Regression of cervical intraepithelial neoplasia by zerumbone in female Balb/c mice prenatally exposed to diethylstilboestrol: Involvement of mitochondria-regulated apoptosis

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Abstract

Background: Cervical cancer is the second most common cause of cancer death in women. We have demonstrated previously that zerumbone (ZER) has an anti-cancer effect towards human cervical cancer cells (HeLa).

Methods: Anti-cancer properties of ZER were investigated using female Balb/c mice exposed prenatally to diethylstilboestrol. Female offspring have been treated with ZER (4, 8 and 16 mg/kg), normal saline and cisplatin (10 mg/kg; positive control). The anti-cancer properties of ZER were evaluated using histopathology, TdT-mediated dUTP nick end labeling (TUNEL) Assay and immunohistochemical staining of Bcl-2-associated X protein (Bax), a key protein in mitochondrial pathway of apoptosis. In addition, laser capture microdissection microscopy isolated RNA was amplified using reverse transcriptase polymerase chain reaction (RT-PCR) based on the specific primer of B-cell lymphoma 2 (Bcl-2).

Results: Treatment with ZER resulted ($p < 0.05$, $\chi^2$ statistics) in the regression of cervical intraepithelial neoplasia (CIN) resembling cisplatin effect (10 mg/kg). TUNEL micrographs showed the absence of apoptosis in cancerous tissues treated with normal saline compared to ZER and cisplatin where abundant apoptotic cells were noticed. A post hoc analysis showed a significant ($p < 0.01$) difference in mean percentage of apoptosis between normal saline treatment (0%), ZER (15.7%) and cisplatin (21.7%). Immunohistochemical staining of Bax protein revealed that ZER modulates the expression of this apoptosis marker. Administration of ZER has also modulated the expression of Bcl-2 gene.

Conclusion: These findings showed that ZER induces apoptosis efficiently in cervical tissues from female Balb/c mice treated prenatally with diethylstilboestrol. This suggested that ZER, a plant-derived compound, could be introduced as a new chemo-preventive agent for CIN in future.

Keywords: Zerumbone; Cisplatin; Cervical intraepithelial neoplasia; Apoptosis; TUNEL assay; Bax protein

Abbreviations: Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; CC, cervical cancer; CIN, cervical intraepithelial neoplasia; CIS, cisplatin; DAB, diaminobenzidine; DES, diethylstilbestrol; HRP, horseradish peroxidase; LCMM, laser capture microdissection microscope; PBS, phosphate buffer saline; PCNA, proliferating cellular nuclear antigen; RT-PCR, reverse transcriptase polymerase chain reaction; TUNEL, TdT-mediated dUTP nick end labelling; ZER, zerumbone.

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**Introduction**

Cervical cancer (CC) is the second most common cause of cancer death in women (Saslow et al., 2007), with an estimated 510,000 newly diagnosed cervical cancer cases and 288,000 deaths. Cancer chemotherapeutic drugs are known to activate various responses to tumour cells. Apoptosis, as one of these responses, is a fundamental cell event as important as growth, differentiation and quiescence. It regulates tissue development, homeostasis and it is a basic defence against cancer (Chiarugi et al., 1997). The mitochondrial pathway of apoptosis is mediated by the B-cell lymphoma 2 (Bcl-2) family proteins (Fulda and Debatin, 2006). Pro-apoptotic Bcl-2-associated X protein (Bax) of the Bcl-2 family regulates the passage of cytochrome c from the mitochondria (Campos et al., 2006).

Most of the earliest pharmaceuticals were plant-derived compounds (PDC), whereby these plants were used initially to treat diseases. PDCs that are useful in clinical oncology include those of flavonoids, coumarins, cinnamates or phenolics. These drugs have been tested in experimental animal studies and demonstrated protection against carcinogens (Twyman et al., 2005). Cancer chemoprevention is the administration of chemical agents to prevent or delay the development of cancer, in which chemical agents are used to prevent cancer (Araldi et al., 2008). However, the number of currently available preventive agents is limited and the chemical agents are costly (Vogl, 2007; Meropol and Schulman, 2007). Natural products have provided a cheaper source with greater chemical structural diversity. In this respect, sesquiterpenes have been demonstrated to stimulate cytotoxicity and apoptosis in several cancer cell lines at low micromolar concentrations within acceptable clinical range of newer anti-cancer drugs (Hamann, 2003). Sesquiterpenes have been found to be abundant in *Zingiber zerumbet*, a plant which is commonly found in Malaysia (Sakinah et al., 2007) and has significant economic properties, as the rhizome can be used as both a spice and a traditional medicine (Chiena et al., 2008). Zerumbone (ZER), the main compound of *Z. zerumbet*, has shown anti-proliferative effects on different cancer cell lines such as HT-29, CaCo-2 and MCF-7 cancer cells (Kirana et al., 2003; Hamann, 2003). This cytotoxicity has been reported to be selective towards cancer cells compared to normal cells (Nobuji et al., 1991; Sakinah et al., 2007). Furthermore, the compound has shown potential in vivo chemo-preventive properties on induced skin cancer in mouse models (Murakami et al., 2004) and colon cancer in rats (Tanaka et al., 2001). In addition, ZER has shown anti-inflammatory (anti-cyclooxygenase-2), suppression of free radical generation, iNOS expression, TNF-α release, anti-HIV (Murakami et al., 2003; Jin-Rui et al., 1997), suppress free radical generation and activates phase II drug metabolizing enzymes (Nakamura et al., 2004).

We have demonstrated previously that zerumbone has anti-cancer effects on human cervical cancer cells (HeLa) (Abdul et al., 2008a; Abdelwahab et al., 2009). Moreover, a mouse model for cervical intraepithelial neoplasia (CIN) was developed successfully in our laboratory (Abdul et al., 2008b). Therefore, this current in vitro study was undertaken to evaluate the anti-cancer properties of zerumbone using this established animal disease model for cervical intraepithelial neoplasia.

**Materials and methods**

**Chemicals and reagents**

Diethylstilbestrol (DES), chemicals for histological staining, cisplatin (CIS) and sesame oil were purchased from Sigma Aldrich (Kuala Lumpur, Malaysia). RNA isolation kit was purchased from Molecular Devices (Canada). DeadEnd™ Fluorimetric TdT-mediated dUTP nick end labeling (TUNEL) Assay and reverse transcriptase polymerase chain reaction (RT-PCR) Kits were obtained from Promega (USA). Bax antibody was purchased from Biovision Laboratories, USA; while proliferating cellular nuclear antigen (PCNA) antibody was obtained from Abcam (USA). Immunohistochemistry kit and Poly L-Lysine-coated slides were purchased from Dakko (USA). Zerumbone (ZER) was extracted from *Zingiber zerumbet* plant using hydrodistillation followed by recrystallization with hexane (Abdul et al., 2008a). The chemical structure of ZER was examined and verified using liquid chromatography mass spectrometry and NMR analyses (Abdul et al., 2008a)

**Animals**

Experiments were performed using inbred female Balb/c mice (18–22 g; n = 6 per group). All animals were kept in a room at a constant temperature 22 °C with 12 h light/dark cycles and had free access to standard diet and distilled water. The protocol of this study was approved by the Animal Care and Use Committee (ACUC), Faculty of Medicine and Health Sciences, Universiti Putra, Malaysia. All experimental procedures were conducted following the Guide to ACUC.

**Induction of cervical intraepithelial neoplasia in mice**

This in vivo model was carried out according to the method developed (Abdul et al., 2008b). Briefly, one male mouse was cohabitate with three female mice for 48 h prior to localize each male with one female. Detection of vaginal plug was considered as the first day of pregnancy (G = 0) and accordingly the pregnant
mice were caged individually. The pregnant mice were given daily subcutaneous injections of diethylstilbestrol (67 μg/kg body weight) dissolved in sesame oil from gestation day 13 to 18. The offspring were weaned at 22 days of age. One female offspring was subjected for confirmation of cervical dysplasia at 52 days of age.

Treatment with ZER and cisplatin

All female offspring exposed to DES in utero were pooled together and were divided into 4 groups. The 5th group consists of mice which were not exposed to in utero DES. The mice were given treatment starting from 52 days of age to 60 days of age (4 dosages on alternate days, i.p.). Group 1 mice were treated with 0.9% of normal saline and acts as a positive control group. Mice in Groups 2 and 3 were given 8 and 16 mg/kg of ZER, respectively. Ten milligrams per kilogram of cisplatin was given to the mice in Group 4. Finally, no treatment was given to the mice in Group 5 as they act as a negative control (normal mice). Following treatment, all the mice were sacrificed at 54 days of age. The cervix were fixed in 10% formalin and embedded in paraffin wax (FFPE). These tissues were processed according to standard H&E staining. Histopathological evaluation of all cervical tissues were examined and confirmed by an independent histopathologist unaware with the experimental design.

In situ TdT-mediated dUTP nick end labelling (TUNEL Assay)

TUNEL Assay was performed on FFPE cervical tissues according to the manufacturer’s instructions (Promega Inc, USA). Briefly, de-paraffinized tissue sections were washed in 0.85% NaCl, immersed in phosphate buffer saline (PBS) for 5 min and fixed in 4% methanol-free formaldehyde. Hundred micro-liters of Proteinase K (20 μg/mL) was added and incubated for 8–10 min at ambient temperature followed by washing in PBS for 5 min. Tissues were incubated with 50 μL of fresh rTdT buffer at 37°C for 60 min at darkness to allow the tailing reaction to occur. This reaction was terminated by immersing the slides in 2× SSC for 15 min at room temperature. Slides were washed twice to remove unincorporated fluorescein-12-dUTP. The samples were stained with propidium iodide solution (1 μg/mL in PBS) for 15 min in the dark followed by washing in deionized water for 5 min. One drop of antifade solution was added to the area containing the treated section and the slides were mounted using glass coverslips. The edges were sealed with rubber cement and left to dry for 5–10 min. Samples were analyzed under a fluorescence microscope (ZIEMSS LSM 70) using standard fluorescein filters.

Immunohistochemistry

Tissue section slides were heated at 60°C for approximately 25 min in hot air oven (Ventcell, MMM, Einrichtungen, Germany). The tissue sections were de-paraffinized in xylene and rehydrated using graded alcohol. Antigen retrieval process was performed in 10 mM sodium citrate buffer boiled in microwave (Model No. EMO6505E, ELBA, 650W, Korea).

Immunohistochemical staining was conducted according to manufacturer’s protocol (Dakocytomation, USA). Briefly, endogenous peroxidase was blocked by peroxidase block (0.03% hydrogen peroxide containing sodium azide) for 5 min. Tissue sections were washed gently with wash buffer and then incubated with Bax (1:500) and PCNA (1:1000) biotinylated primary antibodies for 15 min. The sections were rinsed gently with wash buffer and place in buffer bath. The slides were then placed in a humidified chamber and sufficient amount of streptavidin–HRP (streptavidin conjugated to horseradish peroxidase in PBS containing an anti-microbial agent) was added and incubated for 15 min. Then tissue sections were rinsed gently in wash buffer and place in buffer bath. Diaminobenzidine (DAB)-substrate-chromagen was added to the tissue sections and incubated further for 5 min following washing and counterstaining with hematoxylin for 5 s. The sections were then dipped in weak ammonia (0.037 mol/L) 10 times and then rinsed with distilled water and coverslipped. Positive findings of the immunohistochemical staining should be seen as brown stains under light microscope.

PCNA labelling index (Proliferation Index)

Quantitative evaluation of PCNA immunohistochemical staining (Chandra Mohan et al., 2006) was done based on the number of immunoreactive cells that possess brown color in each microscopic field. Randomly selected fields were scored per slide. The labelling index for PCNA was expressed as the number of cells with positive staining per 100 counted cells.

Laser capture microdissection microscopy and RNA extraction

Laser capture microdissection microscopy (LCMM) (Fend et al., 1999; Sugiyama et al., 2002) was performed using the Arcturus™HS LCM System (PixCell®) and CapSure™ HS LCM system (Arcturus, Mountain View, USA). Briefly the CapSure® HS cap was placed into formalin-fixed paraffin-embedded and H&E-stained (FFPE & HE) slide. The laser spot was then focused, at the recommended starting power (~75 mW) with duration of 1 ms settings for nominal
7.5 μm spot size. The CapSure–ExtracSure assembly was placed in the CapSure-HS Alignment Tray and 10 μL extraction buffer (XB) was pipetted into the buffer well. A new sterile RNase-free 0.5 mL tube was placed onto the CapSure–ExtracSure assembly. The tube was covered by the preheated heating block (42 °C) and incubated for 30 min at 42 °C. Later, the microcentrifuge tube with the CapSure–ExtracSure assembly was centrifuged at 800 g for 2 min to collect the cell extract.

To precondition the RNA purification column, a 250 μL of conditioning buffer was added later removed by centrifugation at 16,000 g for one minute. To proceed with the RNA extraction, 10 μL of 70% Ethanol (EtOH) was pipetted. This mixture was added to the preconditioned RNA purification column and centrifuged for 2 min at 100 g to bind RNA molecules to the column and followed immediately by centrifugation at 16,000 g for 30 s to remove flowthrough. Hundred micro-liter of wash buffer 1 (W1) was then added into the purification column and centrifuged for 1 min at 8000 g followed by DNase treatment using DNase enzyme. Hundred micro-liter of wash buffer 2 (W2) was then added twice into the purification column and centrifuged for 1 min at 8000 g and 2 min at 16,000 g, respectively. RNA purification column with the bound RNA molecules was transferred into a new 0.5 mL microcentrifuge tube. The RNA was eluted with 17 μL of elution buffer and centrifuged for one minute at 1000 g to distribute the elution buffer (EB) in the column, and then centrifuged for 1 min at 16,000 g to elute the RNA. The entire sample was used immediately for RT-PCR or stored at −80 °C until further use.

**Reverse transcriptase polymerase chain reaction**

The Reverse Transcriptase Polymerase Chain Reaction was carried out by the Access RT-PCR System (Promega, USA) according to the manufacturer’s directions. Kit components were pipetted in sterile nuclease-free tubes (nuclease-free water (23 μL), AMV/Tfl 5X reaction buffer (10 μL), dNTP mix (1 μL), upstream primer (5 μL), downstream primer (1 μL) (Bel-2 5’ primer GATGTCCAGCCAG CTGACACCT; 3’ primer CACAAAGGCATCCCAGCTC), 25 mM MgSO4 (2 μL), AMV reverse transcriptase (1 μL), Tfl DNA polymerase (1 μL) and RNA template) and mixed by gentle pipetting. The RT-PCR products were visualized on 1% agarose gel with ethidium bromide.

**Statistical analysis**

All descriptive and inferential statistical analyses have been performed using SPSS version 15.0 (SPSS Inc., Chicago, USA). Data have been checked for homogeneity of variance and normal distribution prior to running the respective statistical analysis. Probability value less than alpha (0.05) was considered as indication of significance.

**Results**

**Histopathological examination**

CIN-associated hyperproliferation lesions such as hyperchromatism, nuclear atypia and abnormal nuclear:cytoplasm ratio were noticed in normal saline-treated group (model positive control) and accordingly, lesions in this group were classified as CIN III (Fig. 1B). Cisplatin was able to regres the proliferation induced by DES, corresponding to CIN I (Fig. 1C). ZER at dosage of 4 mg/kg did not show any remarkable regression towards proliferating CIN lesions (Higher nuclear:cytoplasmic ratio (hyperchromatism) with cytoplasmic swelling and clarity (Fig. 1F)). Dosage of 8 mg/kg has shown anti-proliferative properties (CIN II) (Fig. 1E). Dosage of 16 mg/kg of ZER (Fig. 1D) has effects that mimic the anti-cancer effects of cisplatin at dosage of 10 mg/kg. Both compounds, at their respective effective dosages, regressed the progression of cancerous cervix tissue of stage CIN III to CIN I, the later stage representing cervix tissue with mild cytoplasmatic clarity and disoriented epithelial cell arrangements. The statistical analysis of the association between the response to treatment and the grading of CIN is shown as in Table 1. All experimental animals’ cervical tissue slides were investigated blindly under light microscope and the grading of CIN was reported. The statistical analysis (Chi-square test) concluded that the regression of CIN lesions was associated significantly (P<0.05) with both CIS (10 mg/kg) and ZER at dosage of 16 mg/kg.

**Immunohistochemical staining of PCNA**

Table 2 and Fig. 2 show the effects of treatment with ZER on cell proliferation marker (PCNA). Zerumbone reduced the expression of PCNA in a dose-dependent manner (Table 2). The percentage of PCNA immunoreactive positive cells (brown nuclei; Fig. 2A) in normal control group was insignificantly (P>0.05) different as compared to those in ZER-treated groups (8 and 16 mg/kg) (Fig. 2A and B). However, the percentage of PCNA-positive cells from cancer control (Fig. 2F) group was much higher (P<0.05) compared to normal control (Fig. 2A) and ZER-treated groups (Table 2) as well as the intensity of immunoreactive brown nuclei (Fig. 2). The absence of PCNA immunoreactivity in the cytoplasm and cell membrane clearly proved the specificity and sensitivity of the immunohistochemical
In situ TdT-mediated dUTP nick end labelling of mice cervical tissue

Zerumbone and cisplatin show increased number of apoptotic cells, evidently with higher green fluorescence. Treatment with ZER at 16 mg/kg (Fig. 3C) and cisplatin at 10 mg/kg (Fig. 3E) dosages induced noticeable apoptosis to the cervix tissues, in comparison to normal saline (Fig. 3B), which showed no apparent apoptosis. Normal cervical tissue demonstrated very few apoptotic cells (Fig. 3A). Fig. 4 shows the mean percentage of apoptosis in cervical tissue after treatment with ZER, cisplatin and normal saline. There was a significant mean difference between the treatment groups ($P < 0.01$). Post hoc comparison test (one-way ANOVA) showed a significant difference in mean percentage of apoptosis between normal saline treatment group (0%), with ZER (15.7%) and cisplatin (21.7%) treatments groups ($P < 0.01$). A significant mean difference was also found between treatments groups of ZER at 16 mg/kg and cisplatin at 10 mg/kg dosages as compared to the normal group of female mice, the mean apoptotic percentage being 2.7% ($P < 0.01$). No significant difference in mean apoptotic percentage was identified between treatments groups of 16 mg/kg ZER and 10 mg/kg cisplatin. However, a significant mean difference was found between treatments groups of ZER at 8 mg/kg (8%) and cisplatin at 10 mg/kg dosages (21.7%) with $P < 0.01$. Higher dosage of ZER was needed to induce apoptosis at similar rate as cisplatin.

Expression of pro-apoptotic protein (Bax)

Immunohistochemical results demonstrated that treatment of cancerous mice with ZER and cisplatin cause over-expression of pro-apoptotic protein, Bax (Fig. 5B). In addition to this, the expression of Bax protein in normal and cancerous cervical tissues (Fig. 5A and B) was found to be down-regulated compared to Bax expression in ZER-treated cervix. No brown staining is observed in negative control tissues (data not shown).

Bcl-2-specific mRNA

Expression of Bcl-2-specific mRNA was also studied using RT-PCR that amplified the LCMM products. Fig. 6 depicts the results of RT-PCR products. The signal intensities were noticeably observed to be lower in both ZER (16 mg/kg) and cisplatin (10 mg/kg)-treated groups when compared to cancerous and normal cervical tissues (Fig. 6). Expression of Bcl-2 was gene was concurrently with β-actin as housekeeping gene, which showed that all group were able to exert the band that corresponds to 242 bp (data not shown).

Discussion

Previously we have demonstrated the in vitro anti-proliferative properties of ZER on cervical cancer cells...
ZER has been administered to female Balb/c mice induced with cervical carcinogenesis using diethylstilbestrol as an in vivo animal model. However, it has been previously reported that prenatal exposure of diethylstilbestrol resulted in various reproductive tract abnormalities in women (Iguchi et al., 2008). In addition, DES is known to induce some tumorgenesis, primarily cervical, since gestation is a period of high sensitivity towards chemical carcinogenesis due to factors such as organogenesis coupled with global proliferative growth (Waalkes et al., 2006). Elsewhere, studies have shown an elevated incidence of reproductive tumors in the female offspring of prenatally exposed mice to DES (Titus-Ernstoff et al., 2008). In this respect, abnormalities to the reproductive tract of mice exposed to DES have been verified in an in vivo model in our laboratory, which correspond to CIN stage III (Abdul et al., 2008b).

Histopathological findings (H&E) demonstrate that ZER was able to suppress the proliferation of CIN lesions. The evaluation of CIN grades, done according to the distribution of lesions amongst cervical epithelia using light microscopy (Fadare and Rodriguez, 2007), was verified by an independent investigator unaware of the experimental design of this study. The anti-proliferative activity of ZER was further confirmed by the immunohistochemical staining of PCNA, proliferation marker and its quantitative index, which showed statistical significant ($P < 0.05$) difference between cancer group and ZER-treated group. Up-regulation of PCNA is closely associated with the progression of CIN (Branca et al., 2007).

Apoptosis is characterized by the generation of DNA fragments through the action of endogenous endonucleases. The DNA of apoptotic cell is cleaved into multimers of 180–200 bp fragments (Bhalla, 2003). The TUNEL assay measures fragmented DNA by catalytically incorporating fluorescein-12-dUTP at $3^{\prime}$OH DNA ends using enzyme Terminal Deoxynucleotidyl Transferase (TdT). When the cells undergo apoptosis, fluorescein-12-dUTP will exert yellow to green fluorescence. Viable cells meanwhile exert the red background staining of propidium iodide. In this present study, apoptosis has been quantified by counting fluorescein positive cells (bright green). Our data indicated a significant difference in the mean percentage of apoptosis that was noted between normal saline-treated group and all other treatment groups of the cancer mice. No fluorescein positive cells were found in normal saline-treated cervical cancer tissue, confirming the growth of dysplastic cells (abnormal cells). In contrast, cervical cancer tissues treated with 8 mg/kg ZER, 16 mg/kg ZER and 10 mg/kg cisplatin induced significant apoptosis.

The present data showed that ZER is involved in modulating the level of Bax, a pro-apoptotic factor. The susceptibility of tumor cells to the induction of apoptosis by chemotherapeutic agents is controlled by the ratio of Bcl-2/Bax proteins in the mitochondria (Daniela et al., 2003). When cells received death signals, Bax moves to the mitochondria and generates a catastrophic transformation of mitochondrial function which includes release of cytochrome $c$ to the

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Induced CIN with DES</th>
<th>Remarks</th>
<th>CIN Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Mice</td>
<td>No</td>
<td>Vehicle Control</td>
<td>Normal</td>
</tr>
<tr>
<td>DES Induced</td>
<td>Yes</td>
<td>CIN Control</td>
<td>CIN3</td>
</tr>
<tr>
<td>Normal Saline + DES</td>
<td>Yes</td>
<td>Negative Control</td>
<td>CIN3</td>
</tr>
<tr>
<td>CIS + DES</td>
<td>Yes</td>
<td>10 mg/kg of ZER*</td>
<td>CIN1</td>
</tr>
<tr>
<td>ZER 1 + DES</td>
<td>Yes</td>
<td>4 mg/kg of ZER</td>
<td>CIN3</td>
</tr>
<tr>
<td>ZER 2 + DES</td>
<td>Yes</td>
<td>8 mg/kg of ZER</td>
<td>CIN2</td>
</tr>
<tr>
<td>ZER 3 + DES</td>
<td>Yes</td>
<td>16 mg/kg of ZER*</td>
<td>CIN1</td>
</tr>
</tbody>
</table>

*Statistical significant association.

<table>
<thead>
<tr>
<th>Group*</th>
<th>Treatment</th>
<th>PCNA Labeling Index (Mean $\pm$ SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal Control</td>
<td>$5 \pm 2.20$a</td>
</tr>
<tr>
<td>2</td>
<td>ZER 4 mg/kg B.W.</td>
<td>$45 \pm 13.6c$</td>
</tr>
<tr>
<td>3</td>
<td>ZER 8 mg/kg B.W.</td>
<td>$23 \pm 7.31b$</td>
</tr>
<tr>
<td>4</td>
<td>ZER 16 mg/kg B.W.</td>
<td>$7 \pm 3.32a$</td>
</tr>
<tr>
<td>5</td>
<td>CIS 10 mg/kg B.W.</td>
<td>$6 \pm 3.3a$</td>
</tr>
<tr>
<td>6</td>
<td>Normal Saline + Cancer</td>
<td>$60 \pm 2.3c$</td>
</tr>
</tbody>
</table>

*Groups with different letters (a–d) are statistically significant different at the probability level of 0.05. Post-hoc multi-comparison analysis was performed. ANOVA statistical technique was used to analyze that difference between groups at 0.05 as level of significance.
neighboring cytosol, loss of transmembrane potential and induction of mitochondrial permeability transition events that resulted in apoptotic cells (Zou et al., 1997; Liu et al., 2004). From data obtained, treatment of CIN mice with ZER elicits the up-regulation of Bax expression. Therefore, the increase of Bax protein in CIN tissues seems to contribute to its apoptotic effect. Previous data has mentioned that ZER was able to increase the level of Bax in HepG2 cancer cells (Sakinah et al., 2007). In correlation to our findings, ZER was also found to suppress colonic tumor marker formation in rats (Tanaka et al., 2001), which induces apoptosis in colon cancer cell lines (Murakami et al., 2003) and shows anti-tumor initiating and promoting activities in mouse skin (Murakami et al., 2004).

**Fig. 2.** Representative photographs from the cervix showing effect of ZER on immunoreactivity of PCNA. (A) Normal control mice cervix. (B) DES+ZER (4 mg/kg)-treated mice cervix. (C) DES+ZER (8 mg/kg)-treated mice cervix. (D) DES+ZER (16 mg/kg)-treated mice cervix. (E) Cisplatin treated cervix (10 mg/kg). (F) DES treated cervix. (G) cervical cancer tissue mice stained with ARK immunohistochemical kit but without PCNA primary antibody. PCNA immunoreactivity was not detected in the nuclei.

**Fig. 3.** *In situ* TdT-mediated dUTP nick end labelling of mice cervical tissue (A) Normal mice cervical tissues. Exhibits less apoptotic cells (pink arrows). (B) Cancerous mice treated with normal saline. Cells proliferate aggressively without any presence of apoptosis. (C) DES+zerumbone (16 mg/kg). More apoptotic cells (pink arrows) were noticeable in the cervix tissue. (D) DES+zerumbone (8 mg/kg). Increased apoptotic cells were noticed with distinctive nuclear fragmentation (yellow arrows). (E) Treatment of cisplatin at 10 mg/kg dosage. Increased apoptotic cells were noticed (pink arrows) with nuclear fragmentations (yellow arrows) (magnification: 20×). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

**Fig. 4.** The mean percentage of apoptotic cells (±SD) in cervical tissue sectioning of female Balb/c mice induced with cervical cancer after treatments with ZER, cisplatin and normal saline. The findings showed that there was a significant mean difference between the treatments groups with \( P<0.01 \) (analyzed using post hoc comparison test—one-way ANOVA).
Laser capture microdissection microscopy is a technique for isolating pure cell populations from a heterogeneous tissue section. This technique is applicable to molecular profiling of diseased and disease-free tissue, permitting correlation of cellular molecular signatures with specific cell populations (Yin et al., 2007). The principle components of LCMM technology are (i) visualization of the cells of interest via microscopy, (ii) transfer of laser energy to a thermolabile polymer with formation of a polymer-cell composite and (iii) removal of the cells of interest from the heterogeneous tissue section. In this study, LCMM was utilized to extract cell from cervical tissues of CIN and normal female Balb/c mice. LCMM was used previously as a tool in the extraction of cells from cervical tissues and from paraffin-embedded tissues (Patel et al., 2008). In this study, the Bcl-2 gene bands were not equally appeared in the cancer and normal control mice groups. The current findings therefore suggested that injections of ZER in cancerous female Balb/c mice decreased the expression of this gene which consequently is responsible for the initiation of mitochondrial caused apoptosis. These results are similar with earlier findings that demonstrated apoptosis is induced through the modulation of the Bax/Bcl-2 ratio. Previous report has suggested that the alpha,beta-unsaturated carbonyl group in ZER may play some pivotal roles in the anticancer properties of this natural compound (Murakami et al., 2003).

The current study demonstrates that apoptosis is the main cause of zerumbone’s anti-proliferative properties, as evidenced by TUNEL assay and immunohistochemistry. Moreover, it also strongly suggested the ability of this natural compound to modulate Bax, the mitochondrial protein and the expression Bcl-2 gene. Even though cisplatin has often been used as the preferential drug for treating cervical cancer, the side effects afforded by cisplatin are extremely discouraging. Since zerumbone, a plant-derived compound is able to induce apoptosis and inhibit the progression of CIN; this compound could possibly be developed into a new chemotherapeutic drug for treating cervical cancer in future.

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