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Original Research Article

Zerumbone (ZER), a Potential Anticancer for Breast Mediates Cancer Cell Death Through Targeting β-catenin Signaling Pathway in Tumor Regression in Sprague Dawley Rat Mammary Gland Tumors

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Abstract

Background & Objective: Canonical WNT/Wingless pathway regulates expression of target genes by modulating intracellular β-catenin leading to cancer growth and survival. Here, we studied inhibition of β-catenin using zerumbone (ZER) or β-catenin-siRNA in Sprague-Dawley rat cancer model. ***Methodology:*** Rat mammary gland tumor model was induced with the wild-type LA7 breast cancer cell line or β-catenin-knockdown-LA7 cells. LA7-rats were treated with ZER or β-catenin-siRNA as a positive control and the levels of β-catenin expression and its target gene were assessed using real-time PCR, immunohistochemistry and TUNEL assay. ***Results:*** We found good correlation between β-catenin inhibition and induction of apoptosis when using ZER or β-catenin-siRNA. In this aspect, ZER and β-catenin-siRNA inhibited cellular proliferation as reflected by reduced growth of breast cancer and apoptosis induction in rat mammary gland tumor. Further, our studies demonstrate that treatment with ZER and β-catenin-siRNA affected β-catenin-dependent gene expression. β-catenin downregulation by ZER or knockdown by β-catenin-siRNA improves the morphological and histological feature of the breast tissues as confirmed by subtle changes of the genes involved in cell-apoptotic pathways. ***Conclusion:*** This indicates that ZER targets similarly to β-catenin-siRNA and possibly be a useful anti-breast cancer by affecting expression of down-stream targets that are key components in cancer development. ZER can be a promising anticancer candidate for treatment of breast.

Keywords: Zerumbone, siRNA, β- catenin, apoptosis, downregulation, knockdown.

1. INTRODUCTION

Cytoplasmic β-catenin initially known as a membrane-associated protein, binds to the carboxyl terminus of E-cadherin at plasma membrane, later recruits α-catenin and other structural proteins to form cell-cell junctions [1]. β-catenin also acts as a transcription coactivator, a key component of downstream signaling in the Wnt/ Wingless pathway, which is implicated in the embryonic development [2]. Non-phosphorylated β-catenin enters the nucleus and associates with the T-cell factor and lymphoid enhancer factor (TCF/LEF-1) family of transcription factors, in turn, activates the transcription of β-catenin target genes of cell growth and proliferation regulators, cell death pathways modulators and cell–cell communication [3, 4]. This pathway is involved in disease development when dis-regulation of β-catenin occurs. Therefore, inappropriate activation of the Wnt/ β-catenin pathway might activate certain genes that ultimately establish the oncogenic phenotype. In this respect, the current study focuses on the dis-regulation of β-catenin and associated proteins in breast cancer, rectifying this by the use of β-catenin siRNA or zerumbone (ZER), a natural compound of medical herbal plant, which were found to delay progression of breast cancer through apoptosis induction via up-regulating Bax and down-regulated Bcl2 [5, 6]. This approach to use ZER or β-catenin siRNA to possibly regress breast cancer progression is currently investigated *in vivo*.

The advent of RNA interference (RNAi)-directed knockdown has revolutionized somatic cell genetics, allowing inexpensive, rapid analysis of gene function in mammals [7]. The role of β-catenin in maintaining malignant breast cancer phenotype has not been studied, though its role in regulating cellular function was investigated in other cancers [8, 9]. Thus, the current study investigates if reducing levels of β-catenin protein in induced rat breast cancer may possibly affect β-catenin apoptosis related genes and consequently decreased *in vivo* cancer cell proliferation. The study also investigates if the use of β-catenin siRNAs introduced into breast cancer cells by transfection binds specifically to cellular mRNA of interest that activates RNA degradation, which leads to a substantial decrease of the corresponding protein levels [8]. In addition, ZER was investigated as a useable inhibitor of breast cancer growth. Several studies have shown that ZER can delay the progression of tumor cells, including breast cancer, by up-regulating Bax and down-regulating Bcl2 [5, 6]. We investigate the role of blocking β-catenin by ZER or β-catenin siRNA on cancer development, either by direct injection into rat breast or by silencing the β-catenin gene following transfection of β-catenin siRNA into rat breast cancer cell. Our results provided evidence that silencing β-catenin by ZER or β-catenin siRNA restricts tumor growth, which generates histological and morphological features atypical of apoptosis through elevation or down-regulation of genes related-cell death. This pointed to a direct linked between β-catenin and apoptosis pathway, which suggests that ZER have near similar therapeutic affect to β-catenin siRNA in the treatment of breast cancer through the suppression of Wnt/β-catenin target genes and pathway. This is the first documented evidence of ZER targeting β-catenin to induce apoptosis in breast cancer. Thus, this study strongly recommended the use of ZER, a natural plant metabolite as an ideal novel alternative treatment for breast cancer by being able to induce cell death in cancer cells.

2. MATERIALS AND METHODS

2.1. Cell culture, preparation, and induction of cancer cells

LA7 rat mammary gland tumor cells were purchased from ATCC (VA, USA). The cells were grown in Dulbecco's Modified Eagle's medium (DMEM) at 37°C in a humidified atmosphere of 5% CO2 supplemented with 10% fetal bovine serum (FBS), 100 µg/ml streptomycin and 100 IU/ml penicillin. For LA7 transfection, β- catenin double-stranded siRNAs (sense sequence AUUUAAAGAUGGCCAACAAGCCCUCUU and antisense sequence GAGGGCUUGUUGGCCAUCUUUAAAUUU), and Cylophilin B siRNA (positive control) sense sequence GCAUGGAUGUGGUACGGAAUU and antisense UUCCGUACCACAUCCAUGCUU were designed and synthesized by Dharmacon Research Inc., (Singapore, C). These siRNA were dissolved in PBS and then aliquot and stored at –20°C. LA-7 cells were plated in 75 ml flask in media containing 10% fetal bovine serum to 30–50% confluence. Transfection of the β-catenin siRNA oligonucleotides was performed using Dharmcon transfection reagent 1 in a final β-catenin siRNA concentration of 100 nM for 24h. When non-transfected cells reached 90% confluence or 24 h after transfection, the medium was removed and cells were washed with phosphate buffered saline (PBS). The cells were trypsinized and collected immediately by centrifuging at 100 g for 10 min at 4°C, washed twice with PBS, resuspended in 300 µl serum-free medium and counted using a hemocytometer. All harvested cells were used within one hour of preparation to induce human breast model in rat.

2.2. Animal Treatment

All study protocols and experimental design were approved and conducted according to the guidelines for The Institutional Animal Care and Use Committee (IAUC), Faculty of Veterinary Medicine, University of Putra, Malaysia (UPM). Six to eight weeks old female Sprague-Dawley (SD) rats, weighing 180-200 g were purchased from Takrif Bistari Enterprise, Malaysia. Rats were housed in plastic cage and allowed to acclimate to standard conditions (12-hour light/dark cycle) for one week. During treatment period, rat had free access to food and water and their weight were controlled weekly. After one-week acclimation period, rats were anesthetized using an intraperitoneal injection with ketamine-HCl (75 mg/Kg body weight). Then three hundred microliter of 6×10^6 cells LA7 or β-catenin knockdown LA7 cells were inoculated subcutaneously into the mammary fat pad (right flank) of each rat using syringe and 26-Gauge needle. This cell line was used as an animal model for human breast carcinoma because they are similar both in hormone sensitivity and the histopathology (Jordan 1993). Treatment dose consisted of subcutaneous injections of 20 pmol β-catenin siRNAs or cyclophilin B siRNA three times weekly into the right mammary fat pad. Intraperitoneal (i.p.) injection of ZER 100 mg/kg body weight/3 times weekly [10] and 25mg/kg b.w tamoxifen (Sigma) administered three time per week starting 24 h after injection of the LA7 cells for one month. ZER and tamoxifen were dissolved in sesame oil.

The animals were randomly divided into treatment groups, with 4 animals/group. In the first experiment, 20 rats were randomly and equally divided into four treatment group. Animals in treatment group 1 were induced by Knock-down LA7 cells transfected with β-catenin siRNA that specifically targeted β-catenin sequence. Treatment group 2 were induced with LA7 cells that were transfected with cyclophline B siRNA as positive control with a non-specifically targeted sequence to β-catenin. Treatment group 3 were induced with LA7 (non-transfected LA7) to develop mammary gland tumor (tumor control). Treatment

group 4 were not induced and served as normal controls. Treatment group 5 were induced with LA7 (non-transfected LA7) to develop mammary gland tumor and treated with ZER. Each treatment group was consisting of four rats (n= 4).

In the second experiment, 36 rats were injected with LA7 cells to develop mammary gland tumor. These rats were randomly divided into three groups of 12 rats each. Group one was treated with β-catenin siRNA direct against β-catenin, group 2 was treated with siRNA directed against cyclophline B (positive siRNA), group 3 served as a tumor control, group 4 was treated with ZER, and group five serve as normal control. Each treatment group consists of four rats. Treatment protocol and doses were administered as mentioned above. Rats were carefully observed for morbidity and mortality throughout the experiment. Tumor size was determined at weekly intervals, and tumor volumes were calculated using width (*x*) and length (*y*) (*x*2*y* / 2) [11].

At the end of the treatment period, rats were sacrificed by heart punch under anesthesia, the blood sample was taken, and the entire breast was removed. The breast was washed and divided into five parts of equal size. Tissues were fixed in 10% buffered formalin for histopathology, immunohistochemical analysis or tunnel assay. The remaining breast tissues were snap frozen in liquid nitrogen and stored at -70 ºC for RNA extraction.

2.3. Histopathology

Tissues fixed in 10% formalin were embedded in paraffin and stained with hematoxylin and eosin (H&E). Tissue sections were examined under the microscope by the histopathologist blinded to the treatment, based on nuclear polymorphism, tubule formation and, mitotic score following the described method (Bloom and Richardson 1957). Five random sections from each breast tissue sample were examined. Cell distribution in each slide was scored and cellular morphological changes were classified and categorized using Nottingham Grading System [12]. Mean tumor scoring was calculated using SPSS software version 16.0 (IBM SPSS Inc., Chicago, USA).

2.4. Transmission Electron Microscope

For transmission electron microscope tissue were cut into 1mm3 and fixed in glutaraldehyde overnight at 4°C. The tissues were washed within three different changes of sodium coccadylate buffer (pH 7.4) for 10 minutes each and fixed in 1% osmium tetraoxide at 4°C for 2h. The sample was subsequent to three time washing in sodium coccadylate buffer (pH 7.4) for 10 minutes and dehydrated with a gradient series of acetone as follow 35%, 50%, 75%, 95%, and 100%. The sample were then infiltrate with acetone and resin and embedded in beam capsule with 100% resin, and polymerize for 48 h at 60°C. After staining with toluidine blue, 1 µm tissue sample was examined under light microscope in order to select the area of interest. The selected part was cut with ultramicrotome, placed into a grid, dried and stained with uranyl acetate for 15 minutes then washed with distilled water, followed by lead stain [13]. The sample was then washed twice with distilled water. The samples were viewed under transmission electron microscopy (Phillips, Eindhoven, Netherlands).

2.5. Malondialdehyde assay

Malondialdehyde (MDA) concentration in the serum was used as an indicator of lipid peroxidation and oxidative stress. The MDA concentrations were determined according to the standard method [14]. Briefly, three hundred milliliters of sera was mixed with 1/12 H_2SO_4 and 10% Sodium tungstate, and centrifuged at 3,500 rpm for 10 min. The supernatant was discarded and 0.05 M HCl, 37% HCL plus 1% triobarbit uric acid were added and incubated in a water-bath (95 ºC) for 60 min. The tubes were then centrifuged and the supernatants were obtained to determine MDA concentrations spectrophotometrically at 532 nm. The protein concentrations of the supernatants were estimated using the Lowry method [14]. Results were expressed as MDA nmol/mg protein (n = 3).

2.6. Immunohistochemistry

Tumor samples fixed in 10% neutral buffered formalin were paraffin-embedded and sectioned 4 µm for immunohistochemical analysis. The primary rabbit polylonal antibody against β- catenin, p53, and parp1 (Santa Cruz Biotechnology, Santa Cruz, CA) and MDM2 (Abcam), was applied at concentrations of 1:100 for 1 hr at room temp. Negative control sections were treated in an identical manner, with the exclusion of the primary antibody. Immunostaining was performed using Anti-Rabbit IgG Detection System-HRP kit (Bethyl Laboratories, Inc, Montomery, TX) at room temperature for 1 h according to the manufacturer's instructions. Peroxidase activities were detected after incubating the samples with 3,3′-diaminobenzidine (DAB) by the appearance of a brown precipitate that is insoluble in alcohol. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted using Permount mounting medium (Fisher Scientific). All tumor specimens were stained simultaneously to avoid interassay variation. β-catenin immunostaining was assessed by two independent observers in a blinded manner [15].

2.7. Tunnel assay

Fluorometric TUNEL assay measures nuclear DNA fragmentation, a hall mark of apoptotic cells by incorporating flourescein-12-dUTP(a) at 3`-OH DNA ends using the terminal recombinant enzyme deoxynucleotidyl transferase (rTdT). This assay was performed in situ using Dead End TM Fluorometric TUNEL system, according to the manufacturer's instructions (Promega Inc., Madison, USA). The breast

tissue section were deparaffinized in xylene and rehydrated in a gradient of descending alcohol concentrations, washed in 0.85% NaCl, immersed in PBS for 5min and fixed in 4% methanol-free formaldehyde [16]. The tissue sections were incubated in hundred microliters of proteinase K (20µg/mL) for 8–10 min at room temperature, washing in PBS for 5 min and incubated with 50µL of fresh rTdT buffer at 37 ◦C for I hour in the dark to allow tailing reaction to occur. This reaction was terminated by immersing the slides in saline-sodium citrate solution for 15 min at room temperature. The slides were washed twice with PBS to remove unincorporated flourescein-12-dUTP and stained with propidium iodide solution (1µg/mL in PBS) for 15 min in the dark, washing in deionized water for 5 min three times, and Anti-Fade solution was added to the treated sections and the slides mounted using glass cover slips. The samples were analyzed under a confocal microscope (ZIESS, LSM 70) using standard fluorescein filters.

2.8. Real time PCR

Massinger RNA expression was analyzed using CFX96 real time PCR detection system (Bio-Rad, USA) with SYBR Green as described by manufacture. Total RNA was isolated from tissue using RNeasy Lipid Tissue Mini kit (QIA gene, Inc., Valencia, CA). Total RNA (10 µg) was used for reverse transcription and real time PCR using Sensi MixTM SYBR One-Step kits, according to the manufacturer's protocol in a total volume of 50 µl using standard Real time PCR conditions as described above [17]. All primer allowed to anneal at 60ºC. Primer sequences used in this assay are listed in Table 1.

Table 1: Primer sequences used in real-time RT-PCR assay to validate several proteins expressions in rat mammary gland tumors treated with ZER or SiRNA

Gene name	PCR primer sequences
β-catenin	Forward 5`-GCTTGTTGGCCATCTTTAAATC-3` Reverse 5`-GATTATGCAGCGTGGTGATG-3`
P53	Forward 5`-GTGGATCCTGAAGACTGGATAACTGTC-3` Reverse 5`-AGTCGACAGGATGCAGAGGCTG-3`
Mdm2	Forward 5`-CGGCCTAAAAATGGTTGCAT-3` Reverse 5`-TTTGCACACGTGAAACATGACA-3`
Bid	Forward 5`-CCCACACTGGTGAGACAACT-3` Reverse 5`-TGTCGTTCTCCATGTCCCTA-3`
P21	Forward 5`-TGGACAGTGAGCAGTTGAGC-3 Reverse 5`-ACACGCTCCCAGACGTAGTT-3

2.9. Statistical Analysis

All descriptive and inferential statistical analyses were done using SPSS version 16.0 (IBM SPSS Inc., Chicago, USA). The Data were expressed as mean±SD. Analysis of data was performed using student T test. $P<0.05$ was considered statistically significant.

3. RESULTS

3.1. β-Catenin and ZER inhibit tumor formation in rat induced breast cancer tumors

Induction of breast tumors in rats started three days following LA7 cells injection as recognized by early palpation of the mammary gland [18], visible 6-8 days post injection. Majority of the tumors were soft and rubbery; moreover, tumors becoming irregular, lobulated, and more adhesive to the skin than to the body wall as they grew. There was no reduction on body weight of rats treated with ZER or β-catenin siRNA. The tumor volumes in non-treated control group increase rapidly, reaching an average volume of 2500±512 mm^3 at the end of treatment period. Our results demonstrated that all treatment groups had a significant ($P <0.05$) reduction in tumor volume when compared with untreated controls (Table 2). LA7 tumor bearing rat treated with β-catenin siRNA or ZER showed reduction of more than 99% and 96% tumor size, respectively. This data strongly implicates β-catenin in tumor formation and the significant of ZER as anticancer near like the effect of β-catenin SiRNA in regressing rat mammary gland tumor progression (Table 2).

Table 2: Effects of ZER and β-catenin siRNA treatments in induced LA-7 mammary gland tumor of *Sprague Dawley* rat groups in respect to tumor size (mm^3). Values are presented as mean ± SD of four rats in each group. Data showed significant difference between treatment and non-treatment LA-7 mammary gland tumor rat

groups, specifically during the 21st and 28th day treatment. Small letters (a,b) signifies a. significant increase and b. significant reduction in tumor size between treated and non-treated rats (P < 0.05)

Treatment groups (n=4)	Treatment (Days)			
	7	14	21	28
	Tumor size (mm^3)			
Non-treated tumor rats	401± 150	860 ± 520	1620a ± 520	2500a ± 512
LA-7 tumor rats treated with 100mg/kg ZER	200 ± 52	175 ± 10	22.5b ± 5.0	2.5 b ± 0.7
LA-7 tumor rats treated with siRNA	302 ± 25	180 ± 82	18b ± 2	2.3b ± 0.1
Non-treated transfected LA-7 tumor rats	150 ± 50	250 ± 120	6 b ± 2	3.0 b ± 0.03

3.2. β-Catenin and ZER treatment of mammary tumors restore normal mammary gland histology

LA7-induced mammary gland tumors in female *Sprague Dawley* rats are known to be invasive adenocarcinoma subtype with no tubular structures (Score 3). In this type of tumors, cells were characterized by hyperchromatic nuclei displaying marked variation in size and shape (Score 3), high mitotic activity (Score 2), and necrosis consistent with adenocarcinomas (Fig. 1B and Table 3). Our results showed that treatment of the disease animals with β-catenin siRNA or ZER reversed tumor growth and enhanced normal breast tissue structures (Fig. 1C-E) with few neoplastic cells [19]. Moreover β-catenin siRNA or ZER treatment rats showed a clear decreased in mitotic events number compare to tumors in untreated rats or rats treated with positive siRNA control (cyclophilin B, Fig. 1F). Breast lesion scores in the cancerous rats treated with ZER or β-catenin siRNA were significantly lower than non-treated control tumor rats (P < 0.05). Normal rats treated with ZER showed normal breast morphology (Fig. 1A). The results further showed treated groups had improved mammary gland structures compare to untreated control rats (Nottingham Grading System for breast tissue; Table 3).

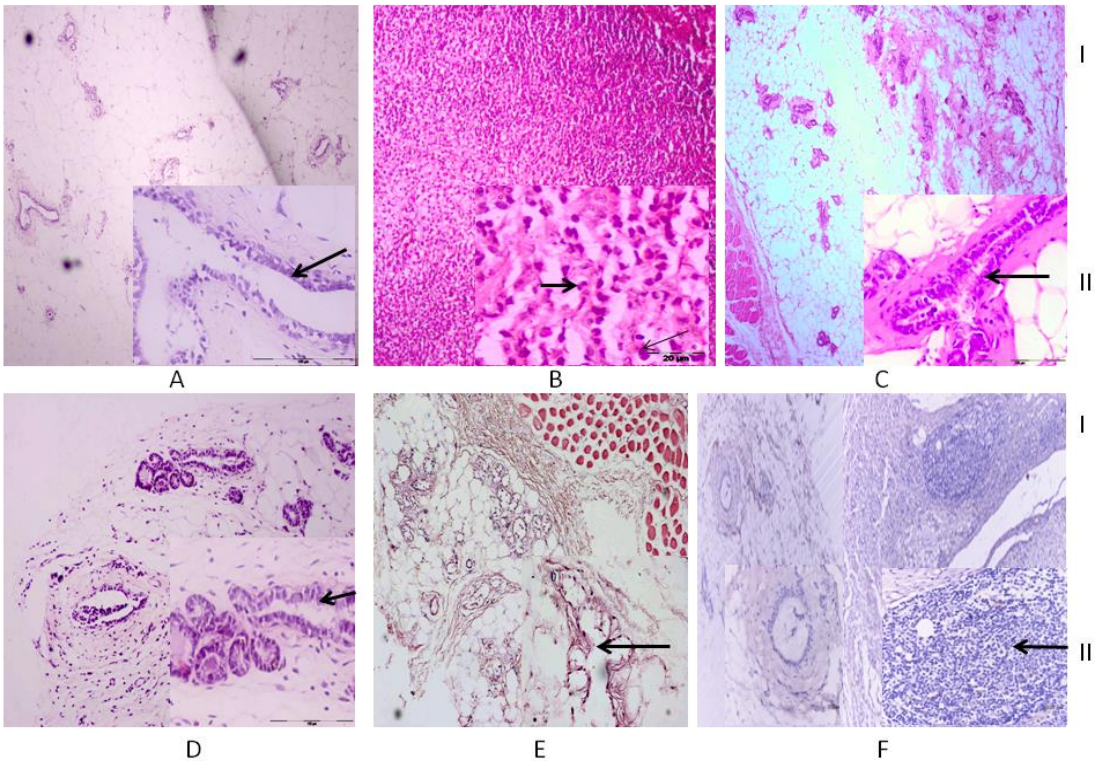

Figure 1: Histopathology of rat mammary gland tumor treated with ZER or β-catenin siRNA. (A) Normal untreated rat breast showing normal cellular structure. (B) Untreated breast tumor (positive control) showing hyperchromatic nuclei, indicating high mitotic, with high variation in shape and size. (C) Rat tumor treated with ZER, (D) Rat tumor induced with β- catenin siRNA transfected LA7 cells show near-normal structure and (E) Rat mammary gland tumor injected locally with β-catenin siRNA showing highly improved structure with tubular pattern, and absence of mitotic activity near to normal structure compare to (F) Mammary gland tumor treated locally with cyclophine B siRNA (positive siRNA) and untreated rats. Micrographs magnification 10x. Inserts magnification 50x.

Table 3: Histopathological Scoring for rats Mammary Gland Breast Tissues using Nottingham Grading System (P < 0.05). The histological sections from the first thoracic mammary gland were scored on a 4-point scale ranging from 0 (normal) to 4 (severe changes). Histopathological changes were further ranked according to

distribution of changes (0.25 focal), 0.5 (locally diffuse) and 0.75 (diffuses)

Treatment Groups	Nuclear polymorphism	Tubule formation	Mitotic count	Grading
Non-treated tumor rats	3 point (Score 3)	No (Less than 10%) Score 3	10 point (Score 2)	Score 8 Grade 3 (poorly-differentiated)
Non-tumor rats	Normal	Normal	Normal	Normal
LA-7 tumor rats treated with 100mg/kg ZER	1 point (Score 1)	Yes (more than 75%) Score 1	0 point (Score1)	Score 3 Grade 1 (well-differentiated)
Non-treated transfected LA-7 tumor rats	2 point (Score 2)	Yes (more than 75%) Score 1	0 point (Score1)	Score 4 Grade 1 (well-differentiated)
LA-7 tumor rats treated with siRNA	1 point (Score 2)	Yes (more than 75%) Score 1	0 point (Score1)	Score 3 Grade 1 (well-differentiated)
LA-7 tumor rats treated with Cyclophline B siRNA	3 point (Score 3)	Yes (10 to 75%) Score 2	10 points (score2)	Score 7 Grade 2 (moderately-differentiated)

3.3. Treatment with ZER and β-Catenin induce apoptosis in mammary tumor cells

LA7 induced rats treated with ZER or transfected LA7 induced rats (non-treated) showed higher numbers of apoptotic tumor cells compared to untreated tumor control rats (Fig.2.1.C and 2.1.D, Fig 2.2). Tumor-negative rats (normal) showed very few or no apoptosis (Fig. 2.1.B, Fig 2.2). Our results of injected β-catenin siRNA have similar rate of apoptosis to ZER treatment in induced tumor rats (Fig. 2.1.E, Fig. 2.2). LA7 induced tumor rats treated with positive control (cyclophlin B siRNA) induces apoptosis but at lesser rate of apoptosis (Fig. 2.I.F, Fig. 2.2) similarly to transfected LA7 induced tumor rats since apoptosis is initiated earlier in transfected LA7.

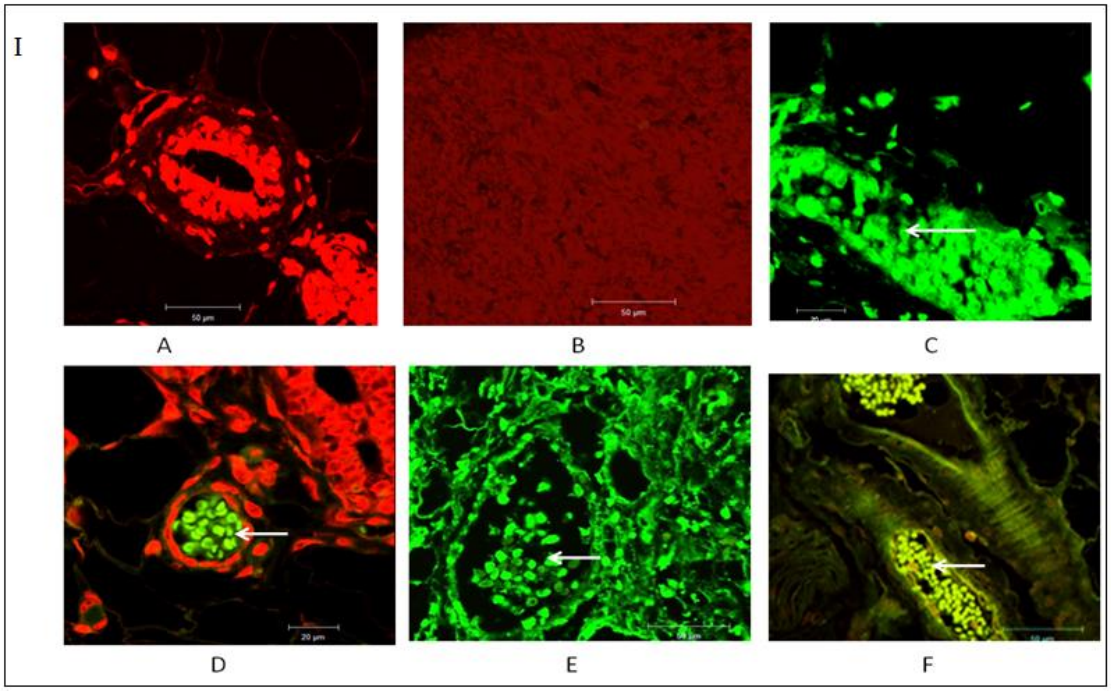

Figure 2(I): TdT-mediated dUTP nick-end labelling (TUNEL assay) for induced LA-7 mammary gland tumor rats. (A) Normal section showing absence of apoptotic cells. (B) TUNEL staining of untreated control LA-7 mammary gland tumor rats showing clear evidence of aggressive cell proliferation without apoptosis. (C) Induced LA-7 mammary gland tumor rats treated with 100 mg/kg body wt. zerumbone showing TUNEL-positive apoptotic cells. (D) Non-treated Transfected LA-7 mammary gland tumor rats showing TUNEL-positive apoptotic cells. (E) Induced LA-7 mammary gland tumor rats treated with 20 pmol β-catenin siRNA showing TUNEL-positive apoptotic cells. (F) Induced LA-7 mammary gland tumor rats treated with cyclophine B siRNA (positive siRNA) showing TUNEL-positive apoptotic cells. White arrow indicates positive apoptotic stain (High magnification 50X).

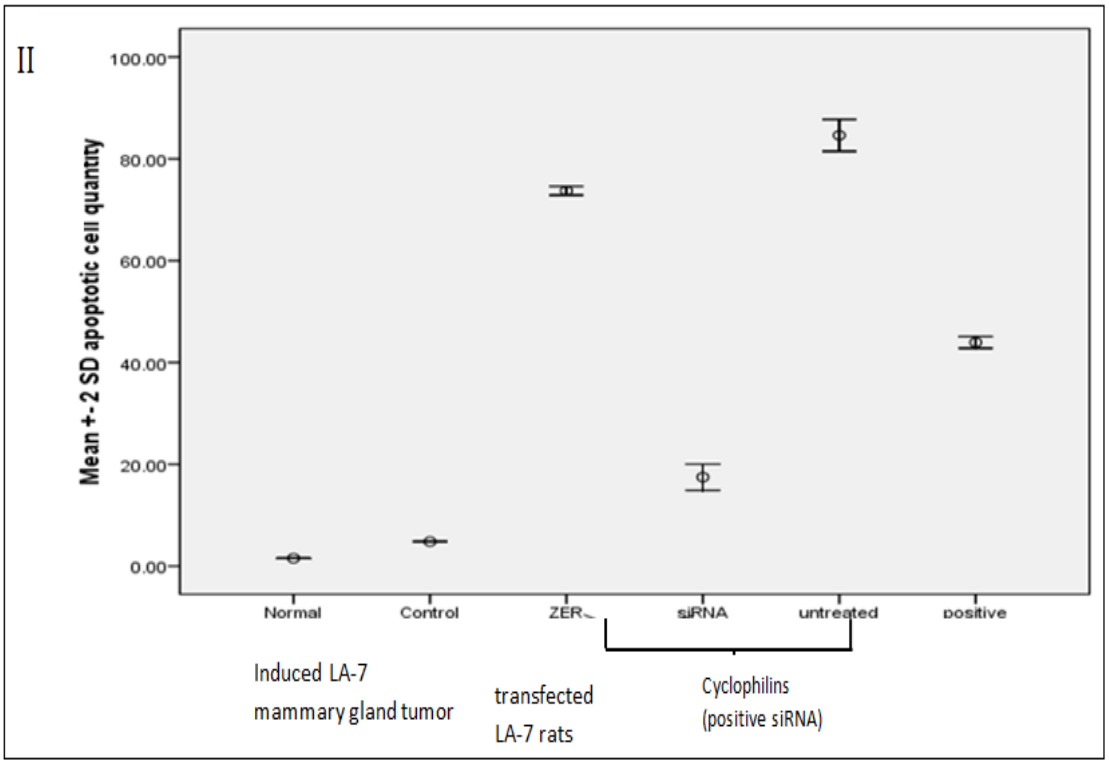

Figure 2(II): Histograph represent percentage cell death from TUNEL assay above. Data are significantly different between treatment groups and untreated control (P < 0.05)

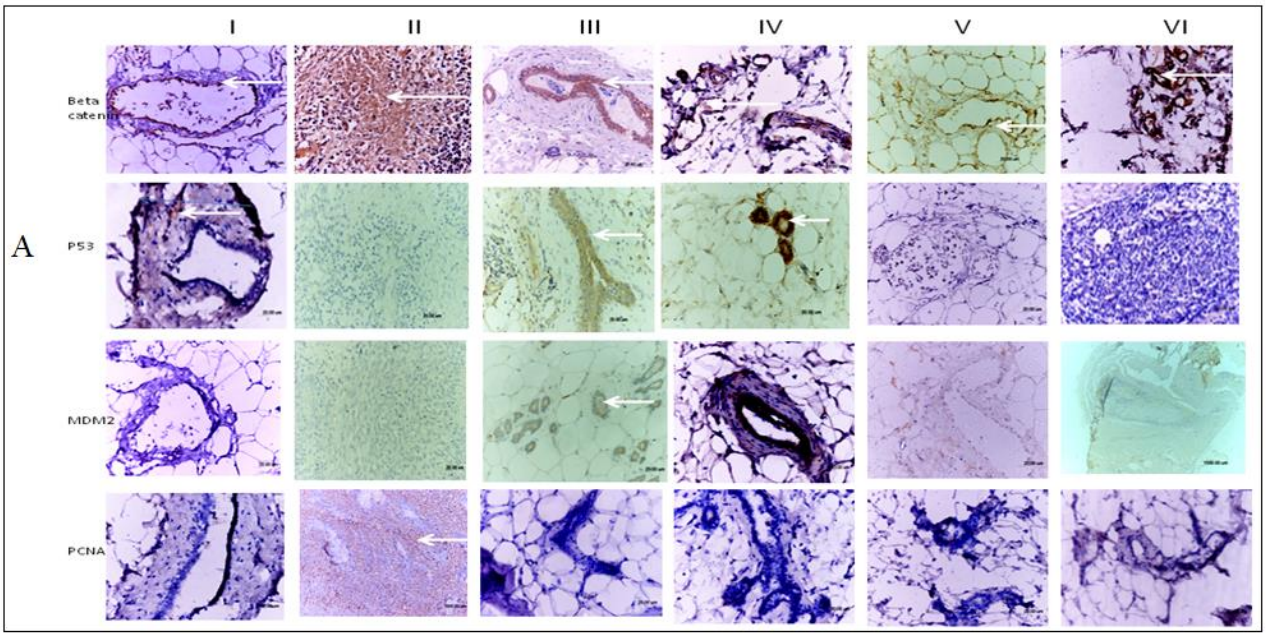

Figure 3(A): Immunohistochemistry analysis of non-treated and treated induced LA-7 mammary gland tumor rats. The panels above represent (I) Normal breast (II) Non-treated LA-7 mammary gland tumor rats (III) Induced LA-7 mammary gland tumor rats treated with ZER (100 mg/kg body wt.) treated rats (IV) Non-treated transfected LA-7 mammary gland tumor rats (V) Induced LA-7 mammary gland tumor rats treated with 20 pmol of β-catenin siRNA (VI) Induced LA-7 mammary gland tumor rats treated with 20 pmol of cyclophine B siRNA (positive siRNA). Levels of proteins expression for β-catenin, p53, MDM2 and PCNA were determined using immunohistochemistry analysis (all positive stain protein expressions indicated by white arrows). PCNA expression decreased in all normal control rats (non-tumor rats) and treatment groups compare to untreated groups of induced LA-7 mammary gland tumor rats. Induced LA-7 mammary gland tumor rats treated with ZER or siRNA showed lesser β-catenin and p53 positive stain than normal non-tumor rats. Induced LA-7 mammary gland tumor rats treated with cyclophine B siRNA showed elevation in β-catenin expression

3.4. ZER and β-catenin siRNA affect oncogene and apoptosis-genes expression in mammary gland tumor rats

The mRNA levels for β-catenin, p53, MDM2, p21 and Bid were determined using real time RT-PCR in rat mammary gland tissues treated with ZER or β-catenin siRNA. Results showed a significant reduction in expression of β-catenin in LA7 induced rat tumor treated with ZER or non-treated transfected-LA7 tumor (Fig. 3A). ZER treatment in LA7 induced tumor rats further showed decreased in p21 but increased in p53 expressions with slight elevation in MDM2 and Bid (Fig. 3A). The RT-PCR assay provided strong evidence of significant ($P < 0.05$) β-catenin reduction to both treatments in LA7 induced tumor rats compare to control (untreated LA7 induced tumor rats) and normal rats [20]. Normal rats had p53, MDM2, p21 and Bid expressions within normal limits (Fig. 3B). In addition, the results further showed increased in both p53 and p21 expression levels in LA7 induced tumor rat treated with β-catenin siRNA and untreated transfected LA7 tumor rats, contrary to the expression of both genes when treated with ZER (Fig. 3A). Meanwhile, the LA7 induced tumor rat injected with β-catenin siRNA showed increased expression in p21 (Fig. 3B). Both treatments are concurrently compared to positive control and normal rats.

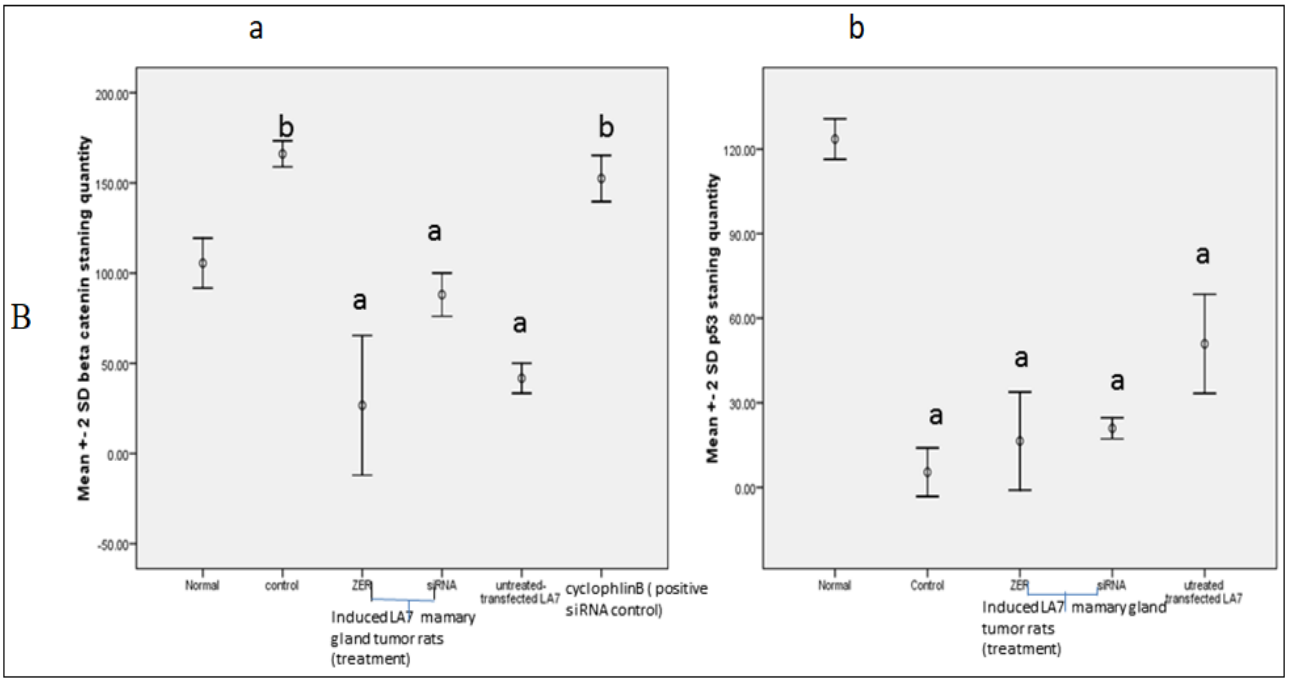

Figure 3(B): Histograph represents percentage of beta catenin and p53 positive stain in induced LA-7 mammary gland tumor rats (a) correlation of β-catenin to other protein expressions compared to ZER or SiRNA treatments (b) correlation of p53 compared to ZER or SiRNA treatments

3.5. ZER and β-catenin siRNA affect oncogenes and apoptosis genes/protein expression

Effect of ZER or β-catenin siRNA on protein expression of β-catenin, p53, MDM2, and PCNA proteins was examined by immunohistochemistry. β-catenin expression showed significant reduction in ZER or β-catenin siRNA treatment ($P < 0.05$, (Fig 4A.III-V and B.a). PCNA could not be detected in breast tissue of normal or treated rats compared to untreated control tumor (Fig. 4.A.II). Furthermore, there was slightly increased in p53 of ZER treatment rats when compare to untreated rats. Normal rats, showing positive β-catenin stain since β-catenin is express in normal growing cell while treated rats (ZER or siRNA) showing less positive stain than the normal rat. Rat with mammary gland tumor treated with cyclophine B siRNA showing increased in β-catenin expression (Fig. 4A.VI).

3.6. Zerumbone deplesion of β-catenin produced apoptotic morphological feature in rat

Transmission electron microscopy was used to study the apoptogenic effect of zerumbone or β-catenin siRNA on rat model. Rats treated with zerumbone or siRNA showed cell death consistent with apoptosis feature like cell shrinkage, cell membrane blebbing, increased cellular granularity, the formation of apoptotic bodies, chromatin condense. siRNA treatment showed apoptosis feature that involved cell shrinkage and chromatin condensation. Moreover, nuclear membrane dilatation and cell membrane blebbing were observed after zerumbone treatment (Fig. 5).

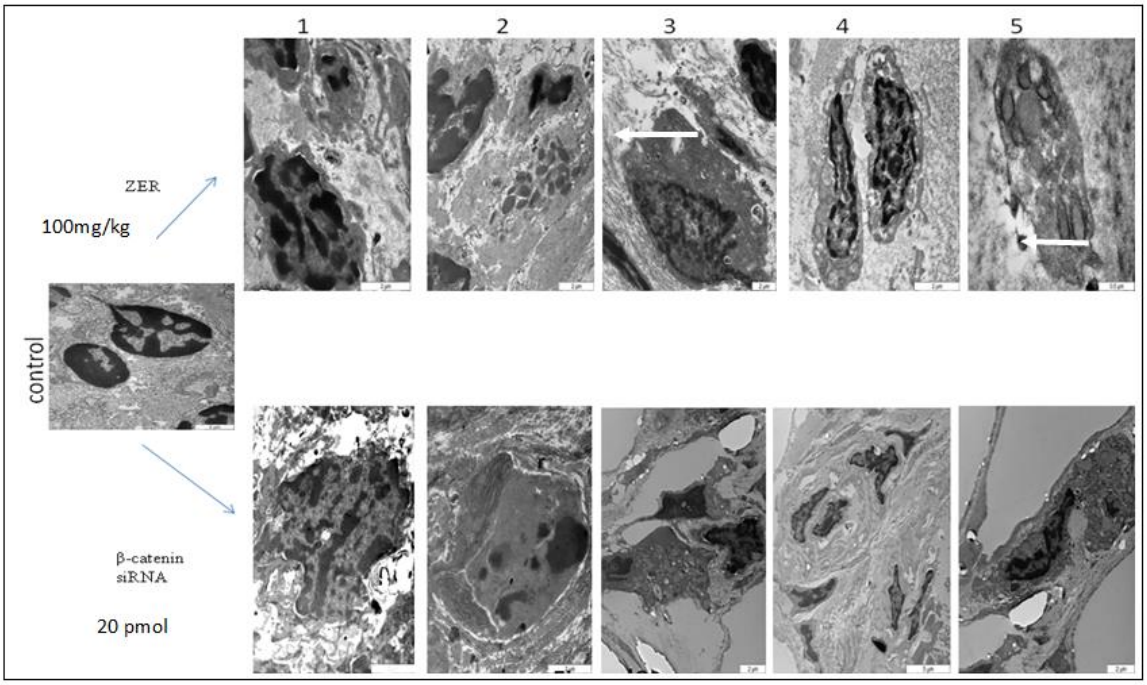

Figure 4: TEM micrographs illustrate apoptotic characteristic phenotypes of induced LA-7 mammary gland tumor rats treated with ZER or β-catenin siRNA. All treatment groups exhibited different stages of apoptosis processes indicated by white arrows (1) Treated LA-7 mammary gland tumor rats undergoing early phase of apoptosis with clear condensed and peripheralized chromatin, whilst cytoplasm is beginning to condense with cell outlines becoming irregular. (2) Clear evidence of late apoptosis showing cell disintegrating into numerous bodies containing mostly nuclear fragments. (3) Nuclear blebs. (4) Nuclear membrane shrinkage. (5) Formation of apoptotic bodies.

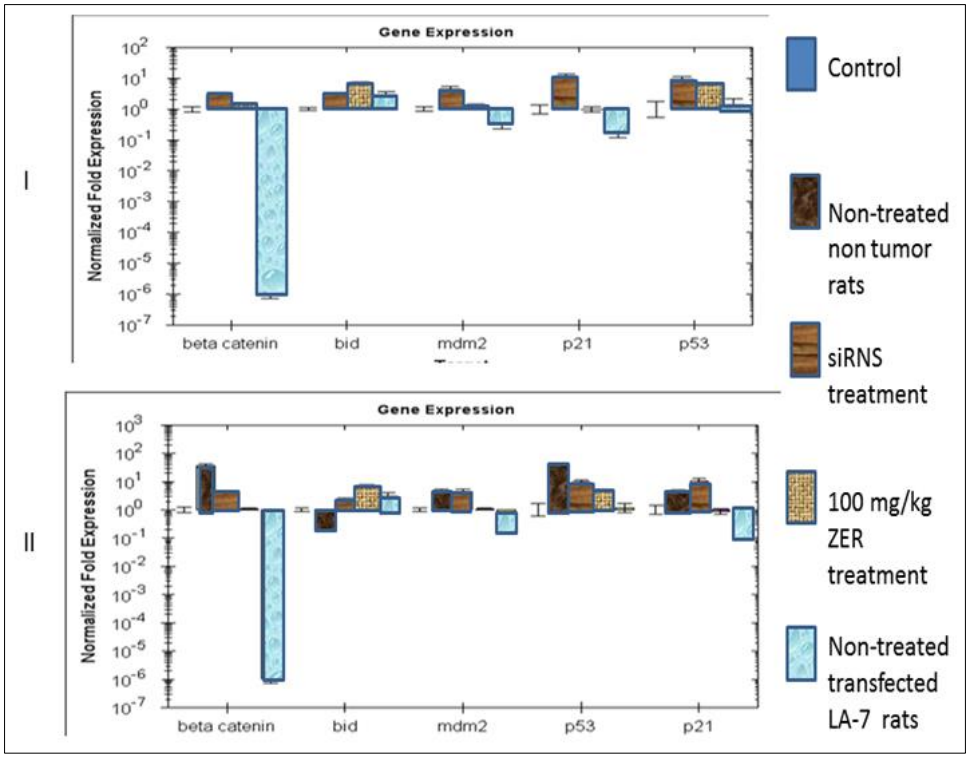

Figure 5(I): Histograph representing proteins expressions for β-catenin, p53, MDM2, Bid and p21 proteins in induced LA-7 mammary gland tumor rats of treated and non-treated rat groups. Levels of mRNA of induced LA-7 mammary gland tumor rats treated with ZER or β-catenin siRNA are analysed using real time PCR. Data is statistically significant showing differences between treatments as compared to non-treatment of induced LA-7 mammary gland tumor rat groups ($P < 0.05$); Figure 5(II): Histograph representing proteins expressions for β-catenin, p53, MDM2, Bid and p21 proteins in induced LA-7 mammary gland tumor rats of treated and non-treated rat groups. Data is statistically significant showing differences between treatment groups and untreated LA-7 mammary gland tumor rats ($P < 0.05$)

3.7. ZER treatment decreased the level of Serum Malondialdehyde (MDA)

Sera obtained from rats at the end of treatment period were used to determine MDA value. The MDA levels were significantly decreased in animals treated with ZER and β-catenin siRNA compare to untreated control tumor rats (Table 4). This data indicated that treatment with ZER and β-catenin siRNA decreased stress levels in rat breast model.

Table 4: Levels of malondialdehyde (MDA) serum of induced LA-7 mammary gland tumor rats treated with ZER and β-catenin siRNA. Values are presented as mean ± SD of four rats in each group. Treatment groups are significantly different compared to untreated group *(P < 0.05)

Treatment groups	MDA nmol/mg protein
Non-tumor rats	2.00 ± 1.02
Non-treated tumor rats	7.672* ± 2.20
LA-7 tumor rats treated with siRNA	0.376 ± 0.175
LA-7 tumor rats treated with 100mg/kg ZER	2.26±0.92
Non-treated Transfected LA-7 tumor rats	1.959±0.35
Positive control (tumor rats treated with CyclophlineB siRNA)	2.982±0.975
Non-tumor rats treated with ZER	2.98±0.950

4. DISCUSSION

An *in vivo* study could provide information parallels to that of a patient's requirements for treatment, in turn, would be useful in selecting the best modalities expected with high probability for a good outcome, increased efficacy and low toxicity. Thus, a typical post-treatment would include histopathological type, the grade of the tumor, the stage of the tumor, and expressions of related proteins and genes for assessment [21]. This has been included in our *in vivo* analysis to verify the outcome of using ZER and β-catenin siRNA for treatment.

Our *in vivo* study provided evidence that tumors in untreated control groups grew rapidly; compare to all treated groups including ZER, which show significant ($P < 0.05$) reduction in tumor size (Table 2). This reduction in tumor volume had strong correlations with the reduction of β-catenin cytoplasmic levels [22]. Moreover, immunohistochemistry expression for PCNA and p53 (Fig. 4), together with histopathological indices (Table 2) can be reliable markers for tumor grades or stages, and be useful for possible early detection and prognosis of breast cancer, prediction of recurrence time and hence, severity of the cancer pathology [23]. In this study PCNA, which is associated with the DNA proliferation and carcinogenesis [24]. was down regulated in treated group compare to untreated control group.

Histopathological examination detected adenocarcinomas being a feature of mammary gland tumor. As shown in Fig 3B, adenocarcinoma in the rats induced with malignant tumors is derived from the epithelium in glandular tissue, thus possibly providing the adenomatous appearance. The nucleoli are often polemorphic and mitotic figures being abundant. Necrosis is a common characteristic of adenocarcinomas and hence, necrotic tumors is softer and possibly fluctuant [25]; The LA7 induced tumor rat treatment group injected with β-catenin siRNA showed significant regression in tumor foci and dramatically improvement to the overall breast structure compare to untreated control (Fig. 1E). Similarly, LA7 induced tumor in rats treated with ZER or untreated knock down LA7 tumor bearing rats (Fig. 1C and 1D) showed pathological evidence [26] near similar to the normal non-induced tumor rats with lower number of mitotic figure (Fig 1A).

A significant reduction to MDA protein levels in tumor rats administrated with ZER or β-catenin siRNA were detected. In this respect, ZER treatment was able to reduce levels of serum MDA in these rats, and hence, assume that ZER's capability to reduce lipid peroxidation and oxidative stress is likely through modulation in the cancer cells [27]. Interestingly, ZER did not influence the levels of MDA proteins in the serum of normal rats (Table 5). This is in agreement to a previous finding by Hoffman, 2002 [28], who suggested that ZER prevents proliferation of cancer cells but rarely affecting normal cells proliferation. This anti-proliferative effect of ZER is due to the presence of α, β-unsaturated carbonyl group, which is crucial in differentiating between normal and cancer cells proliferation through a Michael adduct with GSH, later increasing intracellular redox potential, in turn, discontinuing cancer cells proliferation. Normal cells have lower quantity of intracellular redox potential, thus the effect of ZER on their proliferation is comparatively much weaker.

Our Real time PCR and immunohistochemistry assay analysis confirmed β-catenin reduction in all treatment groups of rats induced LA-7 breast tumors treated with β-catenin siRNA at approximately 51% in direct injection of β-catenin siRNA, 70% with ZER and 90% in β-catenin siRNA transfected LA7 induced breast tumor rats. The β-catenin reduction follows together with reductions in tumor volumes (Table 3). Moreover, these assays showing decreased in p53 level in induced tumor rats

treated with ZER compare to normal control (Fig. 3B). The change in this gene is significant to changes of β-catenin, as ZER reduction of β-catenin reduced p53 to approximately 28% from control. This unique ability of ZER to up-regulate p53 related genes but down-regulate p53 itself may be explained by its ability to down-regulate of NF-kB, which is required for p53-mediated apoptosis as previously investigated [29], confirming a possible link between NFKB and β-catenin observed previously in our microarray assay analysis (unpublished data). Collectively, these findings obviously demonstrate the impressive antitumor capability of ZER and suggest it as a potential therapeutic application breast cancer having elevated Wnt/ β-catenin levels.

Previous studies have identified ZER's effect on several *in vitro* and *in vivo* cancer models [6, 10, 27]. These studies suggested that the inhibitory effect of ZER occurred through the reduction in anti-apoptotic Bcl-2 level and up-regulation of pro-apoptotic Bax without involving p53 [6]. These studies also attributed the apoptogenic effect of ZER to the presence of α, β-unsaturated carbonyl group since compounds possessing this reactive group have been shown previously to display tumor cell growth inhibition and stimulation of cell differentiation, apoptosis, heat shock protein and glutathione S-transferase activities [10, 27]. Our study has shown for the first time that ZER greatly inhibit the proliferation and reduced tumor growth in *Sprague Dawley* rats through reduction of β-catenin similar to that observed in β-catenin siRNA treatments. In addition, we manage also for the first time to apply the use of ZER to identify apoptosis genes (Bid, MDM2, p53, and p21) triggered by β-catenin down-regulation in breast cancer rat models and further verify apoptotic features mediated by Wnt/β-catenin pathway inhibition in breast tumors pathologically.

Several molecular pathways targets are being explored in designing new medicines for clinical trials. Over-expression of the Wnt signaling pathway and the presence of a co-receptor low-density lipoprotein receptor- related protein 6 (LRP6) reported recently as a distinctive subtype of breast cancer [30], could possibly be the potential targeted of ZER anticancer therapy.

Although ZER showed encouraging results as an anticancer, additional pharmacokinetic studies are fundamental to further evaluate the effective doses and toxicity profile of ZER prior to introducing ZER as a new generation of anti-cancer drugs in the market for breast cancer treatment.

Conflict of Interest: Authors declared that there no conflict of interest.

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