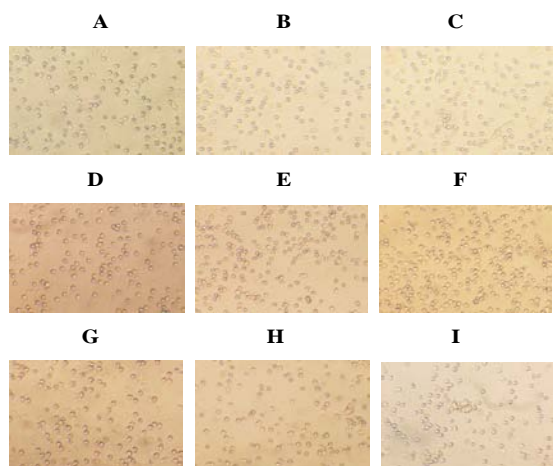


Fig.3: Effect of zerumbone and cisplatin on lymphocytes. Cells were treated for 24h: (A) control, (B) 19 µg/ml zerumbone, (C) 38 µg/ml cisplatin; for 48h: (D) control, (E) 10 µg/ml zerumbone, (F) 24 µg/ml cisplatin and for 72h: (G) control, (H) 5 µg/ml zerumbone, (I) 15 µg/ml cisplatin

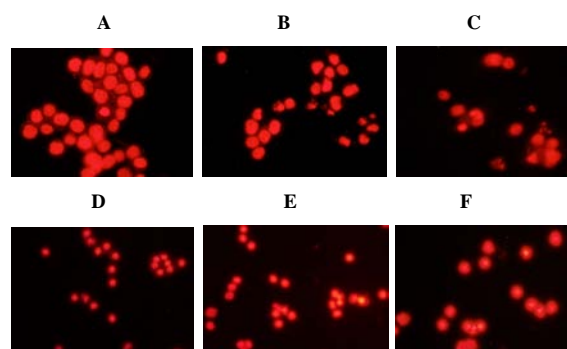


Fig.4: Morphological alterations of COLO205 and lymphocytes treated with zerumbone and cisplatin (A) untreated COLO205, (B) COLO205 treated with zerumbone (19 µg/ml), (C) COLO205 treated with cisplatin (38 µg/ml), (D) untreated lymphocytes, (E) lymphocytes treated with zerumbone (19 µg/ml) and (F) lymphocytes treated with cisplatin (38 µg/ml)

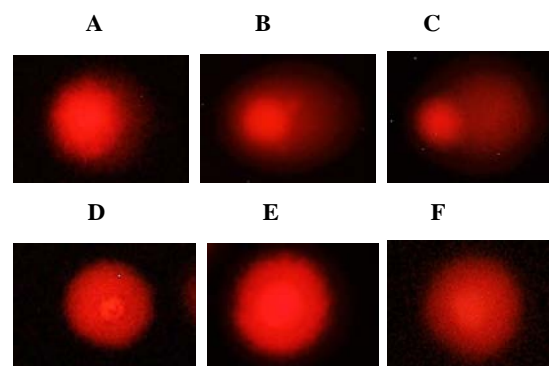


Fig.5: Genotoxic effect of zerumbone and cisplatin on COLO205 cells and lymphocytes, (A) untreated COLO205, (B) COLO205 treated with zerumbone (19 µg/ml), (C) COLO205 treated with cisplatin (38 µg/ml), (D) untreated lymphocytes, (E) lymphocytes treated with zerumbone (19 µg/ml) and (F) lymphocytes treated with cisplatin (38 µg/ml)

Genotoxic effect of zerumbone and cisplatin on cancer cells and lymphocytes

Comet assay is a simple, rapid and reliable technique for the detection and quantification of DNA damage. The assay determines the extent of DNA damage in individual cells and this can be assessed from the length of DNA migration [15]. Comet assay was performed to analyse the extent of DNA damage caused by zerumbone and cisplatin on cancer cells and normal human lymphocytes. The tail length, % tail DNA and olive moment are considered as DNA damage parameters [20]. The zerumbone-treated COLO205 cells showed a significant DNA damage with the tail length of 85.61, percentage DNA in tail of 27.42 and olive moment of 25.14 when compared to the untreated control cells. The cisplatin-treated COLO205 cells also showed a significant DNA damage with the tail length of 69.36, percentage DNA in tail of 22.27 and olive moment of 18.38 when compared to the untreated control cells. In contrast, the treated lymphocytes did not demonstrate any obvious DNA damage when compared to the untreated lymphocytes. The zerumbone-treated lymphocytes illustrated tail length of 7.23, percentage DNA in tail of 6.88 and olive moment of 1.64. And the

cisplatin-treated lymphocytes illustrated tail length of 6.91, percentage DNA in tail of 6.23 and olive moment of 1.46 (fig.5; table 2). The genotoxic agents have the ability to interact with DNA and cause DNA damage. Our findings illustrated that zerumbone can cause DNA damage on colon cancer cells. Previous studies have evaluated the genotoxic effect of zerumbone as dose-dependent on Chinese hamster ovary cells and human peripheral blood lymphocytes. It has also reported that zerumbone is a cytotoxic but

not a clastogenic substance in human lymphocytes [21,22]. Zerumbone carried no obvious structural alerts and no particular mechanism had been identified such as nucleoside analogues that can cause genotoxic effect, making it necessary for further studies to better understand the molecular mechanisms of action of zerumbone. The present results provide an additional evidence that zerumbone has a genotoxic and cytotoxic effects on colorectal cancer cells, while subsidized effects were observed on human lymphocytes.

Table 2: Effect of zerumbone and cisplatin on the extent of DNA damage on COLO205 and lymphocytes

Concentration	DNA damage		
	Tail length	% Tail DNA	Olive moment
COLO205			
Untreated Control	20.95±0.17	15.97±2.47	5.77±0.11
Zerumbone (19 µg/ml)	85.61±1.86	27.42±0.63	25.14±1.23
Cisplatin (38 µg/ml)	69.36±1.35	22.27±0.26	18.38±1.31
Lymphocytes			
Untreated Control	1.77±0.17	1.94±0.11	0.57±0.04
Zerumbone (19 µg/ml)	7.23±0.65	6.88±0.46	1.64±0.21
Cisplatin (38 µg/ml)	6.91±0.21	6.23±0.11	1.46±0.11

The values are expressed in mean±SD, n=3, P<0.0001

CONCLUSION

In the present study, zerumbone and cisplatin exhibited a potent antiproliferative activity against cancer cells, but with negligible effect on normal lymphocytes. Fluorescent microscopic and genotoxic studies have revealed that zerumbone is a strong inducer of apoptosis and caused DNA damage in the cancer cells but with minor effect on lymphocytes. These findings have demonstrated that zerumbone can be a potential phytochemical to treat colon cancer. Further studies have to be carried out in animal models and clinical trials have to be conducted.

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AUTHORS CONTRIBUTION

The study was designed by RT and MLN, as the supervisor provided conceptual and technical guidance for all aspects of the project. RT performed the experimental work and assessed the data. The manuscript was prepared by RT and its critical revision was done by MLN. Final approval of the manuscript was made by RT and MLN.

CONFLICT OF INTERESTS

There is not any conflict of interest which should be disclosed.

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