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# CYTOTOXIC EFFECT OF *ZIZIPHUS SPINA-CHRISTI* EXTRACT ALONE AND IN COMBINATION WITH DOXORUBICIN ON BREAST CANCER CELLS

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*Ziziphus Spina-Christi (L.) (ZSC) is a traditional Arabian medicinal plant used to treat inflammatory symptoms, swellings and pain since long. Triple negative breast cancer (TNBC) is a form of cancer with a poor prognosis owing to the paucity of therapy alternatives. Two of the most critical pathways of TNBC development are Wnt/β-catenin signaling and autophagy. In the present study, we intended to identify the possible mechanisms of the cytotoxic effects mediated by ZSC extract on MDA-MB-231 breast cancer cells and to improve the efficacy of DOX in combination with ZSC. The MTT test was used to estimate cell viability and $IC_{50}$ values. Apoptosis was detected using AnnexinV-FITC detection kit. ELISA was used to measure caspase-3 levels. Cell cycle and the level of autophagosome marker LC3-II were analysed using flow cytometry. Acidic vesicular organelle (AVOs) formation was observed by fluorescence microscopy. Real-time PCR was used to monitor changes in gene expression of β-catenin and autophagic adapter NBR1. It was shown that ZSC treatment dose-dependently inhibited MDA-MB-231 cell viability and induced apoptosis with accompanying elevation of caspase-3 level. Besides ZSC caused a significant elevation in LC3II level and downregulation of NBR1 gene expression with subsequent downregulation of β-catenin gene expression, indicating the inhibition of the oncogenic Wnt pathway. ZSC and DOX combination had synergistic cytotoxic effect by more effective suppression of Wnt pathway and induction of apoptosis and autosis.*

*K e y w o r d s: Ziziphus Spina-Christi, DOX, breast cancer cells, Wnt/β-catenin signaling, apoptosis, autophagic adapter NBR1, autophagosome marker LC3-II.*

Triple negative breast cancer (TNBC) is a unique form of breast cancer with a poor prognosis owing to its aggressive biological activity and paucity of therapy alternatives [1]. TNBC patients do not express the estrogen receptor (ER), progesterone receptor (PR) as well as the human epidermal growth factor receptor 2 (HER2) [2] Accordingly, chemotherapy with doxorubicin (DOX) is now the most prevalent treatment for TNBC [3]. Due to acute and chronic DOX-induced toxicities such as myelosuppression, immunosuppression, and dose-cumulative cardiotoxicity, DOX has a low therapeutic index, and its clinical applicability is limited [4]. Resistance to anticancer drugs, particularly doxorubicin, is still a leading cause of chemotherapy failure in cancer patients [5]. One of the processes implicated in drug resistance's intricate and multifaceted nature is the activation of proliferation and survival signaling pathways [1].

The Wnt signaling pathway has developed to perform a number of roles in normal development and physiology. It affects cell fate, differentiation, proliferation, and stem cell pluripotency, among other biological processes [6]. The pathway dysfunction has also been found to be a crucial pathway in a variety of malignancies, including TNBC [7]. Ultimately, that pathway stimulation in some tumors and its relation to chemotherapy resistance is an

**List of abbreviations:** AO – acridine-orange; AVOs – Acid vascular organelle; DOX – Doxorubicin; ER – Estrogen receptor; HER2 – The human epidermal growth factor receptor 2; LC3 – Microtubule-associated protein 1A/1B-light chain 3; MTT – 4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide; Nbr1 – Neighbor of BRCA1; PBS – Phosphate buffer solution; PI – Pidium iodide; PR – Progesterone receptor; SL – Sidr Leaves; TNBC – Triple negative breast cancer; ZSC – *Ziziphus Spina-Christi* (L.).

intriguing topic that has gotten a lot of interest in recent years [8]. In TNBC, it boosts cancer progression, differentiation, and metastasis by promoting cell survival, proliferation, and stem-like behavior [9]. This encourages the cytoplasmic accumulation of *β-catenin*, the main Wnt signaling mediator. The buildup and translocation of *β-catenin* to the nucleus increases the transcription of a variety of downstream oncogenes [10]. Notably, Wnt signaling interacts with a variety of signaling pathways, including autophagy, which is involved in drug resistance induction [11]

Autophagy is a multi-step mechanism in which damaged cytosolic content is trapped in a double-layered membrane, transferred to the lysosome for breakdown, and digested to generate energy and construct the foundation for cell survival [12]. Autophagy dysfunction has been linked to a number of clinical conditions, including cancer. It's usually recognized as a two–edged sword, with opposing functions in tumor suppression and growth stimulation [13]. Autophagy inhibits tumorigenesis in its early stages. In advanced stages of cancer, however, it gives stress tolerance and therapeutic resistance [2].

Importantly, Wnt signaling and autophagy appear to be negatively proportionate, with Wnt signaling inhibition resulting in autophagy activation in colorectal cancer, breast cancer, multiple myeloma, and liver cancer [14]. Both Wnt signaling and autophagy are essential in TNBC, and each has been identified as a promising therapeutic target [15]. Subsequently, the establishment of consistent targets can facilitate the creation of more effective and less toxic treatments.

Medicinal herbs and their phyto-compound derivatives are increasingly being identified as effective cancer treatment and/or preventative agents [16]. *Zizphus spina-christ*i (L.) (ZSC), popularly known as Sidr, is a Rhamnaceae botanical family multipurpose plant [17]. It is a traditional Arabian medicinal herb, has been used by Egyptians (Bedouin and Nubian) to treat inflammatory symptoms and swellings, pain, and heat since long [18]. Phytochemical analysis revealed ZSC contains a range of bioactive components, including essential oils such as geranyl acetate, methyl palmitate, and methyl stearate, alkaloids like spinanine-A, tannins, phytosterols e.g. beta-sitosterol, flavonoids – for example quercetin derivatives, saponins such as betulinic acid, and triterpenoid sapogenins [19]. The antibacterial, anticancer, anti-inflammatory, antihypertensive, hepatoprotective, antidiabetic, antioxidant, and anti-obesity properties of ZSC are enhanced by these bioactive substances [18, 19]. As a result, natural ZSC extracts may hold promise as an adjuvant treatment for TNBC prevention or treatment. Consequently, combination therapy with ZSC, a very successful innovative non-toxic drug that can reduce chemotherapy doses and control novel metabolic targets, is also being sought.

Indeed, understanding the pathways involved in cancer cell proliferation and resistance could lead to new treatment options [20]. Therefore, in the present study, we intended to identify the possible cytotoxic mechanisms mediated by ZSC extract on MDA-MB-231 breast cancer cells. Meanwhile, this study attempted to improve the efficacy of DOX by combining it with ZSC via targeting the interaction between apoptosis, autophagy and Wnt pathway.

## Materials and Methods

*Reagents and Chemicals.* MTT, 4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (AO), and propidium iodide (PI) were acquired from Sigma-Aldrich (Sigma-Aldrich Co., USA). Tocris Bioscience provided the doxorubicin (DOX) (Ellisville, MO, USA).

*Plant materials and preparation of the methanolic extracts.* Sidr Leaves (SL) *(Ziziphus Spina-Christi*) were found in an Egyptian botanical garden. An experienced taxonomist from the Botany Department of the Faculty of Science at Damietta University in Egypt verified the leaves. SL were cleaned under running water and then dried in the oven at 40°C. An electric grinder was used to grind the dried leaves into a fine powder. The cold maceration process was developed to extract dried powdered leaves. Methanol was used to soak 500 g of powder, which was then filtered through Whatman No. 1 filter paper after being kept in a shaking water bath at 40°C for 8 h. The supernatant was collected in a dark, sealed bottle and concentrated to dryness using a rotary evaporator under low pressure and temperature conditions before being kept in a refrigerator between 2 and 8°C [21]. Dilution was maintained until the extract concentrations were 10, 100, 200, 500, and 1000 µg/ml, respectively.

*Gas chromatography-mass spectrometry (GC-MS) analysis.* A volume of 1 µl of ZSC methanol extract was loaded into the GC-MS (Model; QP 2010S, Japan) for total ion chromatographic analysis using

a Hamilton syringe in the split injection technique. The retention index and morphologies of mass spectra of the bioactive components in the extract were compared using Wiley Registry of Mass Spectral Data's, New York (Wiley 8) and Fatty Acid Methyl Esters Library version 1.0 (FAME library) sources [22].

*Cell culture*. The American Type Culture Collection provided MDA-MB-231 (ATCC®HTB-26TM) (ATCC, Manassas, VA, USA). The cells were grown at 37°C, 5% $CO_2$, in 10% DMEM (Lanza Bioproduct, Belgium), supplemented with 10% fetal bovine serum (Sera Lab, UK).

*MTT assay.* Cells were cultivated in a 96-well plate overnight before being treated with a variety of drugs at different doses. Cells were incubated with 20 L of MTT at a final concentration of 0.5 mg/l. After 4 h, the media was discarded, and the cells were liquidated in 20 µl DMSO per well, with the blue dye dissolved for minutes at 37°C. The absorbance at 570 nm was then measured using a microplate reader (BioTek, USA). The results were adjusted to the controls and expressed as a percentage of viability. The half-maximal inhibitory action on cell proliferation ($IC_{50}$) values shows the concentration that induced half of the maximal inhibitory activity on cell proliferation.

*Design of experiment.* The cell line was categorized into four groups as follows: untreated cells are represented by the *control group*; *DOX group* – after being cultivated for 24 h, cells were treated with Doxorubicin $IC_{50}$ (85.73 g/ml); *ZSC group* – after 24 h of culture, cells were treated with the $IC_{50}$ of ZSC extract (243.59 g/ml); *ZSC+DOX group* – cells were treated with a combination of ZSC extract and DOX, which inhibited cell growth by 50% after 24 h of culture.

*Cell morphology.* Breast cancer cell line cells were grown overnight at 37°C in 5% $CO_2$ and then treated or not for 24 h as stated previously. Under an inverted microscope with amplification of 406, cell morphology was investigated.

*Transition electron microscopy (TEM).* In all groups, TEM was used to explore autophagosomes and late autophagic compartments. To prepare for autophagosome imaging with the JEM-1400 plus Electron Microscope (Jeol, Tokyo, Japan), cells were fixed, sectioned, and stained.

*Combination treatments.* The implication of ZSC+DOX combined treatment was investigated using Chou and Talalay's (1984) technique. Serial dilutions of the combined extract and medication (at their $IC_{50}$ ratios) were made. The MTT test was used to estimate the growth-inhibitory activity of various combinations. The mutually exclusive drug equation was used to compute the combination index (CI) values.

*Analysis of cell cycle.* The cells were plated in 6-well plates ($5\times10^5$ cells/100 mm), cultured overnight, and then treated in triplicate with the same doses and controls as outlined earlier. The treatments lasted 24 h, and each well was serviced individually into flow tubes. Fixation and permeabilization of cells were performed for 1 hour at -20°C with 70% cold ethanol. Two folded washing was applied on cells in cold phosphate buffer solution (PBS) 1X. For staining, a 100 g/ml solution of propidium iodide (PI) was utilised at 37°C for forty minutes. Cells scattering was investigated using FACSCalibur (Becton Dickinson), San Jose, CA, USA and Cell Quest (Becton Dickinson) and ModiFit LT (Verity, Top).

*AnnexinV-FITC/PI staining.* Apoptosis was detected using the AnnexinV-FITC detection kit (KeyGEN BioTECH). Before being treated with medication, cells were allowed to grow in 6-well plates and incubated for 12 h. After treatment, roughly $10^6$ cells were gathered and washed away twofold with PBS. Afterwards, the cells were centrifuged and resuspended in 300 µl of binding buffer. The cells were then treated in the dark for 15 min at room temperature with 5 µl of Annexin V and 2.5 µl of PI, confirmed by flow cytometry within 1 h. A BD FACS Calibur flow cytometer was used to measure apoptosis, and data were calculated using BD Cell QuestTM Pro (version 5.2) software.

*Determination of caspase-3.* Cancer cells ($1\times10^6$) were seeded onto 6-well plates, then treated for 24 h before being suspended. Caspase-3 levels were checked according to the criteria provided by the Human caspase-3 ELISA Kit at 450 nm (Sun Red Biotechnology, Shanghai), using a Bio-Tek SynergyTM HTMulti-Mode Microplate Reader.

*Acidic vesicular organelles (AVOs) identification and counting.* Autophagy results in the formation of acidic vesicular organelles with double membranes (autophagosomes, AVOs), which could be identified via certain dyes, particularly acridine-orange (AO). AVOs were counted using flow cytometry after cells were stained with AO. Cells were grown on 6-well plates, and then treated for 24 h as stated above before being collected in tubes and washed in PBS. The suspension was stained for 15 min with AO (1 g/ml), washed twice in PBS, sus-

pended in PBS, and flow cytometry was performed. The fluorescence was read using the FL2 channel (PE) of a FACSCalibur (Becton Dickinson), which was then analysed using FACSDiva 6.0 software. The generation of AVOs is related to the intensity of AO red fluorescence. AVOs were detected using a fluorescence microscope after treating cells with AO (1 g/ml) for 15 min.

*Semi quantitative analysis of LC3 by flow cytometry.* Cell suspension was prepared after 24 h of treatment as reported earlier. The MAP LC3B Ab CUSABIO USA kit was used to determine the levels of LC3. Cell permeabilization was achieved by slowly adding ice-cold % methanol to prechilled cells. The cells were gently vortexed to a concentration of 90% methanol, after which they were analyzed using the appropriate filter [23]. Quantitative detection was done by flow cytometry (Becton Dickinson, CA, USA).

*Real-time PCR analysis.* For Real-time PCR analysis, the iCycler-iQ Optical (Bio-Rad) was employed. The TRIzol method is used to extract total RNA, which is identical to the abcam® mRNA extraction protocol. A reverse-transcriptase enzyme was synthesised utilising the technique supplied by the HISenScriptTM RH [-] cDNA Synthesis Kit (iNtRON, Korea) to turn the acquired RNA into cDNA [24, 25]. The primers listed below were used in the experiment.

*β-actin*
F: 5′-TGGCACCACACCTTCTACAATGAGC-3′
R: 5′-GCACAGCTTCTCCTTAATGTCACGC-3′

*NBR1*
F:5-TCGACGCAATCTCCACCAAT-3′
R:5-GCA GGA CGT GGT TAG TGT CA-3′

*β-catenin*
F: 5′-GATACCTCCCAAGTCCTGTATGAG-3′
R: 5′-GCATCAAACTGTGTAGATGGGATC-3′

For PCR amplification, SYBR Green with low ROXTOPrealTM qPCR 2XpreMIX was utilized (Enzynomics, Korea). The comparative (Ct) method (2-ΔCTequation) was used to assess the relative mRNA expression levels (CT of gene of interest - CT of housekeeping gene).

*Statistical analysis.* SPSS 22.0 is used for statistical analysis. The quantitative variables were compared using data from three different studies (triplicates). Yet, the results of six ELISA assays were used in this study. The information was presented as a mean standard deviation of the mean (SEM). To compare the means, valid post hoc tests of analysis of variance were used. *P* values less than or equal to 0.05 were considered statistically significant.

## Results

*Identification of bioactive compounds.* Twenty-five chemicals were discovered in a methanol extract of ZSC leaves (Fig. 1, *A*). The presence of the following main compounds in the extract was responsible for its antioxidant and anti-proliferative properties: [4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-] (flavonoid fraction) (Line: 8), n-Hexadecanoic acid (saturated fatty acid) (Line: 21) and [2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one] (Line: 6).

*Cell proliferation analysis.* Using standard MTT assay, we first assessed the effects of therapy on cell proliferation. The proliferation of cells decreased in a concentration-dependent manner after DOX treatment (Fig. 1, *B*). After 24 h of treatment, the $IC_{50}$ was found to be 85.73 µg/ml. Treatment with ZSC extract showed a reduction of cellular viability dose dependently (Fig. 1, *C*). The $IC_{50}$ values were observed at 243.5 µg/ml after treatment for 24 h.

*Monitoring of cells by inverted microscope.* After 24 h of treatment, viable cells decreased significantly and dose-dependently, as examined with an inverted microscope. Most cells shrank and rounded before detaching from the culture plates (Fig. 2, *A*), but viable cells decreased only slightly.

*Monitoring of cells by TEM.* As shown in (Fig. 2, *B*), few vacuoles were observed in control cells. Treatment with DOX, ZSC, or both enhanced the presence of autophagosomes packed with debris.

*Cell cycle kinetics.* Flow cytometry analysis of untreated TNBC cells revealed the presence of 13.3% cells in the sub G1 phase and the majority of cells (51.2%) in the G0/G1 phase. In comparison to the untreated control, DOX treatment caused an accumulation of cells in the sub G1 fraction (21.3%) and cycle arrest in the G0/G1 phase (53.1%). ZSC treatment increased cellular debris in the sub G1 phase (36.7%) and caused cell arrest in the G0/G1 phase (50.1 percent). Cells treated with ZSC+DOX produced more cellular debris in the sub G1 phase (43.7%) and induced cell accumulation in the G0/G1 phase (36.7%) and G2/M phase (36.7%) (13.7 percent), cell cycle analysis was done on triplicated and all changes in cell cycle phases in treated groups are significant ($P = 0.0001$), compared to control group (Fig. 3).

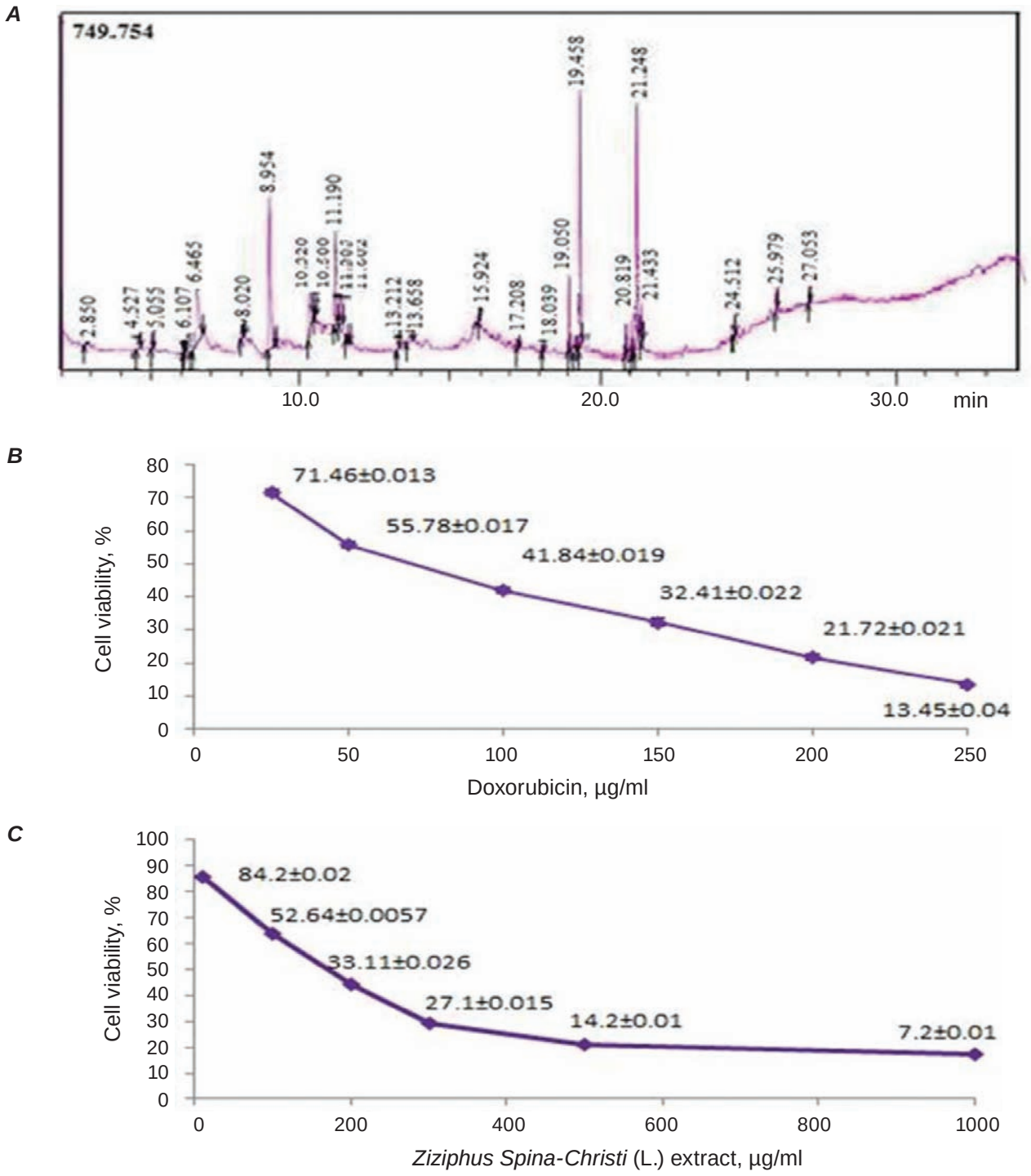


*Fig. 1.* ***A*** *– GC-MS analysis of extract of Ziziphus Spina-Christi (L.) (ZSC).* ***B*** *– The effect of treatment with doxorubicin (DOX) on the proliferation of MDA-MB-231 cells by the MTT assay.* ***C*** *– The effect of treatment with ZSC on the proliferation of MDA-MB-231 cells by the MTT assay*

*Determination of apoptosis.* Untreated cells had unimpaired cells (lower left quadrant) (89.65%), early apoptotic cells (lower right quadrant) (0.7%), late apoptotic or necrotic cells (8.75%) (Upper right quadrant) and primary necrotic cells (0.9%) (Upper left quadrant) (Fig. 4). DOX treatment of cells resulted in a significant shift ($P$ = 0.0001) of cells into the early and late apoptotic quadrants, as well as a decrease in cells in the primary necrotic and living quadrants. Likewise, ZSC therapy resulted

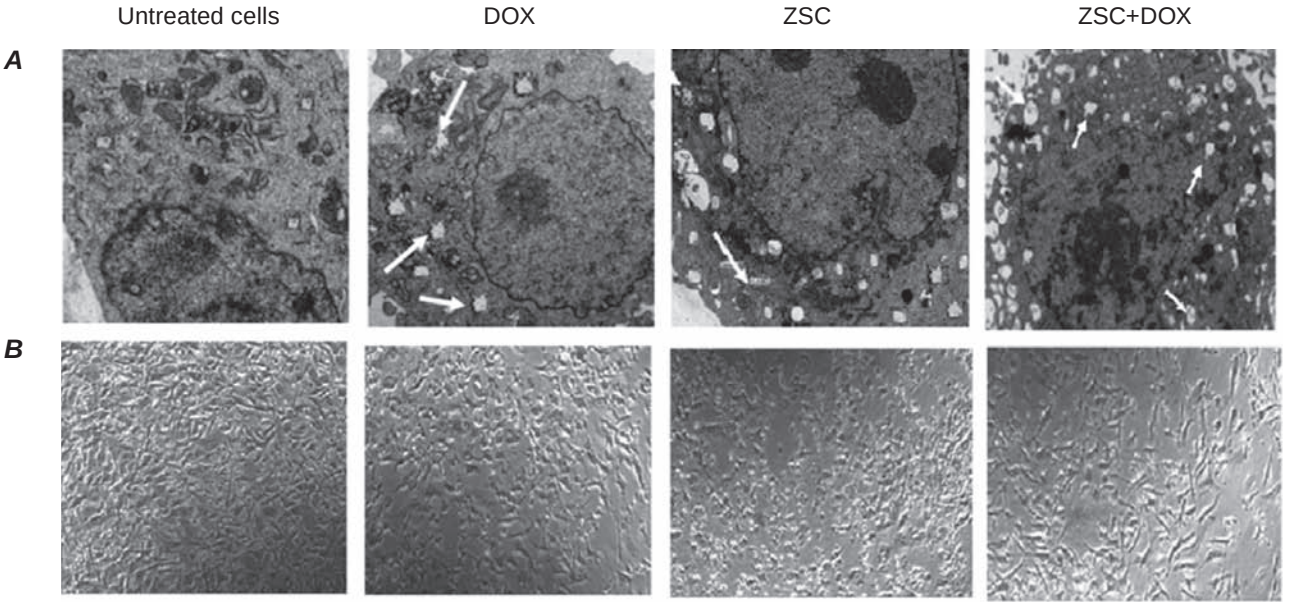


*Fig. 2. The effect of treatment on cells. (**A**), by (TEM). (**B**), by inverted microscope*

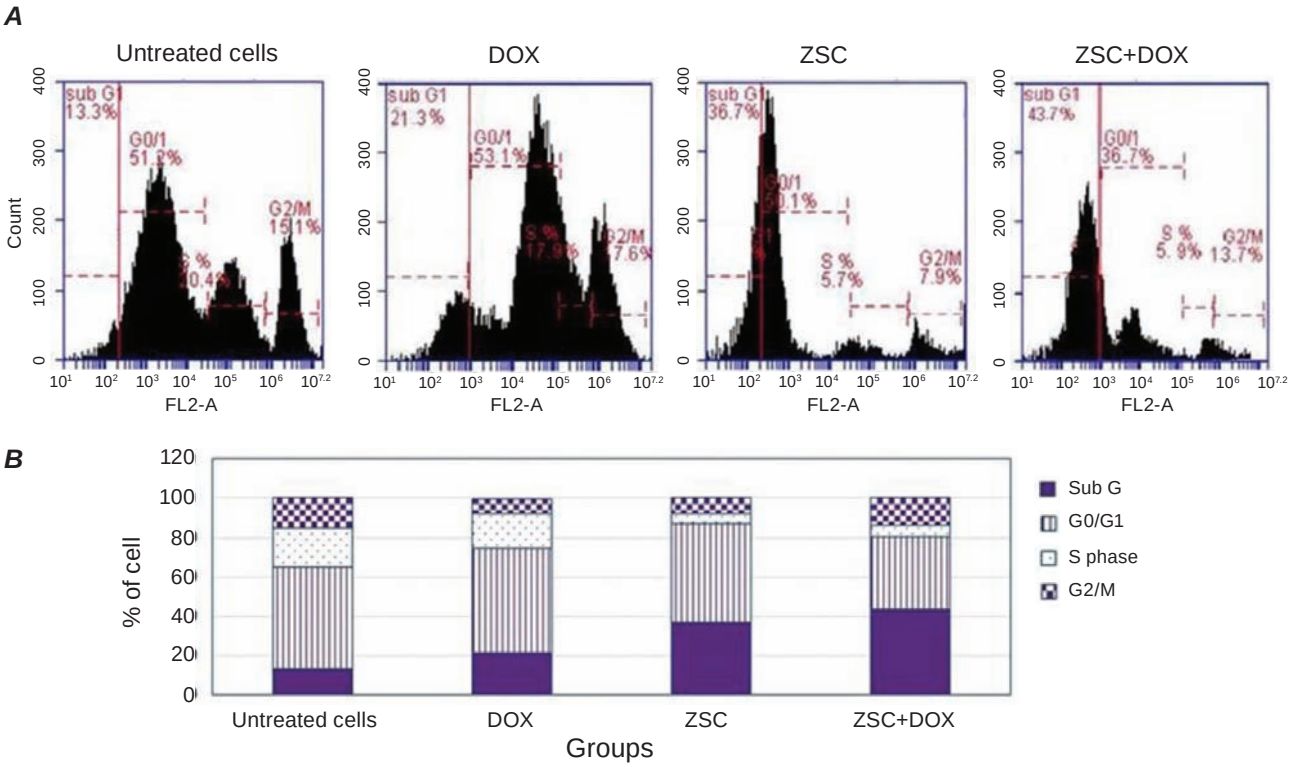


*Fig. 3. **A** – Histographs of cell cycle distribution showing the effect of treatment in different cell cycle phases. **B** – Bar graphs representing the distribution of cells in different cell cycle phases*

in a 23.4% shift of cells to the late apoptotic cells quadrants ($P$ = 0.0001). After treatment with (ZSC+DOX), there was a 44.6% increase in late apoptotic cells quadrants ($P$ = 0.0001) compared to control cells.

*Detection of caspase 3.* Treatment of cells with DOX, ZSC and ZSC+DOX showed a significant elevation in caspase-3 levels *(*$P$ = 0.0001) by (25.05, 20.9 and 39.26%; respectively) comparative to un-

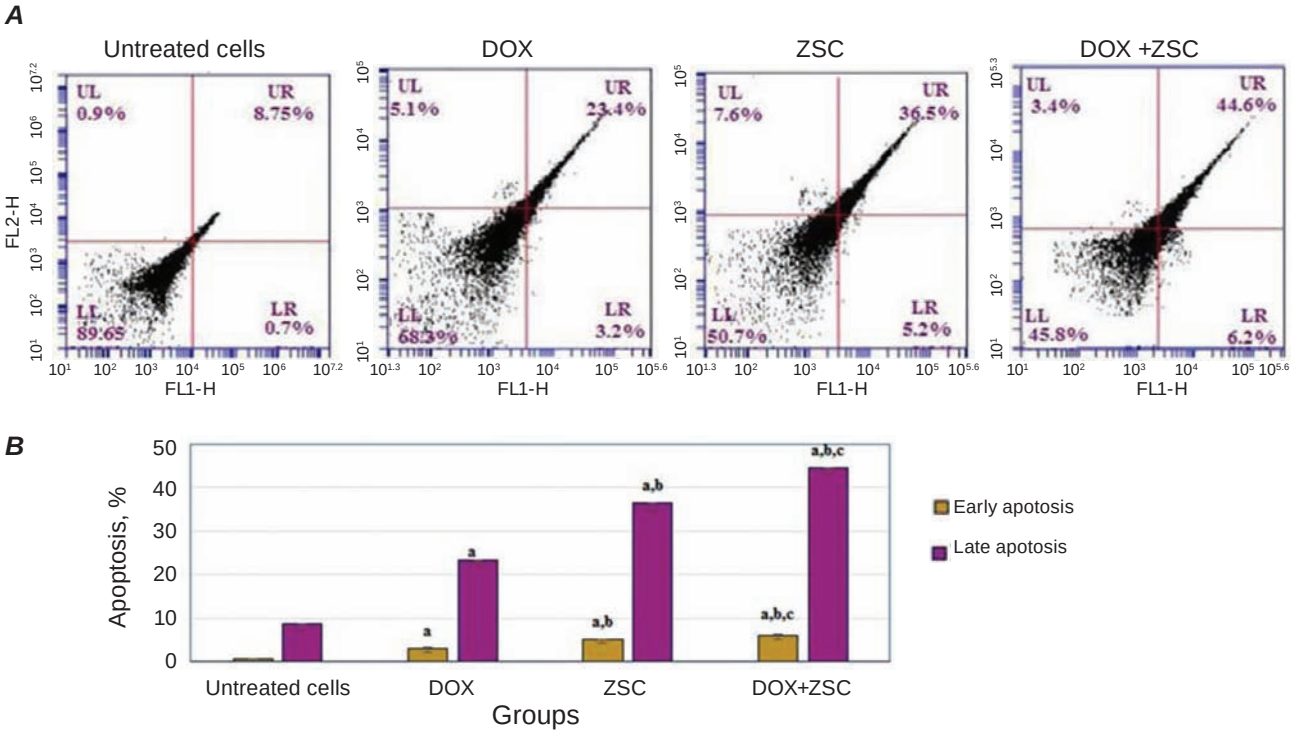


*Fig. 4. (**A**), Flow cytometry analysis of apoptotic cellular death in MDA-MB-231 cells (**B**), Bar chart demonstrating early and late apoptosis. Significant results are explicated comparative to untreated cells as (a), comparative to DOX as (b) and (c) compared to ZSC treated cells. Statistical variance was defined as significant when P < 0.05*

*Table. Effect of treatment on caspase levels and the relative gene expressions of NBR1 and β-catenin in MDA-MBA-231 cell line, the number of repetitions of the experiment (mean ± SE, n = 3)*

| Groups of the cell line | Caspase-3 (ng/$10^6$ cell) | Relative gene expressions of *NBR1* | Relative gene expressions of *β-catenin* |
|---|---|---|---|
| Untreated | 11.63 ± 0.12 | 6.120 ±0.008 | 3.550 ± 0.032 |
| DOX | 14.54 ± 0.012[a] | 5.47 ± 0.02[a] | 2.360 ± 0.034[a] |
| ZSC | 14.07 ± 0.04[a] | 5.23 ± 0.008[a] | 2.160 ± 0.008[a] |
| ZSC+DOX | 16.490 ± 0.008[a] | 4.640 ± 0.017[a] | 1.390 ± 0.009[a] |

Note. [a]$P \leq 0.05$ considered significant comparing to untreated group

treated cells (Table). This activity could be done in the following order: ZSC+ DOX > DOX > ZSC.

*Detection of AVOs.* The formation of autophagolysosomal vacuoles was assessed using AVOs staining by the fluorescent microscope (Fig. 5, *A*); and the flow cytometer (Fig. 5, *B*). Opposed to untreated cells, DOX- and ZSC-treated cells had a non-significant increase in acridine orange-positive cells with intense red fluorescence ($P$ = 0.09 and 0.06) (61.7, 82.7%; respectively). Combination treatment of ZSC+DOX caused a significant elevation in the bright red fluorescence compared to untreated and DOX treated cells ($P$ = 0.02 and 0.025; respectively).

*Detection of LC3 by flow cytometry.* A flow cytometric evaluation of untreated cells grown for 24 h revealed the presence of 33.1% positive LC3 (Fig. 5, *C*). As opposed to untreated cells, DOX, ZSC, and their combination raised the percentage of positive LC3 to 53.00, 78.9, and 91.5%, respectively ($P$ = 0.0001).

*NBR1 gene expression.* Treatment with DOX, ZSC or ZSC+DOX caused a significant suppression

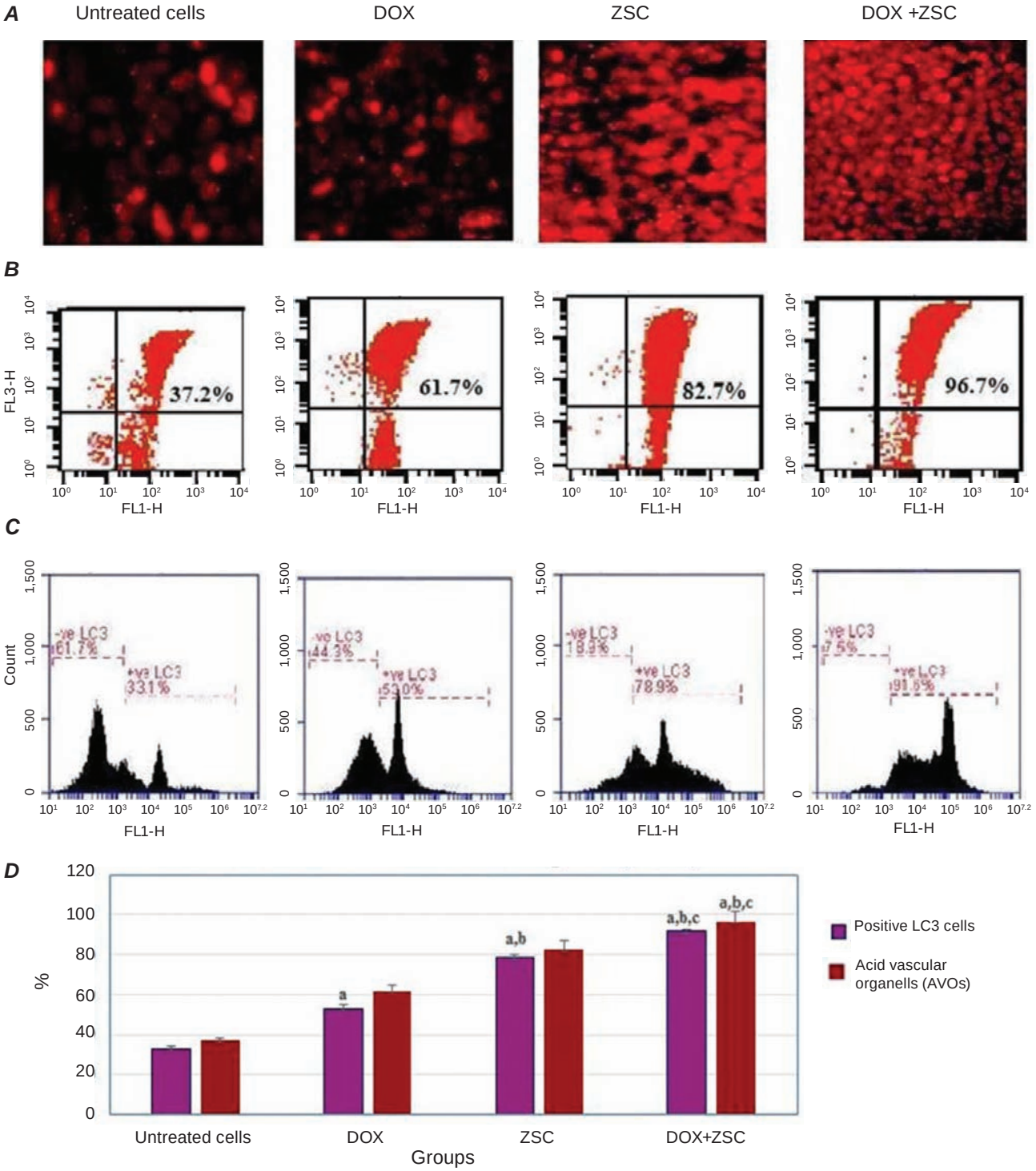


*Fig. 5. Effect of treatment on autophagy.* ***A*** *– Fluorescence microscope image of AVOs formation.* ***B*** *– Flow cytometry analysis of AVOs formation.* ***C*** *– Histographs of LC3 expression.* ***D*** *– Bar graphs representing % of LC3 and AVOs in studied groups. Significant results are explicated comparative to untreated cells as (a), comparative to DOX as (b) and (c) compared to ZSC treated cells. Statistical variance was defined as significant when* $P < 0.05$

of *NBR1* gene expression ($P$ = 0.0001) by (10.62, 14.48 and 24.12%; respectively) (Table). ZSC+DOX > ZSC > DOX is a possible sequence for this activity.

*β-catenin gene expression.* Treatment of cells with DOX, ZSC or ZSC+DOX resulted in a statically significant downregulation of *β-catenin* gene expression ($P$ = 0.0001) by (33.52, 38.96 and 60.75%; respectively) opposed to untreated cells (Table). This activity could be arranged as ZSC+DOX > ZSC > DOX.

## Discussion

In TNBC patients, conventional chemotherapy treatments are unsatisfactory, prompting the development of novel therapeutic techniques. Doxorubicin (DOX) is a TNBC chemotherapeutic drug that works by generating free radicals, which cause significant DNA damage, macromolecule suppression, and mitochondrial dysfunction, cytochrome c release, caspase cascade stimulation, and apoptosis induction [26, 27].

In the present study, treatment of cells with DOX dose dependently caused a significant inhibition of cellular proliferation manner. Besides, it caused a significant accumulation of apoptotic cells in the sub G and G0/G1 cell cycle arrest. Compared to untreated cells, a significant elevation in caspase-3 (pro apoptotic protein) was identified, indicating apoptotic cell death. DOX has been conveyed to trigger distinct cellular signals, including the cleavage of anti-apoptotic proteins (Bcl-2/Bax) and the activation of pro-apoptotic proteins caspase 9, which is followed by caspase-3 cleavage and stimulation [28]. The cell then enters a point of no return, causing a succession of downstream mediators to trigger apoptosis [29, 30]. Nevertheless, the existence of various molecular processes involved in drug resistance (e.g., autophagy, notch, Wnt, etc.) offers a considerable barrier to successful therapy.

As a consequence, herbal-based therapies have been discovered to be one of the most impressive means to manage and/or prevent cancer [16]. This is because of the great prevalence of complex chemical components such as alkaloids, flavonoids, terpenoids, saponin, and phenolic chemicals, which act against cancer by modulating many pathways [31]. These natural chemicals are proven to be naturally available, cheaper, and easier to take orally than synthesized medications, and they have low or minimum adverse effects, as well as being enriched with numerous biologically active components [32].

In the GC-MS spectroscopy of the ZSC methanol extract, the presence of (4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-) was determined. Flavonoids have a prominent influence in the immunoregulatory alterations that occur as cancer develops and progresses [33]. Additionally, the GC-MS spectroscopy study of the extract exposed the incidence of n-hexadecanoic acid and [2,4-Dihydroxy-2,5dimethyl-3(2H)-furan-3-one]. 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3one] has been shown to have antioxidant and anti-inflammatory characteristics [34]. Hence, [2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3one] has been demonstrated to have antioxidant and anti-inflammatory activities [35].

The cytotoxic effects of ZSC extract on cells were investigated using the MTT test in this study. ZSC treatment dose-dependently inhibits cell proliferation. Secondly, it ensued a significant rise in the number of cells at the apoptotic peak and cell cycle arrest, implying apoptosis. The triggering of apoptosis was confirmed by the substantial elevation of caspase-3 levels. The previous observations are in harmony with Abdoul-Azize, Abedini et al and El Seedy et al., who illustrated that flavonoids and fatty acids contained in Rhamanaceae plants disrupt cell cycle as well as caspase-3 activation, which accelerates the release of upstream death cascade components such as cytochrome c from mitochondria. [36-38]. All of the evidence pointed to ZSC's cytotoxic action being due to apoptosis activation. Other molecular pathways underlying ZSC extracts' anticancer activity, though, need to be studied further.

Alternatively, DOX stimulates another signling pathway known as autophagy (type II cell death), to preserve mitochondrial integrity [39]. Autophagy is mediated by an autophagy-related protein that is highly conserved (ATG). The adaptor protein ATG8, also known as (LC3), can recruit specific cargo to the autophagosome via interactions with cargo receptors like (P62 and *NBR1*), resulting in its destruction. Therefore, *NBR1* and LC3 were utilized as indices for measuring autophagic flow.

Treatment with DOX caused significant elevation of LC3 levels and downregulation of *NBR1* gene expression relative to untreated cells suggesting induction of autophagy. Through its cyto-protective mechanism, autophagy activation stimulates survival, mediated doxorubicin-induced cardiotoxicity, and contributes to chemotherapy resistance [40]. As a consequence, autophagy-associated resistance has

emerged as a complicated attribute and a prospective strategy for improving cancer treatment.

The autophagic activity of ZSC was assessed using two autophagy modulators in this work. ZSC caused a significant elevation in LC3 levels and a downregulation in *NBR1* gene expression, supporting autophagy induction. This effect could be attributed to its bioactive ingredients, such as flavonoids, which have been discovered to have anti-cancer properties by influencing autophagy [41, 42]. A previous study reported that the flavonoid wogonin stimulates cancer cell death by suppressing autophagy [43]. Nonetheless, according to Kikuchi et al., several flavonoids have been found to trigger cell death via inducing autophagy [44]. These finding corroborate our hypothesis that ZSC acts as an autophagy activator.

Chemotherapeutics' cytotoxic activity could be improved by decreasing cytoprotective autophagy or promoting type II programmed cell death [13, 45]. Autophagy is usually used to reduce anxiety, though there is indication that it can similarly be used as an alternate cell death mechanism if it is over-activated [46]. Liu et al, discovered that high dosages of autophagy-inducing peptides, nutrient deprivation, and persistent brain ischemia, all of which are autophagy-activated conditions, were found to promote a new type of autophagy-dependent cell death [47]. This type of cellular death, known as autosis, is marked by the appearance of distinct morphological and biochemical characteristics, as well as a vast cytoplasmic vacuolization accompanying autophagy [48]. Autotic cellular death may be stimulated by overabundance of autophagosome formation, instead of lysosomal degradation [49].

Wnt/β-catenin signaling imbalance has been linked to poor clinical outcomes in TNBC, while increasing *β-catenin* in the nucleus improves cell motility, colony formation, stem-like features, and chemoresistance, according to Pohl et al. and Park et al. [9, 50]. In this study, treatment with DOX caused downregulation of *β-catenin* gene expression relative to untreated cells suggesting inhibition of Wnt pathway. The latter result could refer to autophagy caused by DOX. It was conveyed that, autophagy triggering can inhibit Wnt/β-catenin signaling by lysosomal degradation of or *β-catenin* [11, 51]. These findings highlight the crosstalk, synergistic, and antagonistic effects of the Wnt/β-catenin signaling pathway, as well as other pathways, in cancer.

ZSC induced significant downregulation of *β-catenin* gene expression suggesting the inhibition of the oncogenic Wnt pathway in this study. This activity could be modulated by flavonoids like Quercetin, which have the ability to modulate the Wnt/β-catenin signaling pathway and then can play an important role in cancer prevention [52]. Thereby, lowering *β-catenin* levels made cells more sensitive to autophagy-stimulating mTOR inhibitors [53, 54]. All of the above findings indicate that ZSC mediates its cytotoxic activity by inducing apoptosis, autosis, and suppressing Wnt signaling pathways, making it a promising candidate antitumor agent.

In addition, to investigate the possible synergistic effect following the treatment with combinations, the combination index (CI) was calculated. The calculation of CI displayed a synergistic effect when treated with a combination of ZSC and DOX. Growing evidence shows that in clinical studies, combining antitumor drugs with autophagy inhibitors has more effective influence [55-57]. Previous findings indicated that combining ZSC and DOX has synergistic anti-tumor effect, as compared to DOX or ZSC alone, by inhibiting the Wnt pathway and inducing apoptosis and autosis. Our results are compatible with Luo et al. who reported that the combination of an autophagy inducer and Wnt inhibitor had a substantial synergistic effect against tumor growth than an autophagy inducer alone [55].

*Conclusion.* This study is the first to demonstrate pathways that mediate the cytotoxic impact of ZSC. In particular, a ZSC extract exhibited antiproliferative impact against cells through the downregulation of the expression of β-catenin, *NBR1* and the downregulation of caspase-3 and LC3 expression suggesting inhibition of Wnt signaling pathway and activation of apoptosis and autophagy. Moreover, combination handling with DOX showed synergetic antitumor activity and could be used to reduce DOX-induced dose dependent dexterous effect. Furthermore, these results point to the occurrence of crosstalk between the selected targets.

*Conflict of interest.* The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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# ЦИТОТОКСИЧНА ДІЯ ЕКСТРАКТУ *ZIZIPHUS SPINA-CHRISTI* ОКРЕМО ТА В ПОЄДНАННІ З ДОКСОРУБІЦИНОМ НА КЛІТИНИ РАКУ МОЛОЧНОЇ ЗАЛОЗИ

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*Ziziphus Spina-Christi* (L.) (ZSC) – традиційна арабська лікарська рослина, що здавна використовується для лікування запалення, набряків та болю. Потрійно-негативний рак молочної залози (РМЗ) має несприятливий прогноз через обмеженість альтернативних методів лікування. Двома найбільш визначальними шляхами розвитку РМЗ є Wnt/β-катенін сигнальний шлях та автофагія. Метою даного дослідження було визначити можливі механізми цитотоксичної дії екстракту ZSC на клітини раку молочної залози MDA-MB-231 та підвищити ефективність дії доксорубіцину (DOX) у комбінації з екстрактом ZSC. Для оцінки життєздатності клітин та значень $IC_{50}$ використовували МТТ-тест. Апоптоз оцінювали за допомогою набору AnnexinV-FITC. Рівень каспази 3 аналізували методом ІЕА. Клітинний цикл та рівень маркера автофагосом LC3-II досліджували використовуючи проточну цитофлуориметрію. Утворення кислотних везикулярних органел (AVO) спостерігали за допомогою флуоресцентної мікроскопії та кількісно визначали проточною цитофлуориметрією. Для моніторингу змін експресії генів *β-катеніну* та автофагічного адаптера *NBR1* використовували ПЛР у реальному часі. Показано, що застосування ZSC дозозалежно пригнічувало життєздатність клітин MDA-MB-231 та індукувало апоптоз, що підтверджується значним підвищенням рівня каспази-3. Крім того, ZSC спричиняв підвищення рівня LC3-II та зниження експресії гена *NBR1* з подальшим зниженням експресії гена *β-катеніну*, що вказувало на пригнічення онкогенного шляху Wnt. Застосування екстракту ZSC разом з DOX мало синергічний цитотоксичний ефект завдяки більш ефективному пригніченню шляху Wnt та індукції апоптозу і автозу.

Ключові слова: *Ziziphus Spina-Christi*, DOX, клітини раку молочної залози, Wnt/β-катенін сигнальний шлях, апоптоз, автофагічний адаптер NBR1, маркер автофагосом LC3-II.

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