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RESEARCH ARTICLE

Zerumbone induces growth inhibition of Burkitt's lymphoma cell line via apoptosis

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Abstract

Zerumbone (ZER), a natural compound has been extracted from *Zingiber zerumbet* with known pharmacological activities. The aim was to determine the anti-human Burkitt's lymphoma (Raji) cell effect of ZER. The 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide (MTT) assay was used to determine cytotoxic effect while the Annexin-V-fluorescein isothiocyanate/propidium iodide-PI flow cytometric assays was used to determine apoptotic effect of ZER on the human Burkitt's lymphoma (Raji) cell (ATCC CCL-86) cell line. The expressions of Bax, Bcl-2, and c-Myc genes were determined via real-time PCR. ZER suppressed the proliferation of Raji cells with a 48 h IC_{50} value of 5.1 µg/mL. Treated Raji cells also underwent late apoptosis especially after treatment with 100 µg/mL ZER. The apoptotic effect of ZER is associated with increase in Bax and decrease in Bcl-2 and c-Myc gene expressions. These results suggest that ZER inhibited the proliferation of Raji cells through the modification of apoptosis-related gene expressions. Therefore, ZER has potential as a candidate for the treatment of Burkitt's lymphoma.

Keywords: Cytotoxicity, cell proliferation, Burkitt lymphoma.

Introduction

Burkitt's lymphoma is among most common cancers worldwide (Al-Attar et al., 1979; Achmad et al., 2019). Like all cancers, conventional treatment for lymphomas with immunotherapy and chemotherapy often lack efficacy due to the development of cancer resistance to the compounds. Therefore, there is need for the discovery of more effective and safer anticancer drugs than the conventional therapeutics. Currently, the most attractive candidates as anticancer compounds are products from medicinal plants (Albaayit et al., 2021a; Ling et al., 2016).

Anticancer compounds primarily targets the cellular mechanisms regulating proliferation of cancer cells, particularly the apoptotic pathways (Albaayit et al., 2021b; Sharifi et al., 2014). The driving force behind these pathways is the Bax proteins, which are associated with the mitochondrial control of cell proliferation (Glab et al., 2017). The members of the Bcl-2 family are among Bax proteins that function either as pro- or anti-apoptotic molecules (Albaayit et al., 2020a; Sharifi et al., 2014). The Bcl-2 protein, an anti-apoptotic molecule is among the most popular targets for anticancer therapeutics, and in lymphoma this is no exception. Lymphomas, for example Burkitt's lymphoma, are also governed by the expression of the oncogene, c-Myc, that is responsible for the regulation and expression of various apoptosis-related genes. Thus, the c-Myc gene can be modulated to make cancer cells more susceptible to apoptosis (Benassi et al. 2012; Cerquetti et al., 2015; Nguyen et al., 2017).

Zingiber zerumbet (L.) Smith is a perennial herb found in South East Asian countries and its rhizomes had been used traditionally in these countries for different purposes like food flavouring, appetizer, and herbal

medicines (Albaayit et al., 2020b; Attyah & Ismail, 2012). Till date, many manuscripts related to this plant has been published and discussed its ethnomedicinal uses like treatment of diarrhoea, toothache, fever, inflammation, fever, constipation, indigestion, severe sprains, pain relief, antirheumatic, antispasmodic, antiplatelet aggregation, and diuretic agents (Jantan et al. 2008; Bhuiyan et al. 2009; Zakaria et al. 2010; Yob et al. 2011,). Due to its high medicinal value, several researchers had isolated its compounds and described the phytochemical contents of rhizomes of *Z. zerumbet*. Till date many compounds have been identified from different extracts of its rhizomes such as humulene, monoterpenes, zerumbone, humulene monoxide, humulene dioxide, linalool, α-pinene, α-terpineol, β-pinene, camphor, borneol, humulene epoxide-I, humulenol-I, zerumbone oxide, zerumbone epoxide, diferuloylmethane, acetylated rhamnopyranoside, (*Z*)-nerolidol, kaempferol derivatives, *p*-hydroxybenzaldehyde, vanillin, saponins, terpenoids, and zederone (Varier et al. 1945; Balakrishnan et al. 1956; Dev et al. 1960; Ramaswami et al. 1962; Dũng et al.1995). Among all bioactive compounds identified from different extracts of rhizomes of *Z. zerumbet*, zerumbone has got special attention and extensively studied.

Zerumbone (ZER) is a crystalline sesquiterpene isolated from the essential oil of the rhizomes of *Z. zerumbet* (L.) Smith. ZER is unique in structure, containing a cross-conjugated ketone in an 11-membered ring (Albaayit and Maharjan, 2018). Among the pharmacological properties of ZER are the hepatoprotective, anti-inflammatory, antidiabetic, immune-modulatory, analgesic, antiadipogenetic, antioxidant, antifungal, and antimycobacterial effects (Sakinah et al., 2007; Haque et al., 2017; Albaayit et al., 2021c). ZER has also been shown to possess cytotoxic properties toward several cancer cell lines including the hepatic (HepG2), breast (MCF-7), cervical (HeLa), colon (COLO205), and ovarian (Caov-3) cells (Girisa et al. 2019). Murakami et al.1999 had shown the ZER inhibitory activity on human Burkitt lymphoma (Raji) cell line, however till date, its molecular mechanistic study on Raji cell line was not found, therefore this study was carried out to investigate the *in vitro* apoptotic effect of zerumbone on Burkitt lymphoma (Raji cell lines) through MTT assay, flow cytometry, and PCR techniques.

Materials and Methods

Material

Zerumbone crystals were prepared and characterized by using the method described by Mohamad et al. (2014). The crystals were diluted with 0.1% dimethyl sulfoxide (DMSO) to obtain the stock solution.

Cytotoxicity of ZER against Raji cell lines

Raji cell suspension at a concentration of 5×10^3 cells/well in a 96-well plate were treated with ZER at concentrations ranges from 3.1 to 100 µg/mL, while the control was treated with 0.1% dimethyl sulfoxide (DMSO) for 48 h at 37°C and 5% CO_2. 20 µL of MTT solution (5 mg/ml) was added and the plate incubated for 4 h in the dark. Formed purple formazan crystals were dissolved with 200 µL DMSO.

The absorbance of the samples was then measured at 570 nm using an ELISA plate reader (Tecan, California, USA). The half cancer cell growth inhibition (IC_{50}) value was obtained from the graph of inhibition percentage versus concentration (Albaayit et al. 2019). The following equation was used to calculate percentage growth inhibition for each concentration of ZER:

% growth inhibition = 100-(Average reading of test compound/Average reading of control) x100

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Apoptosis

Annexin-V/propidium iodide assay

Apoptosis in Raji lymphoma cell line were also determined by using the Annexin/PI staining technique (Thermo Fisher Scientific). Briefly, 1 ml of 1 × 10^6 Raji cells was seeded into each well of a 6-well plate and the plate incubated for 24 h at 37°C. ZER at either 5.1 or 100 µg/mL was added to the respective wells and the plate incubated for 48 h. After the treatments, the cells were harvested by trypsinization, washed thrice with phosphate buffered saline (PBS), and stained with 5 µL fluorescein isothiocyanate (FITC)-conjugated annexin-V and 5 µL PI for 15 min in the dark at room temperature. The percentage of cells undergoing apoptosis and necrosis were determined by flow cytometrically (FACSCalibur™, Becton Dickinson) (Albaayit, 2021).

Quantitative real-time polymerase chain reaction

RT-qPCR was used to determine expression of apoptosis-related genes in the treated Raji cells. 1 × 10^6 Raji cells/well/0.5mL suspension cells seeded into each well of a 6-well plate. The cells were treated with ZER at 5.1 (IC_{50}) and 100 µg/mL for 48 h. Total RNA was isolated from harvested cells using the TRIzol® Reagent (Bio basic BS410A, Canada). Complementary DNA (cDNA) was synthesized using the cDNA synthesis kit (Thermo Fisher Scientific K0221). 1 µL of cDNA was amplified using real-time PCR and primers listed in Table 1. The amplification of genes was ran with SYBR Green Master Mix (Thermo Fisher Scientific). LightCycler® 480 Gene Scanning Software (Agilent Technologies Stratagene Mx 3000P, Santa Clara, USA) was used to generate the results and calculate the amount of transcripts relative to the control. GAPDH was used as the housekeeping gene control (Hidayat et al. 2016).

Table1: Genes and primer sequences

Gene	Forward primer sequence	Reverse primer sequence
BCL2-associated X (*BAX)*	AAGAAGCTGAGCGATGTC	GGCCCCAGTTGAAGTTGC
B cell lymphoma -2 (BCL2)	GGCATTCAGTGACCTGACATC	AGTCATGCCCGTCAGGAAC
C-Myc	CACAGCAAACCTCCTCACAG	GGTGCATTTTCGGTTGTTGC
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	CCAGAACATCATCCCTGCCT	CCAGAACATCATCCCTGCCT

Statistical analysis

Differences among means were determined by one-way analysis of variance and Tukey's *post-hoc* test using SPSS (Version 19.0; IBM Corporation, Armonk, NY, USA) at $P<0.05$. The results are expressed as mean ± SD. All experiments were performed in triplicates.

Results and Discussion

Burkitt's lymphoma is among the most aggressive cancers. Currently, the mainstay of treatment for lymphoma is chemotherapy, which has many side effects. For that reason, phytochemicals with minimal side effects has been sought as potential anticancer compounds. Among natural compounds shown to have potential in the treatment of Burkitt's lymphomas are β-elemene (Tonglin and Gao, 2018), shikonin (Ni et al., 2018), and resveratrol (Jara et al., 2018).

ZER, the most prominent phytochemical in *Zingiber zerumbet*, was shown to have potent anticancer properties by acting on multiple pathways and suppresses the growth of cancers (Samad et al. 2015; Girisa

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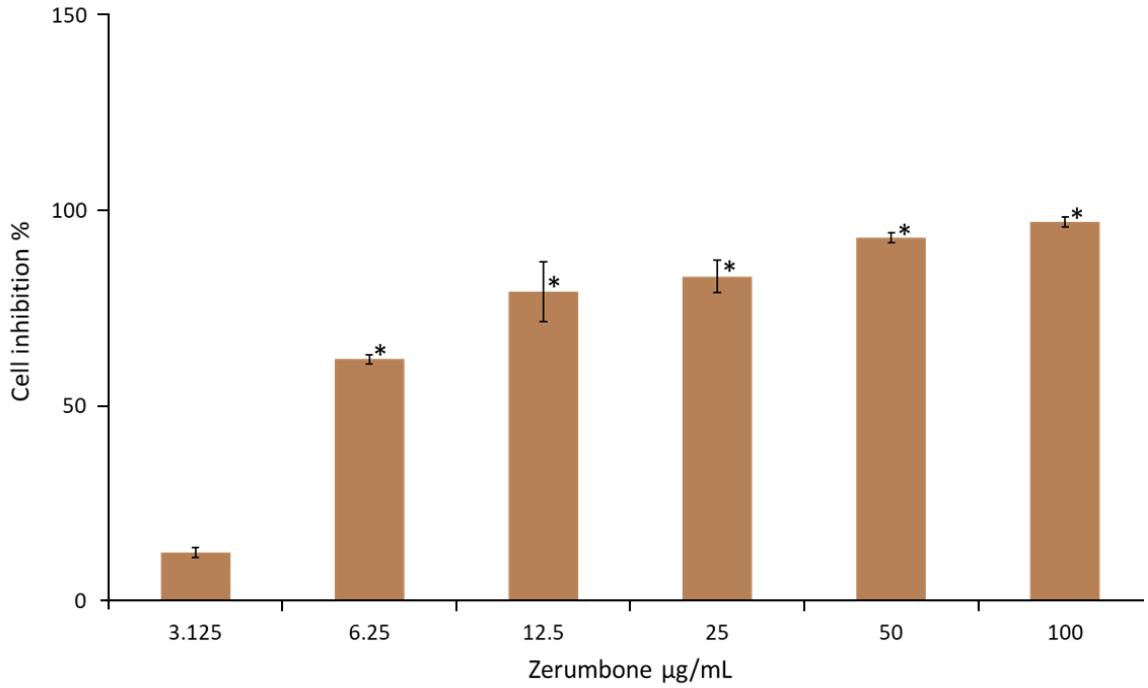
et al. 2019). In different cancer cell lines, ZER downregulated CD1d, IL-1β, TGF- β1, CD44, MMP-3, NF-κB, TNF-α, IL-8, β-catenin, BCl-2, VEGF, MMP-9, PI3K/AKT/mTOR, JAK2/STAT3 genes and upregulated p53, Caspase-3, Bax, Caspase 8, Caspase 9, IL-6, DR4, DR5 genes (Girisa et al. 2019). However, it is still unknown how ZER affects Raji cells. By the MTT colorimetric assay, ZER, in fact, significantly inhibited the proliferation of Raji cells as shown in figure 1. The lowest mean IC50 of 5.1 µg/mL against Raji cells, and hence was chosen for subsequent studies. It is believed that the effect of ZER on Raji cells is attributed to its β-unsaturated carbonyl group (Sadhu et al. 2007). ZER, especially at high doses, inhibited Raji cell proliferation via the induction of late apoptosis. Flow cytometric analysis clearly showed that ZER induced apoptosis in Raji cells (Figure 2). Zerumbone, especially at 100 µg/mL induced the majority of Raji cells to undergo apoptosis.

The Bcl-2 family of proteins, especially the Bax and Bcl-2 proteins are popular targets for anticancer drug candidates (Pfeffer and Singh, 2018). ZER caused significant upregulation in BAX by ~1.3 and 1.8-fold while downregulation in the Bcl-2 gene of the Raji cells by ~1.7 and 2.7-fold at 5.1 and 100 µg/ml, respectively. This suggests that ZER has potential as anti-Burkitt's lymphoma agent (Figure 3).

c-Myc is proto-oncogene protein that both activates and represses target gene via several mechanisms (Dang et al., 2006). Cancer cells have high c-Myc expression (McMahon, 2014) and this is presumably associated with its role as an activator of pro-proliferative genes. The c-Myc gene was significantly downregulated in Raji cells with ZER treatment, further showing that this compound is anti-proliferative toward Raji cells (Figure 3).

Figure 1. Effect of zerumbone on the Raji cell inhibition



After 48 h exposures determined via MTT assay. Values are mean ± standard deviation. *means significantly different (P <0.05) from control (0% inhibition) means.

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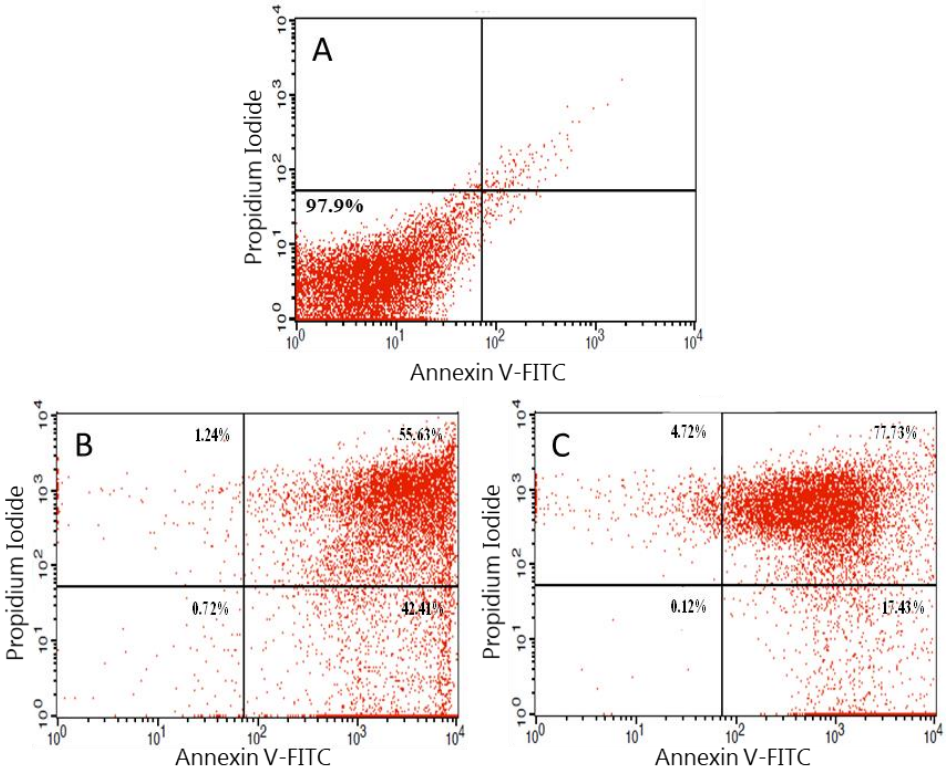
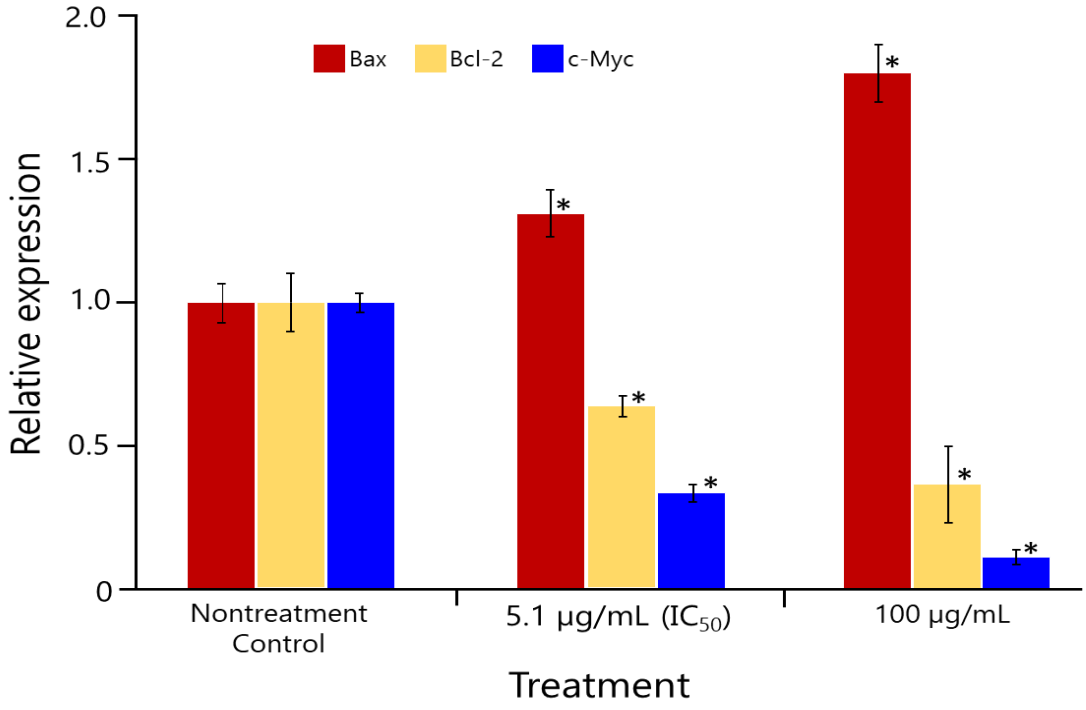
Figure 2. Effect of Zerumbone on apoptosis of Raji cells



After 48-hour treatment. (A) Nontreatment control, (B) 5.1 and (C) 100 µg/mL zerumbone for 48 h. Zerumbone, especially at high dose, induced late apoptosis in Raji cells.

Figure 3. Effects of Zerumbone on Bax, BcL-2 and c-Myc gene expressions



After 48 hour treatment. Zerumbone increased Bax and decreased Bcl-2 and c-Myc gene expressions in Raji cells.

Conclusion

Till date, many *in* vitro and *in* vivo studies had demonstrated the potent anti-tumour effect of zerumbone on different cancer cell lines. However, no significant antiproliferative effect of zerumbone on human Burkitt lymphoma (Raji) cell line had been described. This current study has shown the anti-tumour effect of zerumbone on Raji cell lines by up-regulating the pro-apoptotic Bax, and downregulating the anti-apoptotic Bcl-2 and c-Myc genes. To further confirm the potential candidate of zerumbone in the treatment of Burkitt lymphoma, more molecular studies have to be done regarding its effects on other apoptotic and anti-apoptotic genes, protein expression, cell cycle, and immunocytochemistry.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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