

Efficacy of Molecular Targeting of Cancer Stem Cells in the Treatment and Eradication of Experimentally Induced Triple-Negative Breast Cancer

Nahla H. EL-shaer^{a*}, Muhammad Alaa Eldeen^a, Aml M. Hashem^a, Refaat A.Eid^b, and Nermin Raafat^c

^aDepartment of Zoology, Faculty of Science, Zagazig University, Egypt.

^bDepartment of Pathology, Faculty of Medicine, King Khalid University, Saudi Arabia.

^cDepartment of Medical Biochemistry, Faculty of Medicine, Zagazig University, Egypt.

Nahla H. EL-shaer, Associate Professor of Histology and Histochemistry, Faculty of Science, Zagazig University, Egypt.

Muhammad Alaa Eldeen, Ph.D. Candidate, Faculty of Science, Zagazig University Egypt.

Aml M. Hashem, Professor of Histology and Histochemistry, Faculty of Science, Zagazig University, Egypt.

Refaat A.Eid, Professor of Pathology, Faculty of Medicine, King Khalid University, Saudi Arabia.

Nermin Raafat, Professor of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Zagazig University, Egypt.

Corresponding Author: Muhammad Alaa Eldeen

Email: muhammadalaaelden@zu.edu.eg

ABSTRACT

Background: Triple-negative breast cancer (TNBC) is a destructive form of breast malignancy that deficiencies the opportunities of targeted therapeutics. Developing evidence proposes that breast cancer stem cells (BCSCs), considered the main reason for poor prognosis as they induce resistance, metastasis, and relapse.

Aim of the Study: This study aims at examining the efficacy of Doxorubicin, Cyclophosphamide, Salinomycin, Digoxin, and Nano-silica encapsulated cobra venom in eradicating BCSCs and managing TNBC.

Methods: We performed our study using the TNBCs cell line (MDA-MB-231). Our study involved 5 groups with 5 different interventions; Untreated cells, cells treated by Doxorubicin 3.5 μ M only, cells treated by a combination of Salinomycin 15 μ M and Doxorubicin 3.5 μ M, cells treated by a combination of Digoxin 120 nM and 1 mM Cyclophosphamide and cells treated by a combination of (Naja Haje) venom 3.5 μ M and Salinomycin 15 μ M.

Results: Outcomes of our study demonstrated that the involved therapeutics inhibited the growth of the MDA-MB-231 cell line in a significant way by different efficacies and potencies. Cells treated with the combination of doxorubicin and salinomycin were significantly inhibited at a higher level than those treated with doxorubicin only Digoxin and Cyclophosphamide combination exhibited a lower efficacy than the previous combination in inhibiting the growth of cancer cells. Naja haje venom and Salinomycin combination showed the most significant inhibition of MDA-MB-231 cell line growth.

Conclusion: The current study confirms the potency of these novel approaches in the management and the eradication of triple-negative breast cancer.

Keywords: Cancer Stem Cells, TNBC, MDA-MB-231, Eradication.

Correspondence:

Muhammad Alaa Eldeen

Department of Zoology, Faculty of Science, Zagazig University, Egypt.

Email: muhammadalaaelden@zu.edu.eg

INTRODUCTION

Breast cancer (BC) is a diverse disease with various morphological features, a variety of responses to various therapeutic alternatives, and clinical results. BC classifications have been developed to try to treat patients more effectively [1]. The most devastating form of BC is TNBC. TNBC cells lack expression of estrogen, progesterone receptors, and displaying a lower expression of human epidermal growth-factor-2 (HER2) cells. They are currently missed in targeted treatment options for TNBC hormonal or antiHER2 therapy [2]. Unlike other types of BC, TNBC patients exhibit higher [3].

Cancer stem cells play a major role in inducing heterogeneous tumor formation. Some tumor cells are not tumorigenic. Instead, the heterogeneity of the original tumor can be recapitulated by a small amount of the cancer cells. In solid tumors, CSCs can proliferate and form spheroids in 3D culture. On the other hand, non-CSCs experience apoptosis [4]. Furthermore, CSC's are highly tumorigenic; therefore, even in small numbers, they can be

successively transplanted in rodent models inducing tumor initiation, whereas non-CSC are not able. The incidence of the tumor has been used for the standard CSC frequency estimation method. More than 15 years ago, Al-Hajj recognized a subpopulation of cells with the CD44+/CD24-/Lin- phenotype in BC patients with the potentiality of inducing tumors in rat models [5]. Subsequently, **Ginestier et al.** confirmed that cells with high aldehyde dehydrogenase also can similarly initiate tumors [6]. Hence, the expression of CD44+/CD24- and ALDH are considered the significant hallmarks of BCSCs. Cumulative indication shows that BCSCs are accounted for poor prognosis in addition to cancer formation [7]. While chemotherapeutic agents can eradicate the majority of cancer cells. They have not the potential ability to eradicate BCSCs. This subset of cells has been made the principal reason for therapy resistance and tumor relapse [8].

The majority of conventional therapeutic approaches presently accessible have the potential ability to eliminate

primary tumor bulk but might not have the ability to offer robust clinical outcomes in treated patients [9]. Due to the inadequate application of conventional therapies in eradicating BCSC subpopulation, accordingly, providing an occasion for the mammary tumor relapsing. Utilizing cancer stem cells are highly resistant, aggressive, and have a greater ability of invasion than their non-cancer stem cells complement. Consequently, targeting these populations may be a promising approach for eradicating BC [10].

Thus, it is necessary to develop a new targeted therapeutic strategy for specific biomarkers, signaling pathways, and microRNAs to selectively treat BC more securely and more efficiently [11]. The higher resistance of BCSCs to conventional therapies, when compared with their non-stem cells counterparts of the tumor bulk, highlights the necessity for novel targeting strategies for the stem population. All the previously mentioned.

BCSC characteristics, markers, and mechanisms of resistance could serve as possible aims for a more effective treatment for breast cancer patients to be used alone or in combination with existing therapies [12].

Therefore, we hypothesized that using therapeutic approaches with a potential ability to directly target this small population of cells called BCSCs is a promising tool for eradicating BC and avoiding its frequent and usual relapsing.

Consequently, we conducted this *in vitro* study of experimentally induced TNBC to examine the efficacy of Doxorubicin, Cyclophosphamide, Salinomycin, Digoxin, and Nano-silica encapsulated cobra venom as promising therapeutic approaches for managing TNBC, to determine the precise mechanism underlying this effect and also to demonstrate essential recommendations for further research works in the same scientific scope.

METHODOLOGY

Site of our Study:

This study was performed in the Department of Medical Biochemistry, Faculty of Medicine, Zagazig University, and in Medical and scientific research center, Zagazig University, Egypt between May 2019 and November 2020. This study is an *in vitro* experimental study: Using human TNBC cell line to assess the potency and the efficacy of our interventions.

Cell Culture

TNBC cell line (MDA-MB-231) was obtained from the American Type Culture Collection (ATCC) and was cultured in RPMI (Lonza Bioproducts) accompanied with 10% fetal bovine serum (FBS) (Lonza Bioproducts), Streptomycin 100 mg/ml (Lonza Bioproducts) in addition to Penicillin 100 U/ml. Subsequently, Cells were subcultured two times weekly in a moistened atmosphere at 37 °C, 5% CO₂ at a CO₂ incubator (Heraeus, Langensfeld, Germany).

Cytotoxic effect on MDA-MB-231 cell line using MTT assay.

The cytotoxic activity test was conducted to determine the IC₅₀ and IC₉₀ of each involved therapeutics to detect the doses of each therapeutic approach that we used in our study. The MTT assay is a colorimetric assay that depends on the decreasing of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

to purple formazan [13]. Initially, various concentrations of, Cyclophosphamide, Salinomycin, Doxorubicin, Digoxin, and Silica nanoparticle-conjugated Egyptian cobra (*Naja haje*) venom were added to 104 cells/well. After 48 hrs incubation, 2.5 µg/ml of MTT was added to each well and incubated at 37°C for 4hrs. The formazan crystals that formed were liquified by the addition of 200 µl/well of 10% Sodium dodecyl sulfate. The positive control was used that gives a 100% lethal effect in the same circumstances [14]. In the end, we read the absorbance at 595 nm. Using the formula: (Reading of extract / Reading of negative control) - 1) x 100, the ratio of cell viability modification was assessed.

Experimental Model

TNBCs were distributed in six-well plates (2.5 × 10⁴/well) supplied with culture medium (RPMI in addition to 10% FBS, Penicillin, and Streptomycin). Our study involved 5 groups with 5 different interventions; Untreated cells, cells treated by Doxorubicin 3.5 µM only, cells treated by a combination of Salinomycin 15 µM and Doxorubicin 3.5 µM, cells treated by a combination of Digoxin 120 nM and 1mM Cyclophosphamide and cells treated by a combination of (*Naja Haje*) venom 3.5 µM and Salinomycin 15 µM.

RNA Extraction

After performing trypsinization, the complete RNA amount from cells was extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany) based on the specific guidelines of the manufacturer.

RNA Quantification by NanoDrop Nucleic Acid Quantification:

Initially, and after RNA extraction completion, RNA samples along with the ice water were taken to the spectrophotometer, at that time, the sample reader washed away using ultrapure water before dry them with a Kim Wipe. Based on the software manufacturer guidelines, 2 µL of elution water was used as a (blank), and the system was reset, at that moment the software's setting was readjusted to RNA then blank option. At that point, 2 µL of the sample was loaded and the measure option was chosen. After completing the measurement process, the A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ ratios in addition to the quantity of RNA recovered (in ng/µL) were documented.

Real-time PCR analysis for expression of Bax, P53, Caspase-9, Bcl-2, and CD44 genes

Evaluation of mRNA expression levels were accomplished using real-time PCR. Initially, reverse transcription of complementary DNA (cDNA) was conducted by using (TIAGEN FastQuant RT Kit). cDNA was amplified utilizing Real-time PCR (StratageneMx3005P-qPCR System). The reaction was performed in a 20 µl reaction mixture containing 5 µl of cDNA template, 10 µl Eva Green mix (Jena Bioscience), and 100 pmol/ul primers. GAPDH was used as a reference gene. Primer pair sequences for Bax, P53, Caspase-9, Bcl-2, and CD44 genes were as shown in (Table 1). The Reaction conditions were as follows: 95 °C for 30 s for mediating denaturation and activate polymerase, 95 °C for 15 s to facilitate 40 cycles of denaturation; 58 °C for the 60s for annealing and elongation steps. Gene expression levels were evaluated using the 2- $\Delta\Delta$ CT equation, designated by Livak and Schmittgen [15].

Table 1: Primers sequence of CD44, Bax, Bcl-2, Caspase-9, P53, and GADPH genes.

Target	Ref Seq Accession	Sequence	product size (bp)
Bax	NM_017059.2	5'-CCTGTGCACCAAGGTGCCGGAAC-3' (forward) 5'-CCACCCTGGGTGTCTTGGATCCAGCCC -3'(reverse)	120
Bcl-2	NM_016993.1	5'-TTGTGGCCTTCTTTGAGTTCGGTG-3' (forward) 5'-GGTGCCGTTCAAGTACTCAGTCA-3' (reverse)	144
P53	NM_000546	5'-ACTTGTGCTCTTGAAGCTAC-3' (forward) 5'-GATGCG GAGAATCTTTGGAACA-3' (reverse)	279
Caspase-9	NM_001229	5'-CACTCCCCCTGAAGACGAGTC (forward) 5'-GTGGGCAAAGTATATGGCG (reverse)	1248
CD44	NM_001202557	5'-CGGACACCATGGACAAGTTT (forward) 5'-GAAAGCCTTGCAGAGGTCAG-3' (reverse)	882
GADPH	NM_001256799	5'-TGAAGGTCGGAGTCAACGGATTGGT-3' (forward) 5'-CATGTGGCCATGAGGTCCACCAC-3' (reverse)	460

Evaluating cell viability by Trypan Blue.

All involved groups which untreated or treated with Doxorubicin, Cyclophosphamide, Digoxin, Salinomycin, Naja Haje venom were detached, then stained with trypan blue, and finally calculated with a hemocytometer under the light microscope.

RESULTS**MTT assay for cytotoxicity Percentage**

Cytotoxic effect of Salinomycin, Cyclophosphamide, Doxorubicin, Digoxin, and Silica nanoparticle-conjugated Egyptian cobra (*Naja haje*) on MDA-MB-231 cell line: We conducted an MTT assay to assess the rate of proliferation of MDA-MB-231 cells after treatment with varying concentrations of Salinomycin, Doxorubicin, Digoxin, and Silica nanoparticle-conjugated Egyptian cobra (*Naja haje*).

We examined the in vitro effects of different concentrations of these suggested therapeutic approaches on the viability of MDA-MB-231 cells after 48 hr using the MTT assay (**figure 1**).

The result presented that all of these approaches had a concentration inhibitory effect on MDA-MB-231 cells. Salinomycin, Doxorubicin, Digoxin, Cyclophosphamide, and Silica nanoparticle-conjugated Egyptian cobra (*Naja haje*) venom inhibited the growth of MDA-MB-231 cells depending on concentration. At the concentrations of 3.5 μ M, 15 μ M, 120 nM, 1 mM, 3.5 μ M, 50% viability was detected during the 48 hr treatment by Doxorubicin, Salinomycin, Digoxin, Cyclophosphamide, (*Naja haje*) venom, respectively. whereas maximum cytotoxicity LC90 was observed at a concentration of 5.5 μ M, 25 μ M, 200 nM, 1.5 mM, 6 μ M respectively.

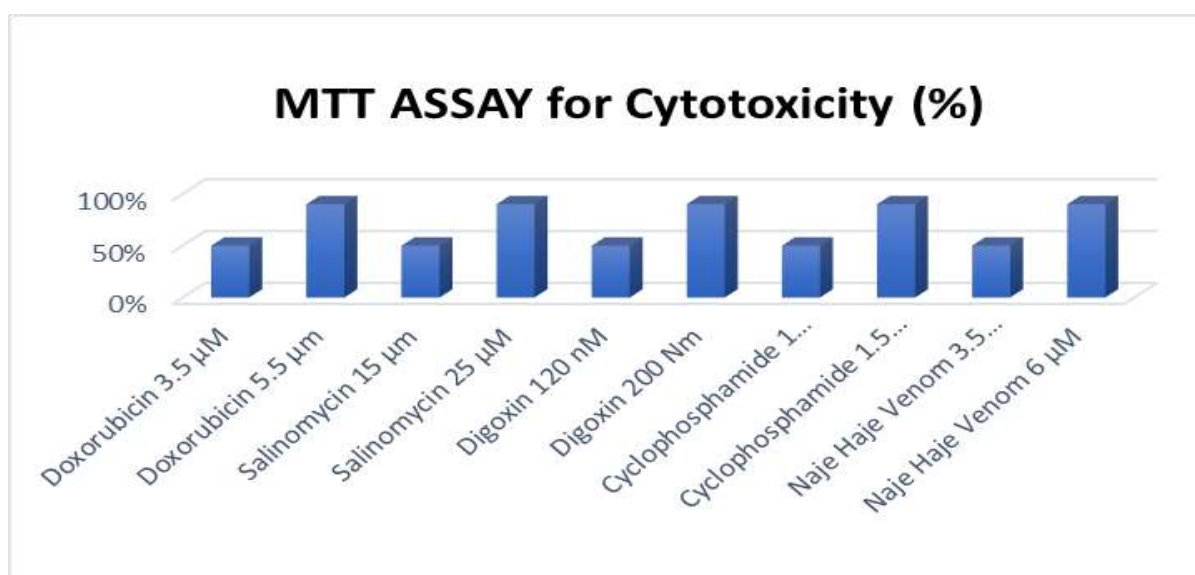


Figure 1: MTT assay for cytotoxicity (%) of the suggested therapeutic approaches in MDA-MB-231 cells: Bar graph showing the cell viability (%). Naja Haje venom showed the most potent cytotoxic effect with 90% cytotoxicity of 6 μ M of Naja Haje venom.

Gene expression analysis

The findings of our study demonstrate a significant difference between all groups as regards pro-apoptotic (Bax & P53) genes, tumor initiator Caspase-9, anti-apoptotic Bcl-2 gene, and cancer stem cells gene CD44.

In comparison with group 1 (positive control group), there was a statistically significant increase in Bax, p53, and Caspase-9 expression in group 2, group 3, group 4, and group 5 (F=988, 880, 1196, P < 0.001 for each). The most significant expression upregulation of Bax, P53, Caspase-9 was noticed in group 5 TNBCs which were treated with Naje Haje copra venom and Salinomycin (F= 2102, 2363, 3635, P < 0.001 for each). In contrast, the least significant upregulation of Bax, P53, Caspase-9 expression was

noticed in group 2, TNBCs which were treated with Doxorubicin only (F= 362, 73, 292, P < 0.001 for each).

Regarding Bcl-2 and CD44 gene mRNA expression, there was statistically significant downregulation in group 2, 3, 4, and 5 when compared with group 1 (F= 559, 903, P < 0.001 for each). The most significant downregulation of Bcl-2 and CD44 expression was noticed in group 5 TNBCs which were treated with Naje Haje copra venom and Salinomycin (F= 1901,7832, P < 0.001 for each).

On the other hand, the least significant downregulation of Bcl-2 and CD44 expression was noticed in group 2, TNBCs which were treated with Doxorubicin only (F= 150, 741, P < 0.001 for each) (**Figure 2**) (**Table 2**).

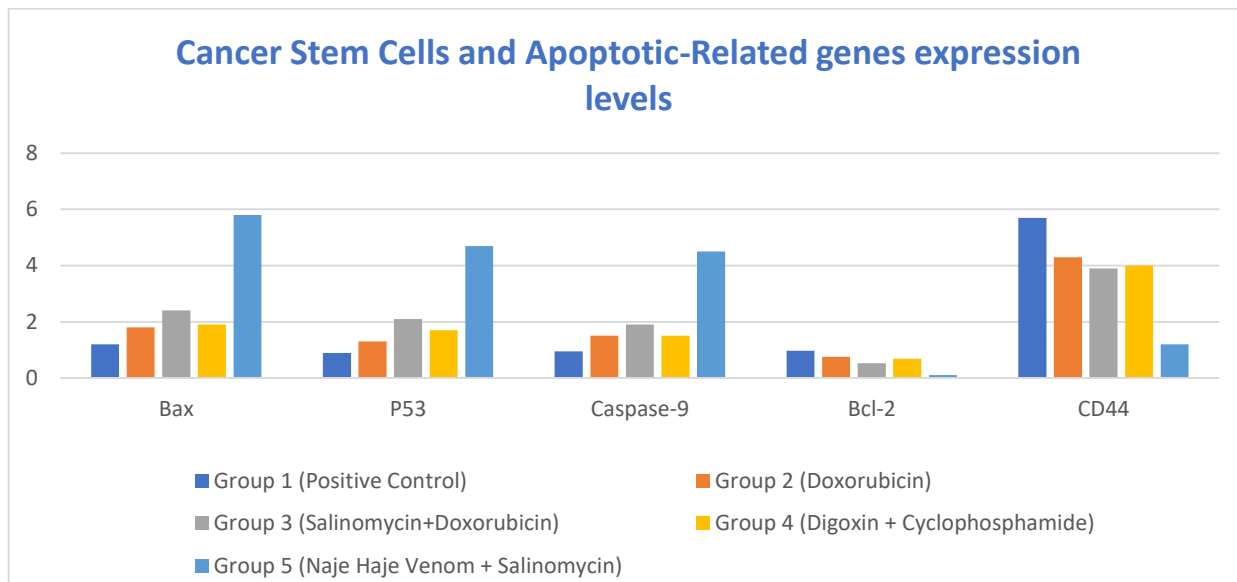


Figure 2: Mean values of Bax, P53, Caspase-9, and CD44 gene expression when normalized to GADPH gene expression in the studied groups at the end of the study. Bax, P53, Caspase-9 expression levels were significantly upregulated in group II (Doxorubicin), group III, (Salinomycin + Doxorubicin), group IV (Digoxin + Cyclophosphamide), and group V (Naje Haje Venom + Salinomycin) (P < 0.001 for each). On the other hand, Bcl2 and CD44 gene expression levels were significantly downregulated in groups 2,3,4, and 5 (P < 0.001 for each).

Table 2: A statistical comparison among the control group and the treated groups regarding pro-apoptotic, anti-apoptotic, and cancer stem cells gene expression levels.

			p-value	95% Confidence Interval	
				Lower Bound	Upper Bound
Bax	Group 1	Group 2	<0.001	-1.5526	-1.2140
		Group 3	<0.001	-2.6960	-2.3574
		Group 4	<0.001	-1.6993	-1.3607
		Group 5	<0.001	-4.7260	-4.3874
		Group 2	<0.001	-.6926	-.3874
P53	Group 1	Group 3	<0.001	-1.6759	-1.3708
		Group 4	<0.001	-1.1426	-.8374
		Group 5	<0.001	-3.8726	-3.5674
		Group 2	<0.001	-.6416	-.3918
		Group 3	<0.001	-1.2482	-.9984
Caspase-9	Group 1	Group 4	<0.001	-1.0349	-.7851
		Group 5	<0.001	-3.6749	-3.4251
		Group 2	<0.001	.1741	.2592
		Group 3	<0.001	.3908	.4759
		Group 4	<0.001	.2675	.3525
Bcl-2	Group 1	Group 5	<0.001	.8175	.9025
		Group 2	<0.001	1.2019	1.5448
		Group 3	<0.001	1.7186	2.0614
		Group 4	<0.001	1.4419	1.7848
		Group 5	<0.001	4.3219	4.6648
CD44	Group 1	Group 2	<0.001	1.2019	1.5448
		Group 3	<0.001	1.7186	2.0614
		Group 4	<0.001	1.4419	1.7848
		Group 5	<0.001	4.3219	4.6648

Table 3: A statistical comparison among the positive control group and the treated groups regarding cell viability.

Group	Cell Viability (%)
+ve Control	97.535 ± 1.097
Doxorubicin only	76.37 ± 0.959
Salinomycin + Doxorubicin	67.41 ± 0.792
Digoxin + Cyclophosphamide	34.0908 ± 0.984
(Naja Haje) venom + Salinomycin	18.0908 ± 0.883
F	273.985
P value	< 0.001

Results of cell viability test.

The finding of this test demonstrated significant inhibition of viable cells in treated groups with a combination of Salinomycin and Doxorubicin, a combination of Digoxin and Cyclophosphamide, and a combination of (Naja haje) venom and Salinomycin when compared to untreated cells or the cells which treated with Doxorubicin only (Table 3).

DISCUSSION

TNBCs are the most aggressive form of BC which is associated with poor prognosis, metastasis, and relapsing. Accumulated evidence confirmed that these characteristics referred to the present high amount of BCSCs [16]. At this time, the only available therapy for TNBCs is conventional chemotherapy which is associated with a high level of tumor recurrence [17].

Relapsing after conventional chemotherapy may be due to the presence of CSCs or other cancer cells which acquire stemness features due to the tumor microenvironment modification induced by chemotherapy [18].

Recently, several novel CSCs targeted therapeutic approaches are developed [19]. For instance, the usage of oncolytic herpes simplex virus type 1 (HSV1) is a promising therapeutic approach for eradicating CSCs [20]. Also, the usage of polyplexes to transport siRNA against the AKT2 gene which considered a cornerstone in tumorigenesis [21]. Additional studies used a gold nanoparticle- encapsulated drug to avoid the drug efflux transport system in CSCs [22] or combined a drug with another pharmaceutical (API) able to conquer CSCs. For instance, dox was combined in liposomes with salinomycin, and this combination exhibited a potential activity against CSCs [23].

In this study, we aimed to follow the previously discussed efforts regarding targeting breast cancer stem cells as a promising and novel therapeutic approach for successfully eradicate BCSCs and manage TNBC. So, we conducted our research study using a TNBCs cell line (MDA-MB-231). Then we started to investigate the efficacy of novel therapeutics like Salinomycin, Doxorubicin, Digoxin, Cyclophosphamide, and Naja Haje cobra venom conjugated in silica nanoparticles. Five treated and untreated groups were involved in our research as follow: Group one: Untreated TNBCs, Group two: TNBCs treated with Doxorubicin only, Group three: TNBCs treated with a combination of Salinomycin in addition to Doxorubicin, Group four: TNBCs treated with a combination of Digoxin + Cyclophosphamide and Group five: TNBCs treated with a combination of Silica nanoparticle-conjugated Egyptian cobra (Naja haje) venom + Salinomycin.

Then, after incubation of the cancer cells with therapeutics for 48 hours, we extracted the RNA and conducted an RT-PCR procedure to examine the expression of the pro-apoptotic (Bax & p53) genes, anti-apoptotic (Bcl-2) gene, the apoptosis initiator caspase-9, and the cancer stem cells (CD44) gene to assess the effectiveness of these suggested therapeutics in targeting and eradicating the tumor initiator cells (BCSCs).

The Bcl-2 proteins are considered as an essential apoptotic-related protein, either as apoptotic activators (Bax) or as apoptotic inhibitors (Bcl-xL) [24]. Bax and Bak are the effectors of the Bcl-2 family as consequent to activation, they modify confirmation, insert into the outer mitochondrial membrane, oligomerize, and induce mitochondrial outer membrane permeabilization (MOMP) [25]. Conversely, Bcl-xL is a powerful inhibitor of apoptosis which heterodimerize with Bax and neutralizes the effects of the latter. In the case of excess expression of Bcl-2, cells are protected against apoptosis. Instead, in the case of overexpression of Bax, cells become more susceptible to undergoing apoptosis.

In this study, we found that there was a significant difference among the studied groups regarding pro-apoptotic (Bax & P53) genes, tumor initiator Caspase-9, anti-apoptotic Bcl-2 gene, and cancer stem cells gene CD44. These significant differences confirmed the efficacy of these therapeutics in achieving a good prognosis, management, and probably eradication of TNBC.

Initially, we used the untreated TNBCs as a positive control group or as a normalization group for evaluating the efficacy of the used therapeutics.

In comparison with group 1 (positive control group), there was a statistically significant increase in Bax, p53, and Caspase-9 expression in group 2, group 3, group 4, and group 5 (F=988, 880, 1196, P<0.001 for each). This displayed the efficacy of the used therapeutics in inducing TNBCs apoptosis and control cancer cell proliferation.

The most significant expression upregulation of Bax, P53, Caspase-9 was noticed in group 5 TNBCs which were treated with Naja Haje cobra venom and Salinomycin (F=2102, 2363, 3635, P<0.001 for each).

This result confirmed that Naja Haje and Salinomycin combination is the most effective therapeutic combination for targeting TNBCs.

In contrast, the least significant upregulation of Bax, P53, Caspase-9 expression was noticed in group 2, TNBCs which were treated with Doxorubicin only (F=362, 73, 292, P<0.001 for each). This result displayed that Doxorubicin alone is the least effective therapeutic approach for targeting TNBCs.

Concerning Bcl-2 and CD44 gene mRNA expression, there was statistically significant downregulation in group 2, 3, 4 and 5 when compared with group 1 ($F= 559, 903, P < 0.001$ for each).

The downregulation of the anti-apoptotic gene expression Bcl-2 confirms the efficacy of the used therapeutics in mediating tumor cell apoptosis and so efficient TNBC management. The downregulation of the cancer stem cells gene CD44 approves the effectiveness of the involved therapeutic approaches in targeting BCSCs and inducing TNBC eradication. The most significant downregulation of Bcl-2 and CD44 expression was noticed in group 5 TNBCs which were treated with Naje Haje cobra venom and Salinomycin ($F= 1901,7832, P < 0.001$ for each). And this result also confirmed the previous conclusion stated that Naje Haje and Salinomycin combination is the most effective therapeutic combination for targeting BCSCs and managing TNBCs. While Doxorubicin alone is the least effective therapeutic approach for achieving the same goal.

So, these findings confirm the previous findings of **Liang *et al.* [26]** who reported that (TNBCs) cells can experience apoptosis by the successive stimulation of caspases 9, -3, and -6. An amplified level of caspase-9 is analogous to Bax gene expression, thus, mutually these proteins increased with increasing doxorubicin dose and incubation time. Upregulation of caspase-9 induced in cells treated with doxorubicin. Bcl-xL interacts with Apaf1 to avoid apoptosis by hindering Apaf1 dependent activation of caspase-9 [27]. These results propose that Doxorubicin induces apoptosis through the mitochondrial-dependent intrinsic pathway. Similarly, these results are compatible with the results of **Sharifi *et al.* [28]** Concluding that an increase in the Bax/Bcl-xL ratio and the caspase-9 level has an essential role in doxorubicin-induced apoptosis in (TNBCs) cells as a reduction in Bcl-xL triggered upregulation of caspase-9 levels in apoptotic cells.

Regarding group 3, our findings are matched with the previous conclusion of **Al Dhaheri *et al.* [29]** who confirmed that Salinomycin triggers double-strand breaks exposed by an accumulation of γ H2AX. The extent of DNA damage, which depends on the concentration of Salinomycin, regulates the response of the cells to these damages. A higher concentration of Salinomycin induces greater DNA damage, MDA-MB-231 cells trigger the apoptotic signaling pathway by activating caspase 3/9. On the other hand, lower concentrations of Salinomycin causes limited DNA damage which triggers through hyperacetylation of histone H3 and H4 which later induces upregulation of p21 expression.

Concerning Group 4, which was treated with a combination of Digoxin and Cyclophosphamide, our results are compatible with the conclusions of previous work by **Winnicka *et al.* [30]** who suggested that digoxin can induce apoptosis for the cancer cells by a cytotoxic mechanism mediated by a continuous increase of intracellular Ca^{2+} . More recent work by **Benítez *et al.* [31]** shows that activating caspases and DNA fragmentation is mediated by downregulation of intracellular K^+ levels which blocks caspase activation and apoptosis. Significantly, cardiac glycosides mutually trigger upregulation of Ca^{2+} and downregulation of K^+ . Furthermore, Cyclophosphamide is a promising therapeutic for BC which exerts its cytotoxic features through the alkylation of DNA. Cyclophosphamide is not cell-cycle phase-specific and metabolizes to an active form mediating protein synthesis inhibition via crosslinking of DNA and RNA [32]. Nearly all of the antineoplastic

characteristics of cyclophosphamide are referred to as the phosphoramidate mustard formed as a result of being metabolized by liver enzymes. The phosphoramidate metabolite forms cross-linkages within and between adjacent DNA strands at the guanine N-7 position. These alterations are permanent and finally lead to apoptosis [33].

Our finding regarding the therapeutic potency of the Egyptian cobra (Naje Haje) venom as anti-cancer agents is compatible with the previous finding of **Abir *et al.* [34]** who confirmed that Cerastes Viper venom possesses anti-cancer potential on human breast cancer in comparison with Cisplatin drug. This is positively related to the cell cycle arrest and apoptotic induction as well as apoptotic gene expression.

This result also agreed with **Gomes *et al.* [35]** and **Chaisakul *et al.* [36]** who reported that the snake venom cytotoxins affect targeting cancer cell proliferation, migration, invasion, neovascularization, and apoptotic activity through arresting cell cycle in the Pre-G1 population.

This similarly agreed with **Ebrahim *et al.* [37]** who reported that TNBCs cell lines treated with Cobra venom showed early apoptotic and late apoptotic as well as necrosis. This result may be explained by the cytotoxicity of snake venom that targeted cellular metabolism alterations and affected cancerous cells by blocking some specific ion channels, inhibiting angiogenesis, and activating intracellular pathways causing apoptosis **Calderon *et al.* [38]**.

This is correspondingly agreed with **Zhaoyu *et al.* [39]** who reported that the presence of two main pathways of apoptosis. The extrinsic pathway is stimulated by binding of ligands to the tumor necrosis factor- α (TNF α) receptor after that oligomerization and enrollment of procaspase-8 through adaptor molecules to initiate the death-inducing signaling complex. Instead, cellular stress is the main stimulator of the intrinsic pathway, causing the cytochrome-c releasing which mediates procaspase-9 activation through the interaction with apoptosis promoting activating factor-1 (APAF-1) and creation of an active apoptosome complex. Bcl-2 family proteins control DNA damage-induced apoptosis by regulating cytochrome c releasing in case of DNA damage.

CONCLUSION

Agents that inhibit cancer stemness may complement the antineoplastic activity of chemotherapy by eradicating drug-resistant CSCs or hindering the capacity of tumor cells to obtain features of CSCs. We found that treatment of triple-negative breast cancer with various anti-cancer agents like, Salinomycin, digoxin, and Silica nanoparticle-conjugated Egyptian cobra (Naja haje) venom combined with the conventional chemotherapy of breast cancer like doxorubicin and cyclophosphamide had significantly greater antitumor activity than treatment with conventional chemotherapeutic agents alone. We speculate that Salinomycin, digoxin, and Silica nanoparticle-conjugated Egyptian cobra (Naja haje) venom can efficiently target BCSCs, inhibit the tumor stemness, and resistance to conventional therapy serve in improving the response to chemotherapy and enhance the survival of breast cancer patients.

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