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Zerumbone (ZER), a Potential Anticancer for Breast Mediates Cancer Cell Death Through Targeting β-catenin Signaling Pathway in MCF-7 Cell Line

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Abstract

Background: Zerumbone is a sesquiterpene which was found to delay progression of breast cancer through apoptosis induction via up-regulating Bax protein and down-regulating Bcl2 protein. ***Purpose*****:** In this study, we aimed to investigate Zerumbone, a natural compound of medicinal herbal plant isolated from *Zingiber zerumbet,* for anti-cancer activity against MCF-7 cell line. ***Methods*****:** Human cancer cell lines of breast MCF-7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). ON target plus smart pool siRNA, the most efficient method is used for preparation of β-catenin siRNA. Cyclophilin B siRNA is considered as positive control. Zrumbone is an ethanol-soluble compound isolated from *Zingiber zerumbet*, extracted by the hydro-distillation (steam distillation) method. Purification of ZER was examined using HPLC. The MCF-7 cells transfected with 100 µM human β-catenin (CTNNB1) siRNA or cyclophilin B siRNA positive control. After transfection, the cells were harvested at 48hrs for mRNA analysis or 48 – 72 hrs for protein analysis. IC_{50} for ZER was determined using MTT assay. MCF-7 transfected with β-catenin siRNA were then treated with ZER IC_{50}. The mRNA expression was analyzed using real-time to ensure the knock down of β-catenin. TUNNEL assay was used to confirm apoptosis and flow cytometry was applied for quantification of apoptotic cells. Western blot analysis was done to evaluate the effect of ZER and β-catenin (CTNNB1) siRNA on the expression of β-catenin and its functional impact on the proliferation and survival of MCF-7 cells. ***Results:*** ZER decreased β-catenin protein expression in breast cancer cell lines. ZER downregulated β-catenin mRNA level in breast cancer cell lines. Depletion of β-catenin by β-catenin siRNA and ZER induce cell apoptosis. Apoptosis caused by ZER was confirmed/examined using by annexin V (annexin V was used as a marker for drug action). ***Conclusion*****:** Zerumbone possesses anti-breast cancer activity, which could be attributed to the reduction of β-catenin protein expression in MCF-7 breast cancer cell lines, downregulation of β-catenin mRNA level in breast cancer cell lines and through annexin V.

Keywords: Zerumbone, Real-time PCR, β-catenin protein and TUNNEL Assay.

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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, 2]. 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 [3, 4]. 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 [5, 6]. 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 [7, 8]. This approach to use ZER or β-catenin siRNA to possibly regress breast cancer progression is currently investigated *in vitro*.

The advent of RNA interference (RNAi)-directed knockdown has revolutionized somatic cell genetics, allowing inexpensive, rapid analysis of gene function in mammals [9]. 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 [10]. 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 [11]. 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 [7, 8]. We investigate the role of blocking β-catenin by ZER or β-catenin siRNA on cancer development, either by MCF-7 cell line. 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 [12]. 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:

Human cancer cell lines of breast MCF-7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in RPMI-1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum heat-inactivated at 56°C for 30 min (from FlowLab, Australia), 50 units/mL penicillin, 50 units/mL streptomycin and 5 μg/mL blasticidin (all from FlowLab, Australia). The cells were maintained under complete media in a 37°C a humidified 5% CO_2 incubator. The cell cultures were frequently examined under an inverted microscope and upon the cells reaching 80% confluence; cells were sub-cultured and plated in 6-well plates for 24 h at 37°C in a humidified 5% CO_2 incubator. The cells were then grown to 50 - 60% confluence and subsequently subjected to β-catenin and/or siRNA inhibition [13].

2.2. β-catenin siRNA:

ON target plus smart pool siRNA the most efficient method that provided guaranteed for potential silencing with unmatched strategies for greater target specificity. ON target plus smart pool human β-catenin (CTNNB1) siRNA (CCAACUUCCUCUCGAAUAU CATJ-017147-09 which extends between 1174 to 1192 of beta catenin, GGUUUGAAGUGUUGACAUU CATJ-017147-10 which extends between 785 to 803 of beta catenin, GAGCCCAGCUAGAAUUAUA CATJ-017147-11 which extend between 1052 to 1070 of beta catenin, and UCGACAACAUGUAAGAAAA CATJ-017147-12 which extends between 903 to 921 of beta catenin; or cyclophilin B siRNA positive control (Dharmacon Research Inc., Lafayette, CO) were dissolved in 1X β-catenin siRNA buffer or annealing buffer [30 mM HEPES-KOH (pH 7.9), 100 mM potassium acetate, and 2 mM magnesium acetate]. The mixture was allowed to homogenize in orbital shaker at room temperature for 30 min, and then aliquoted at 20 μM concentration and stored at –20°C. The MCF-7 cells (1 x 10^5 cells/mL) were plated in 6-well plate in media containing 10% fetal bovine serum to give 30–50% confluence, and transfected with 100 μM human β-catenin (CTNNB1) siRNA or cyclophilin B siRNA positive control using DharmaFECT 1 transfection reagents (Dharmacon) at a final concentration of 100 nM following manufacturer's instructions. After transfection, the cells were harvested at 48hrs for mRNA analysis or 48 – 72 hrs for protein analysis. All experiments were performed in triplicates [14].

2.3. Zerumbone treatment:

ZER is an ethanol-soluble compounds isolated from *Zingiber zerumbet*, extracted by the hydrodistillation (steam distillation) method [8]. Purification of ZER was examined using HPLC. Stock ZER solutions were prepared as 1 mg/mL in absolute ethanol then further diluted in cell culture medium (100 µg/mL). IC_{50} for ZER was determined using MTT assay by plotting absorbance on the y-axis versus treatment on the x-axis (ZER at concentrations of 1.09, 2.19, 4.38, 8.75 and 35.00 µg/mL). MCF-7 Cells or MCF-7 transfected with β-catenin siRNA were then treated with ZER IC_{50} at concentrations 12 µg/ml. All experiments were performed in triplicates.

2.4. Real time RT- PCR

To ensure the knock down of β-catenin, mRNA expression was analyzed using real-time RT-PCR. MCF-7 cells were transfected with β-catenin (CTNNB1) siRNA for 48 h or treatment with or without ZER for 24 and 48 h. Total RNA was extracted using the RNeasy Mini Kit (QIA gene, Inc., Valencia, CA) according to the manufacturer's protocol. Concentration of RNA was determined using spectrophotometer (Utrospec3000) and ratio 1.9 to 2.1 for 260/280 determined the quality of RNA. RNA (10 μg) was used for reverse transcription and real time PCR using Sensi Mix TM SYBR One-Step kits, according to the manufacturer's protocol in a total volume of 50 µl (QIAGEN, Valencia, CA). All PCR reactions were performed in CFX96 real time PCR detection system (Bio-Rad, USA) using standard real time PCR conditions: I cycle reverse transcription at 42°C for 20 min followed by polymerase activation at 95°C for 5 min, then 40 cycle of gene amplification included a 95°C for 15 sec, annealing at 55°C for β-catenin or 60ºC for β-actin for 30 sec, and extension at 72°C for 15 sec. Sequences of primers used in this assay are listed in Table 1. At the completion of cycling, melting curve analysis was performed to establish the specificity of the PCR product [15]. Negative controls consisting of no-template (water) reaction mixtures were run with all reactions. Melting curves were determined for each primer set following all real time PCR runs using the CFX Manager software. Crossing point values for each transcript were normalized to the respective crossing point values for reference gene β-Actin. Data are presented as fold change in gene expression using the ΔΔCt method (9). After 24, 48 h of ZER treatment and 48, 72 h transfection with β-catenin (CTNNB1) siRNA, MCF-7 cells were harvested at different time points for Western blot analysis. Cells were lyses using CytoBuster™ Reagent (Promega, USA) and the total protein content was determined using Bio-Rad protein assay reagent (Bio-Rad, USA). The Western blot analysis was then performed as described previously [16]. In brief, total protein were separated using SDS-PAGE and immunoblotted either by polyclonal antibody to β-catenin (Santa Cruz Biotechnology) or anti-β-actin (Santa Cruz Biotechnology) as control. After extensive washing the membranes were incubated with anti-rabbit IgG-alkaline phosphatase conjugate antibody (Zhongshan Company) for 1 h at room temperature and developed with a ProtoBlot®II system detection kit (Promega, USA) in Western Blue® stabilized substrate for Alkaline Phosphatase. For loading normalization and accurate assessment, β-actin antibodies were used.

Table 1: Primer sequences used in real-time RT-PCR assay to validate several proteins expressions in MCF-7 breast cancer cells treated with ZER or SiRNA

Gene name	PCR primer sequences
β-catenin	Forward 5`-AATCGGTTTGAAGTGTTGACT-3` Reverse 5`-ACCTTTATATTTTCTTACATGTTGTCG-3`
β-actin	Forward 5`-GGCGGCACCACCATGTACCCT-3` Reverse 5`-AGGGGCCGGACTCGTCATACT-3`

***2.5. TdT-mediated dUTP nick end labeling (TUNEL) assay*:**

Apoptotic cells were confirmed with the *in-situ* cell death detection kit, Dead End ™ Fluorometric TUNEL System (Promega, Madison, WI) following manufacturer's instructions. Briefly, MCF-7 cells were treated with ZER or β-catenin siRNA. About $2x10^7$ cells were cytospin onto poly-L-lysine-coated slide and fixed in 4% paraformaldehyde for 25 min at 4ºC and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. DNA fragments were labeled with the TUNEL reaction mixture for 60 min at 37°C in a humidified atmosphere in the dark. The slides were then incubated in propidium iodide 1ug/ml for 15 min at room temperature, rinsed in deionized water, and anti-fade solution was added. Cells treated with DNAase were used as positive control. Samples were analyzed under fluorescence 100X (520nm for green fluorescence and 620 nm for propidium iodide) and confocal microscope 63X. Cells were mounted and examined and number of apoptotic cells (green staining) was counted [17]. Several microscopic fields were randomly counted for each sample. The percentages of viable, apoptotic, and necrotic cells were determined in 200 cells. This assay was performed three times (n = 3).

2.6. Apoptosis Analysis:

Quantification of apoptotic cells was done by flow cytometry (Becton Dickinson, USA). Briefly, the MCF-7 cells (2 x 105 cells) were plated in 25 mL cell culture flask for 24h and later treated with ZER at 12 µg/ml and 12.5 µg/ml respective IC50 values for MCF-7 transfected with β-catenin (CTNNB1) siRNA for 48h as single agent or in combination with ZER. The cells were washed twice with PBS, and then pelleted by centrifugation at 400 × g. The cell pellets were gently washed once with 1X binding buffer, and stained with annexin V stain (Clontec Laboratories) with enhanced green fluorescent protein solution (1:40) and propidium iodide (PI, 50 mg/mL) for 15 min at 4°C in the dark. After staining, the cells suspension was suspended in 500 µL of PBS and evaluated using flow cytometry. Early apoptotic cells showed exposed phosphatidylserine but with intact cell membranes bound to Annexin V-FITC while excluding PI. Cells in necrotic or late apoptotic stages were labeled both with Annexin V-FITC and PI [18].

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2.7. 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. ZER decreased β-catenin protein expression in breast cancer cell lines:

Western blot analysis was done to evaluate the effect of ZER and β-catenin (CTNNB1) siRNA on the expression of β-catenin and its functional impact on the proliferation and survival of MCF-7 cells. After MCF-7 cells were treated with ZER for 24 and 48 h (Fig.1. I. A) or transfected with β-catenin (CTNNB1) siRNA (Fig.1. I. B), bands relating to β-catenin expressions were reduced. Treatments are time dependent, associated to the decreased in β-catenin protein expression in MCF-7 cells. β-actin antibody gave similar protein expression during different treatment.

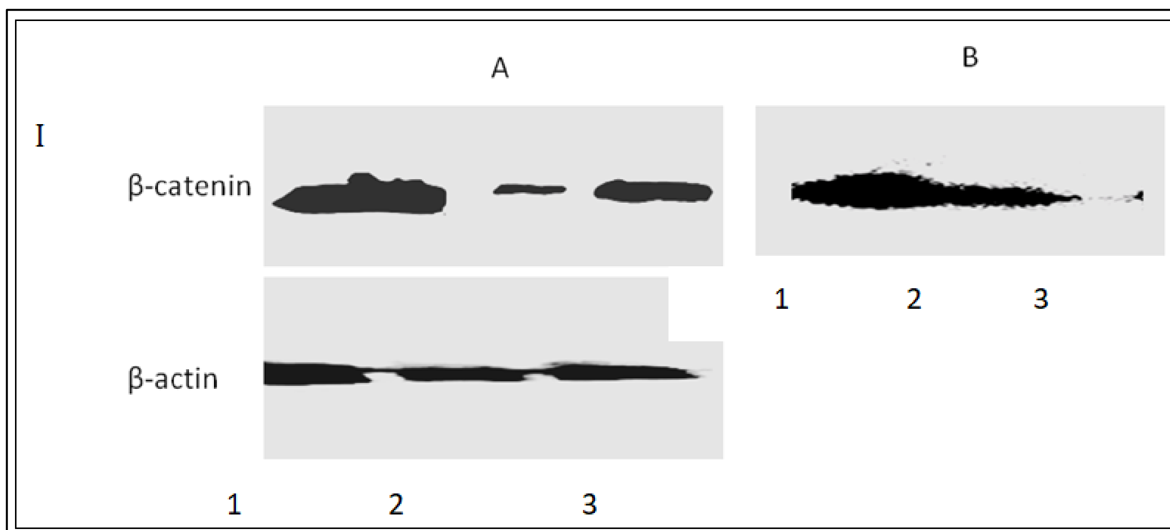

Figure 1(I): Western blot analysis for β-catenin protein expression on MCF-7 cells treated with (A) ZER for 48 h (2), or 24 h (3) while (1) represent nontreated cell (control), (B) MCF-7 cells treated with siRNA, 1= MCF-7 control, 2=MCF-7 transfected with β-catenin (CTNNB1) siRNA for 48h, 3= MCF-7 transfected with β-catenin (CTNNB1) siRNA for 72 h. The reduction in β-catenin expression showed time depending in both siRNA and ZER

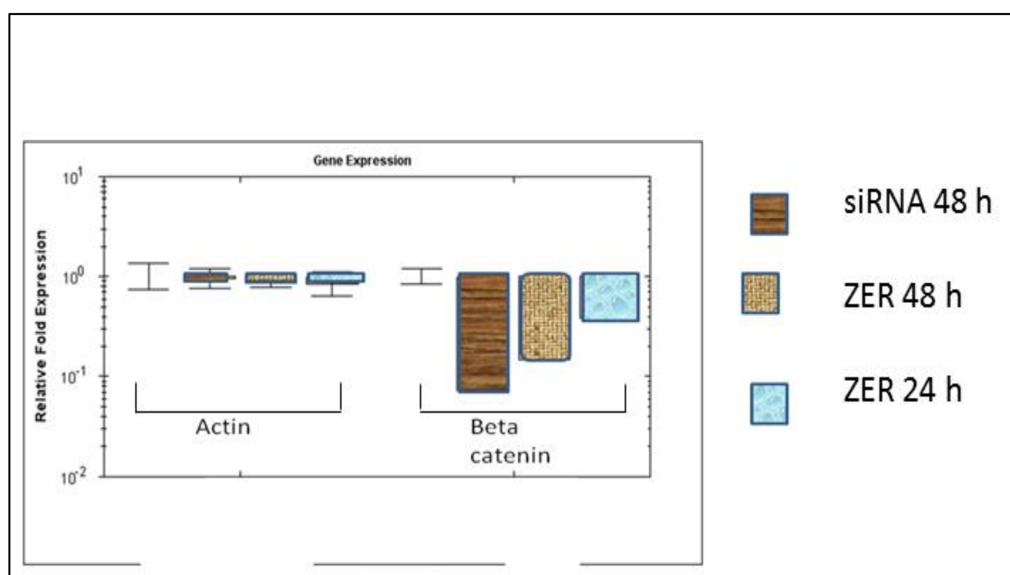

Figure 1(II): Levels of mRNA in MCF-7 cells treated with ZER or siRNA determined by real time PCR. Levels of gene expression were determined by real time RT-PCR for β-catenin and β-Actin. Expression of β-actin on MCF-7 used as internal control. T1=MCF-7 cells were transfected with β-catenin siRNA for 48 h (siRNA), T4=MCF-7 treated with ZER for 48 h and T5= MCF-7 treated with ZER for 24 h

3.2. ZER downregulated β-catenin mRNA level in breast cancer cell lines:

MCF-7 cells transfected with β-catenin siRNA or treated with ZER were analyzed for β-catenin

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expression by real time PCR. Real time RT-PCR detected 45.5% reduction in the mRNA level of β-catenin in MCF-7 cells treated with ZER for 24, 83% in MCF-7 treated with ZER for 48 h, and 93% reduction after β-catenin siRNA transfection (Fig.1.II). The Expression of a housekeeping gene β-actin mRNA was similar between Control, ZER, and siRNA groups.

3.3. Depletion of β-catenin by β-catenin siRNA and ZER induce cell apoptosis

TUNEL assay was performed to determine whether depletion of β-catenin by ZER or β-catenin siRNA could promote cell death. MCF-7 were transfected with β-catenin siRNA or treated with ZER (12 µg/ml) for 24 to 48 hours. The TUNEL assay results indicate 6.6 % apoptotic cells of control non-treated MCF-7 cells, as TUNEL positive (Fig.2a.E). In contrast, TUNEL positive cells amounted to 30% after 24-hour ZER treated MCF-7 cells, 36% in 48-hour ZER treated cancer cells (Fig.2a.A-C) (Fig.2b), whilst 32.4% in β-catenin siRNA-transfected cells treated (Fig.2a.D) (Fig.2b), while Fig.2a.F is positive TUNEL control. This data clearly suggested that depletion of β-catenin by β-catenin siRNA and ZER treatment induce cell death to the cancer cells.

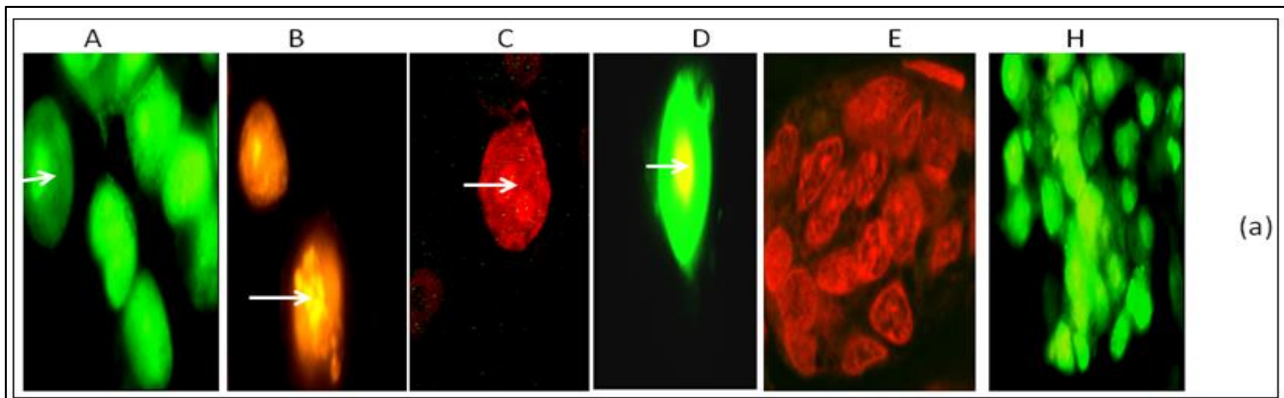

Figure 2(a): Confocal florescence micrographs showing apoptosis induction in MCF-7 cells after ZER and β-catenin siRNA treatments. Micrographs showing apoptosis induction of the two treatments as analyzed using TUNEL assay. MCF-7 cells treated with ZER after 24 h (A) after 48 h (B) and after 72 h (C). MCF-7 cells treated with β-catenin-siRNA after 48 h (D). Untreated MCF-7 cells (E) and DNAase TUNEL assay positive control (H). Bright green fluorescence representing early apoptosis of chromatin condensation (white arrows) after 24h ZER (A) or 48h siRNA (D) whilst blebbing and nuclear fragmentation (white arrows) observed after 48h ZER (B). Orange fluorescence represents late apoptosis after 72h ZER (C). Magnification 100x

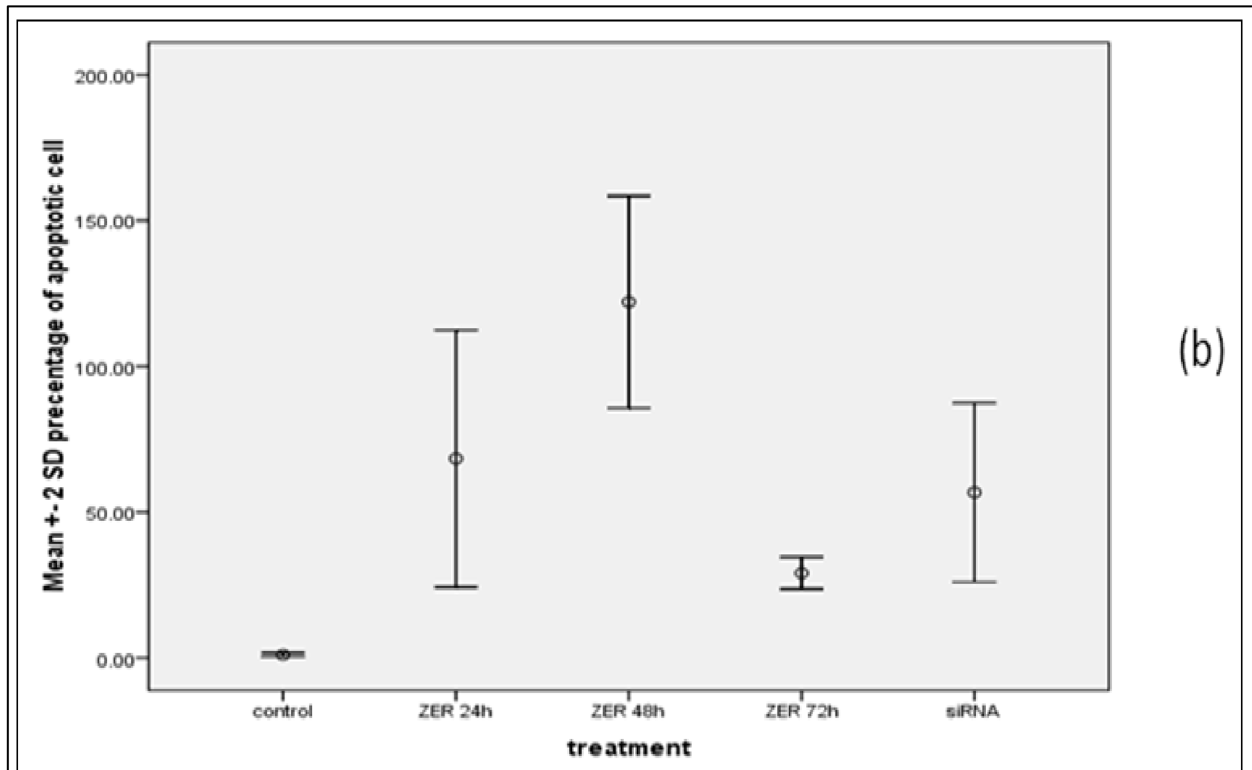

Figure 2(b): Histograph represents percentage of apoptotic cells after treatments with ZER or siRNA. Data are significantly different between treatment groups and untreated control (P < 0.05)

3.4. ZER causes apoptosis in breast cancer cells by annexin V:

Annexin V is used to examine if β-catenin depletion after ZER or siRNA treatments in MCF-7 cells could specifically promote cell death through apoptosis induction. After treating MCF-7 cells with ZER, the analysis showed that rate of apoptosis after 12 h incubation was 3.75% compare to control, later increased to 18.82% late apoptosis after 24 h treatment, whilst early apoptosis did not prevail (Table 2). On the contrary, transfection of MCF-7 cells after 48 h with 100 nM β-catenin (CTNNB1) siRNA showed evidence of early apoptosis at 19.98% and late apoptosis of 24.76% (Table 2). This confirms our initial results that showed an almost complete knock-down of β-catenin expressions after 48h transfection of MCF-7 with β-catenin SiRNA. This result confirms evidence of cancer cell death to apoptosis induction after treatment.

Table 2: Percentage of apoptotic cells in MCF-7 breast cancer cells after treatments with ZER, β–catenin siRNA or cyclophilin B siRNA (positive control). The apoptosis rate for ZER and β-catenin siRNA treatments is highly significant (*P* < 0.01) and (*U* < 0.05), while no significant difference (P>0.01) in apoptosis rate is determined for the positive control compared with untreated cells

Treatment	Percentage of apoptosis in MCF-7 cells			
	Early	Control	Late	Control
ZER (12 hrs)	0.58	1.08	---	1.97
ZER (24 hrs)	0.58	1.08	---	1.97
siRNA β-catenin (48 hrs)	19.98	2.64	2.42	5.16
Positive (cyclophilin B) control siRNA	3.5	5.08	18.82	3.74

4. DISCUSSION

Activation of Wnt pathway can modulate cell proliferation, cell survival, cell behavior, and cell fate. The core molecule of this pathway, β-catenin has been demonstrated from previous studies to increase cellular proliferation [19, 20], likely due to its ability to increase the expression of specific cellular genes [21]. Normally, the level of β-catenin is tightly control by GSK3b, one of destruction machinery that leads to proteasomal degradation of β-catenin and prevent β-catenin/Tcf transcription activity and expression of downstream genes mediated anti apoptotic or proliferated phenotype [22]. Increased β-catenin levels may play a role in the proliferative advantage seen in breast and other premalignant lesions. It has been demonstrated that this pathway not only plays a role in the promotion of cell proliferation and cell cycle progression, but also may provide an important survival function to facilitate cell transformation [23, 24]. Meanwhile, activation of the Wnt/β-catenin pathway inhibits apoptosis in cancer cell lines [25, 26]. In addition, D. Wang et al., 2018 demonstrated that Wnt/β-catenin pathway plays a role in the progression of human prostate cancer, especially to the acquisition of apoptosis-resistant phenotype [27]. The constitutive activation of β-catenin/Tcf transcription has been found in several human cancers. Thus, β-catenin stabilization, as well as the formation of a complex with Tcf transcription factors, is likely to play a critical role in Wnt-mediated anti-apoptosis.

Our study evaluates the functional consequences of ZER or β-catenin siRNA's ability to decrease β-catenin in MCF-7 cells, determined by assaying the β-catenin gene mRNA and protein expression. In addition, the impact of this β-catenin reduction to a variety of other cellular regulatory genes implicated in apoptosis induction was also verified.

In this current study, both β-catenin siRNA transfection in MCF-7 and MCF-7 cell treated with ZER were analyzed for β-catenin expression by real time PCR and western blot analysis. The mRNA and protein levels of β- catenin were significantly reduced during the period of (48hrs) after treatment in both transfected and ZER treated MCF-7 cells.

Previous studies had identified ZER inhibited growth of P-388D cancer cells with apoptosis induction [28]. In our study, TUNEL assay confirmed apoptosis induction by ZER and β-catenin siRNA in MCF-7 cells and rat breast cancer tumor model, possibly due to the reduction of β-catenin cytoplasmic levels. The morphological changes in TUNEL assay analysis were time-dependent especially at early apoptosis during transfection (Fig 1).

These observations are well correlated to the flow cytometry analysis, which showed higher apoptotic cells in transfected MCF-7 cells (early apoptosis), but treatment with ZER to the MCF-7 cells generated lesser apoptotic cells in early apoptosis (Table 2).

5. CONCLUSION

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.

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

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