Original Article

ZERUMBONE (ZER) INDUCES APOPTOSIS IN HEPG2 CELLS VIA MITOCHONDRIAL PATHWAY

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ABSTRACT

Objective: The aim of the study is to determine the anti-cancer effect of ZER on the human hepatocellular carcinoma (HepG2) cell line.

Methods: The anti-cancer mechanisms investigated were apoptosis and anti-proliferation. The assays used in the *in vitro* study were 3-4-5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide (MTT), annexin V and acridine orange/propidium iodide staining, and cell cycle analysis to determine apoptosis. Colorimetric assays were employed for caspase-3 and 9 determinations. The morphological changes were determined by scanning electron microscopy.

Results: Zerumbone inhibited the proliferation of HepG2 cells in a dose-dependent manner and induced cell cycle arrest at the G2/M phase and apoptosis, shown by chromatin condensation, cell shrinkage and formation of apoptotic bodies, in the HepG2 cells in a time-dependent manner. Zerumbone also stimulated caspase-3 and-9 activities in the HepG2 cells.

Conclusions: This study suggests that the induction of apoptosis of ZER on HepG2 was via the mitochondrial pathway.

Keywords: Zerumbone, HepG2, Apoptosis, Caspase-3, Caspase-9.

INTRODUCTION

Liver cancers is the third most common cancer in the world with a frequency of 85 to 90% of all cancer cases in developing countries [1, 2]. This cancer is closely linked to chronic hepatitis B infection and can commonly kill almost all inflicted patients within a year [3, 4]. In 2000, an estimated 564, 000 new cases of liver cancer occurred worldwide with most patients eventually perishing as the result of this disease [5]. Liver cancers are more often presented at the late stages; thus treatments of cancer is not always promising with many side-effects and development of drug resistance [6]. This has led to the search for more amicable compounds for the treatment of the cancer. Several natural compounds from plants have been investigated for their anti liver cancer properties.

Malaysia possesses more than 600 species of ferns and 1, 082 species of angiosperm. Of these 76 species of fern have been documented to have medicinal properties [7]. One of the species, *Zingiber Zerumbet* (L) Smith can be found growing naturally mainly in Southeast Asia. *Zingiber Zerumbet* as a medicinal plant in the treatment many ailments is known since antiquity. The rhizomes of the plant is a traditional medicine for anti-inflammation, anti-rheumatic and anti-spasmodic [8].

Zerumbone is a monocyclic sesquiterpene with chemical formula $C_{15}H_2O$ and molecular weight of 218.340 dalton. The IUPAC name of this phytochemical is (2E, 6E, 10E)-2, 6, 9, 9-tetramethyl cycloundeca-2, 6, 10-trien-1-one [9]. This compound, isolated from the essential of *Zingiber zerumbet* Smith, possesses anticancer activity while practically harmless to normal cells [10]. The antiproliferative effects of ZER on several cancer cell lines like CaCo-2, HT-29 and HepG2 tumour cells occur via the apoptosis pathway and G2/M cell cycle arrest [11]. On cervical cancer cells, ZER not only induces apoptosis, arrests cell cycle at G2/M phase but also down regulates IL-6 [12]. Treatment of hepatic cancer cells with ZER elicit the down regulation of Bcl-2 and up-regulation of Bax expression, which significantly induce apoptosis. The effect of ZER is mediated through the action of Bcl-2 that reacts by blocking and interceding the Bax-induced events at several levels, preventing Bax redistribution after induction of the death signal [13]. In short, ZER balances the Bax to Bcl-2 ratio and enhances the Bax protein expression in liver cancers leading to cancer cell death. Thus, the aim of this study was to determine the antiproliferative effect of ZER and its role in the apoptosis of a liver cancer (HepG2) cell line.

MATERIALS AND METHODS

ZER preparation

Pure colorless ZER crystals were extracted from fresh *Zingiber zerumbet* Smith rhizomes by steam-distillation according to a method described earlier [11].

Growth inhibition of human hepatocellular carcinoma cells

The assay was performed in triplicate and dimethyl sulfoxide (DMSO) was used as the negative control. 3-4-5-Dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide (MTT) assay was used to estimate cell proliferation according to the previously described method [14]. The cells (1×10^4) of passages 4 to 7 were treated with ZER at concentrations of 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml, for 72 h. MTT was prepared by adding 5 mg/ml in phosphate buffer saline. DMSO (0.1% v/v; Sigma-Aldrich, St Louis, MO, USA) was the negative control.

Cellular morphology

Assessment of cellular morphology of ZER-treated HepG2 cells was conducted according to standard procedures. In brief, the treatments were performed in a 6-well plate (Orange Scientific, Belgium). HepG2 cells were plated at seeding density of 2×10^5 cells/ml and treated with ZER at its IC_{50} (6.20 µg/ml) concentration. Untreated cells served as negative control. The plate was then incubated under 5% CO_2 at 37 °C for 24, 48, and 72 h. The morphological changes of treated cells were compared with untreated control under phase contrast inverted microscope equipped with NIS-Elements D software (Nikon).

Quantification of apoptosis using fluorescence microscopy

The effect of the ZER on the morphology of HepG2 cells and quantification of apoptosis was investigated using an acridine orange (AO)/propidium iodide (PI) double-staining method according to the previously described method and examined under a fluorescence microscope (Leica, Tokyo, Japan).

Ultrastructure of HepG2 cells

HepG2 cells treated with IC50 concentration of ZER were cultured for 24, 48 and 72 h, and processed for scanning and transmission electron microscopy according to standard methods. The specimens were viewed under a scanning electron microscope (64000; JEOL, Tokyo, Japan) at an accelerating voltage of 15–25 kV. Sections on copper grids were stained and viewed under a transmission electron microscope (Phillips, Eindhoven, The Netherlands).

Cell cycle

Cell cycle analysis, via flow cytometry, was done to provide evidence of cytotoxicity of the ZER toward HepG2 cells, according to a protocol described earlier, with slight modification [11].

Annexin V-FITC assay

Annexin V-FITC Kit was used according to the manufacturer's protocol (MACS Miltenyi Biotech, USA). Analysis was performed using the Fluorescence-activated cell sorting (FACS) flow cytometer (BD Bioscience, California) equipped with CellQuest Pro software.

Caspase assay

The activities of caspase-3, -8, and-9 in HepG2 cells treated with the ZER were determined by the Colorimetric Assay Kit (Gen Script, USA) according to manufacturer's instructions.

Statistical analysis

The experiments were done in triplicates and the results expressed as mean±SD. Statistical analysis was done using SPSS version 17.0 (SPSS Inc., Chicago, USA). Probability values of less than alpha 0.05 ($P<0.05$) were considered statistically significant.
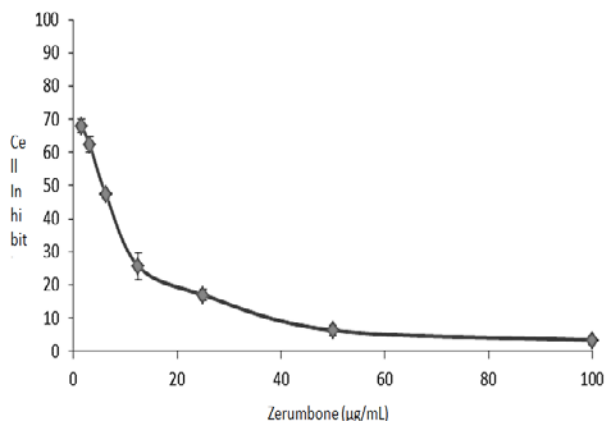

Fig. 1: Effect of ZER on on hepatocellular carcinoma (HepG2) cell line assessed by (MTT) assay
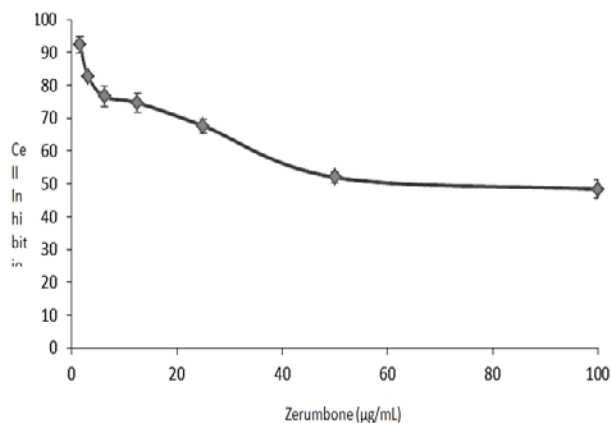

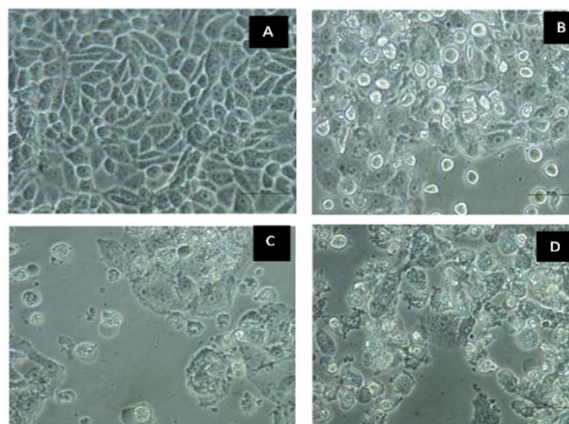**Fig. 2: Effect of ZER on normal liver (WRL68) cell line assessed by (MTT) assay**

Fig. 3: Hepatocellular carcinoma (HepG2) cell line treated with ZER at its IC$_{50}$ concentration (6.20 µg/ml), A= Untreated cells, B = ZER 24 h, C = ZER 48 h, D = ZER 72h
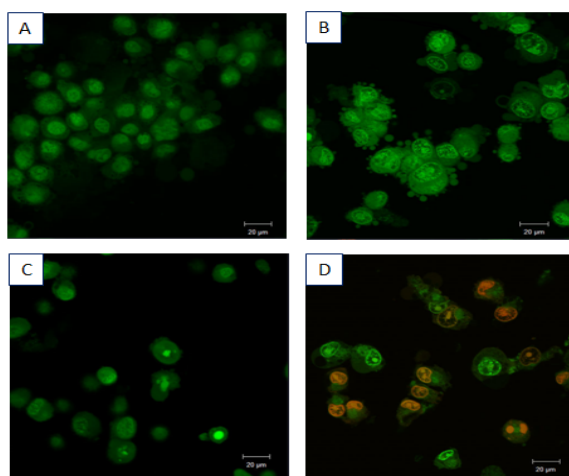

Fig. 4: Acridine orang/Propidium iodide-stained zerumbone-treated HepG2 cells after 72 h. IC$_{50}$ (6.20 µg/ml) zerumbone concentration was used, (A) Untreated HepG2 cells. (B) ZER 24 h (C) ZER 48 h and (D) ZER 72h. V=Viable cells, B= Cell membrane blebbing, C=Chromatin condensation, LA=Late Apoptosis (400 × magnification)
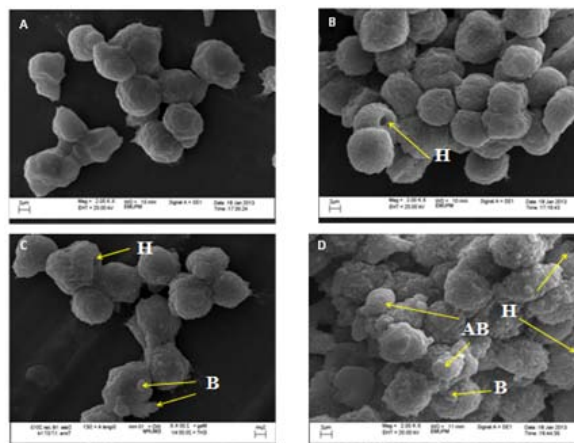

Fig. 5: Morphology of untreated and zerumbone-treated hepatocellular carcinoma (HepG2) cell line. Zerumbone (ZER) concentration at its IC$_{50}$ (6.20 µg/ml) (A) Untreated cells, (B) ZER-treated at 24h (C) ZER-treated at 48h (D) ZER-treated at 72 h. H=hole, B=membrane blebbing, AB=apoptotic body
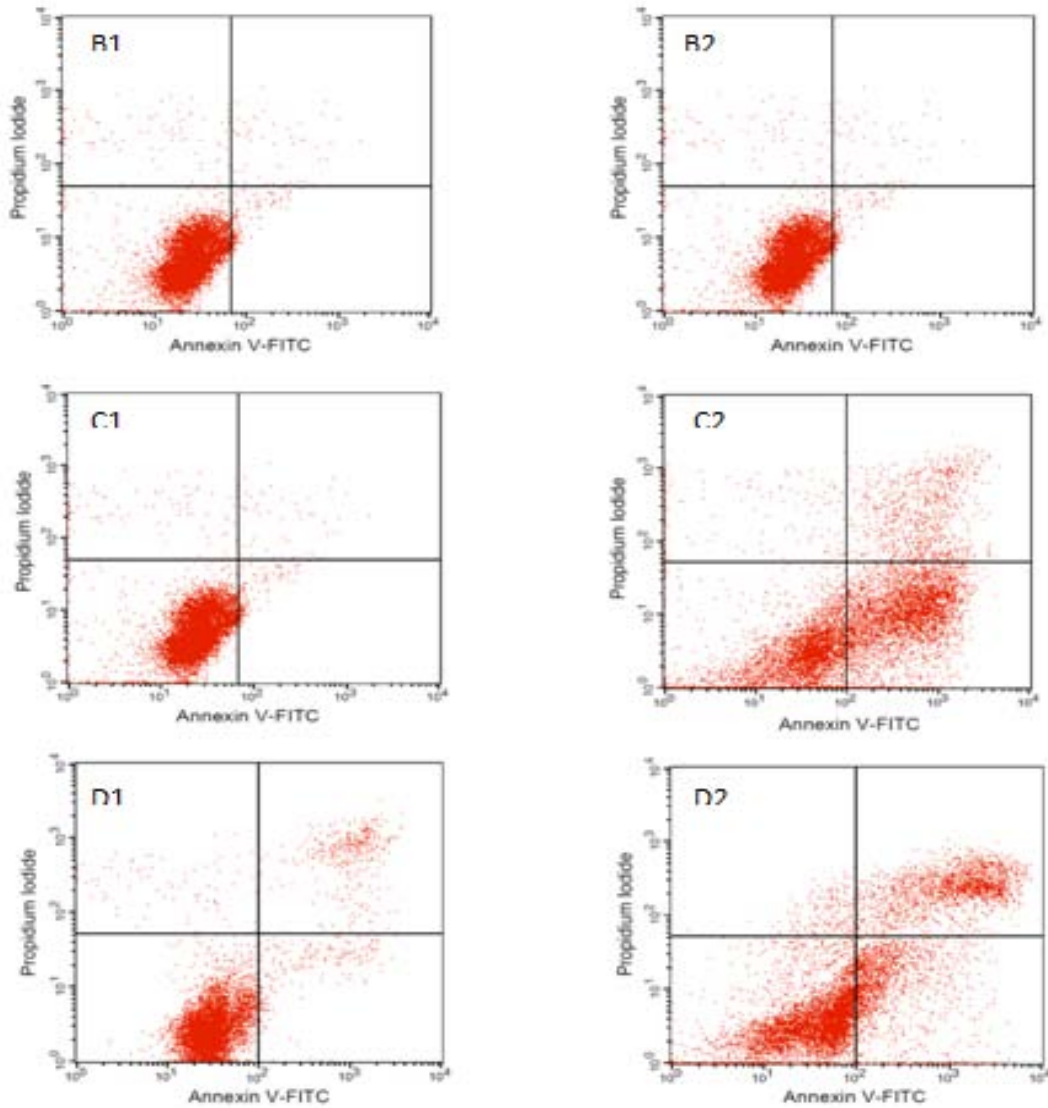
Fig. 6: Flow cytometric analysis of zerumbone-treated hepatocellular carcinoma (HepG2) cells after staining with FITC-conjugated Annexin-V and propidium iodide
A1 to D1 represents untreated control cells at 6, 12, 24 and 48 h. A2-D2 cells treated with ZER at 6, 12, 24 and 48
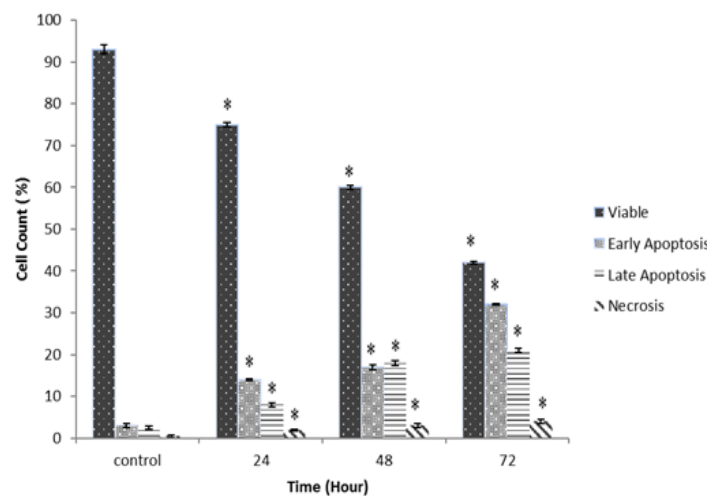

Fig. 7: Quantitative analysis of untreated (control) and ZER-treated HepG2 cells. Zerumbone treatment at IC_{50} concentration (6.20 μg/ml) showed time-dependency
N=3, percentage of cell count±SD, *significant difference (P<0.05) compared with control

Table 1: Cell cycle distribution of hepatocellular carcinoma (HepG2) cell line treated with zerumbone

Time (h)	Cell cycle (%)			
	Sub G1	G0/G1	S	G2/M
0	8.01±1.16	63.001±1.42	23.00±3.52	6.00*±3.52
12	6.00±2.25	50.00±1.70	22.00±2.25	22.00*±1.03
24	6.00±1.15	46.00±1.91	23.00±2.62	25.00*±1.59
48	6.00±1.40	42.0±1.62	23.00±1.82	29.00*±1.18
72	4.00±1.01	45.0±2.24	17.00±1.82	34.00*±0.98

n=3, mean±SEM, *significant difference ($P < 0.05$) compared with 0 h (Control).
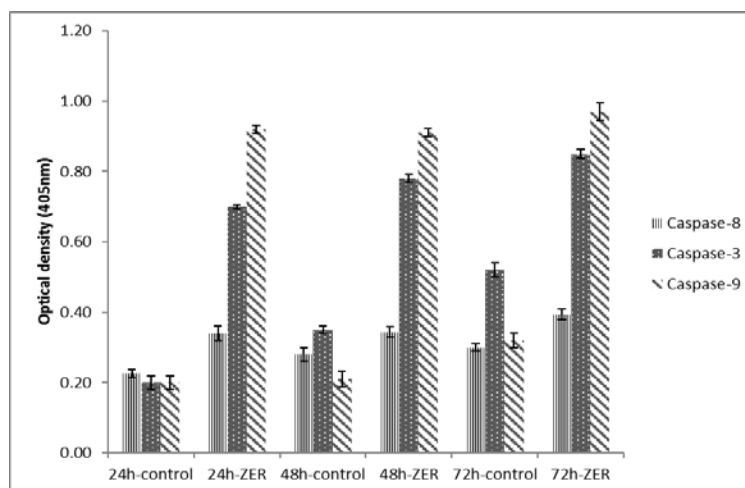

Fig. 8: Caspases activity in hepatocellular carcinoma (HepG2) cells treated with ZER after 24, 48 and 72 h. Cells treated with IC_{50} concentration (6.20 µg/ml) ZER

RESULTS AND DISCUSSION

Zerumbone is one of the bioactive compounds known to possess various health benefits and has also been shown to have anticancer properties [11]. In the current study, the effect of ZER on the HepG2 cell line was investigated specifically on cellular proliferation, cell cycle analysis and regulation of apoptosis *in vitro*. The results from MTT assay showed that the ant proliferative effect of ZER is towards the HepG2 (fig. 1), while remaining innocuous towards WRL68 cells, a human hepatic normal cell line (fig. 2).

Anti-proliferative and apoptosis-inducing ability is the main properties targeted for therapeutic compounds to be effective as anti-cancer agents. Among the gauge used to ascertain the anticancer effect of a compound is through determination of IC_{50} concentration. The lower, the IC_{50} the more effective is the compound in combating cancers. In the case of ZER, the IC50 concentration on the HepG2 cell line was 6.20µg/ml at 72 h of incubation but show no such cytotoxicity towards normal WRL68 cells (IC_{50} cannot be determined). The low IC_{50} concentration indicates that ZER is highly cytotoxic to hepatocellular carcinomas. This value is also within the range of effective anti-proliferative agents.

One of the most significant adversities associated with use of chemotherapeutics is the side-effect. While many anti-cancer agents are effective in killing cancer cells, they are also often toxic to normal cells and tissues. At the same time, development of resistance towards these agents is alarming [15]. Thus, there is pressing need for the development of safer and more effective therapeutic agents [16]. At least for some cancers, ZER seems to meet the requirements of a good anticancer agent, because it is cytotoxic to cancer cells and not to normal cells.

There were a few possible mechanisms that could be attributed to the anti-proliferative effect of ZER towards HepG2 cells.

In the present study, it is proposed that ZER inhibits the cell cycle progression and subsequent induction of apoptotic cell death of the liver cancer cells. This is clearly evident by the morphological changes in ZER-treated HepG2 cells that include distinctive nuclear morphology, chromatin condensation and fragmentation, loss of cell volume or cell shrinkage, plasma membrane blebbing and production of apoptotic bodies, which are all features of apoptosis [17]. These effects of ZER were time-dependent, with increase in apoptotic features with duration of HepG2 exposure to the compound.

Cell death can occur by a spectrum of morphologically and biochemically pathways associated with apoptosis and necrosis. Using the double-staining method of two fluorescence dyes, AO and PI, it was also shown that ZER at its IC_{50} concentration induced apoptosis of HepG2 cells, further confirming the observed morphological changes (fig. 4).

Ultrastructural changes are another mean of determining the effect of ZER on the HepG2 cells. In this study, distinct ultra-morphological alterations were observed in ZER-treated HepG2 cells. At 24 h of ZER-treatment, the HepG2 cells showed membrane blabbing and hole formation with concomitant cell shrinkage, increase in surface irregularities and from 48 h onwards apoptotic body formation. Formation of cytoplasmic extension and blebs were also time-dependent. On the contrary, untreated normal HepG2 cells showed no morphological changes and remained relatively round to oval in shape (fig. 5).

The effect of ZER on the HepG2 cell cycle was determined in this study. Based on the growth inhibition of ZER on HepG2 cells, cell cycle analysis showed that the effect of ZER is mediated through changes in the specific phase of HepG2 cell cycle. After 12, 24, 48 and 72 h incubation periods of HepG2 cell treatment with at IC50 concentration of ZER, there was an increase in the G2M phase of the cell cycle and this effect was time-dependent (table. 1). The cell cycle describes a series of events leading to division and replication [18, 19]. Normal cells divide at controlled and limiting rate while malignant cells divide at an uncontrollable manner and they usually lose checkpoint pedals that control replication of defective cells. This

effect of ZER on the arrest of HepG2 cell cycle at the G2M phase is a testament to its growth inhibitory effect on cancer cells. It is suggested that ZER causes a G2M cell cycle arrest and apoptosis in cancer cells via the Fas-and mitochondria-mediated pathway [20].

Further evaluation of the anti-hepatocellular carcinoma effect of ZER was determined by FITC-Annexin V assay. The FITC Annexin V assay can quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. This assay also differentiates between apoptotic and necrotic cells and between early and late apoptosis [21]. It was shown that ZER significantly increased the number of HepG2 cells in early and late apoptosis, which was dose-dependent (fig. 6). However, the study showed that the anticancer effect of ZER is not totally through apoptosis. There seems to be a very small portion (<10%) of the ZER-treated HepG2 cells that was destroyed through necrosis (fig. 7).

Apoptosis-induction is the most important effect that cancer chemoprevention and chemotherapeutic agents aims to achieve. Therefore, the fact that the primary anti-liver cancer effect of ZER is through apoptosis rather than necrosis suggests that this compound has great potential to be developed an effective cancer chemotherapeutic. In addition, the effect of ZER seems to gain strength with duration of exposure, making the compound useful for long-term treatment of liver cancers.

Cancer progression of benign to malignant phenotype is linked to apoptosis and associated signalling pathways and cellular events [22]. Among the mediators in apoptosis signalling pathways are the caspases. The caspases are largely categorized into initiators (caspase-2, -8, -9, -10), effectors or executioners (caspase-3, -6, -7) and inflammatory (caspase-1, -4, -5) [23]. The caspases undergo proteolytic activity and they have the potential to cleave proteins at the aspartic acid residues. Each caspase is specific for a particular amino acid. When caspases are triggered, they become irreversibly committed to induction of cell death. The activation of caspase-9, the initiator caspase, in response to a variety of death stimuli, lead to the downstream mitochondrial cytochrome c release with subsequent triggering of effector caspases [23]. Caspase-9, the well-characterised caspase, plays a crucial role in the regulation of apoptosis, while caspase-3, which is an execution caspases activates cytoplasmic endonuclease, which together with proteases degrades nuclear material cytoskeletal proteins [24]. In this study, ZER significantly ($P<0.05$) stimulated both caspase-3 and-9 dose-dependently, while showing less effect on caspase-8 activity (fig. 8).

CONCLUSION

In conclusion, we found that ZER-induced apoptosis was accompanied by the stimulation of caspase-3 and-9 in HepG2 cells, implying that ZER induced apoptosis via the mitochondrial death pathway. Therefore, we believe that ZER is a promising molecule in cancer chemoprevention or chemotherapy and further efforts to explore this therapeutic strategy are needed.

ACKNOWLEDGEMENT

CONFLICT OF INTERESTS

The authors have no any conflicts of interest in this work

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