RESEARCH ARTICLE

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The Synergistic and Anticancer Potential of *Withania Somnifera* (Ashwagandha) Ethanol Extract as an Adjuvant with Doxorubicin in MCF7 Breast Cancer Cell Line

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Abstract

Background: Recent approaches in breast cancer treatment involve exploring natural extracts either as substitutes for traditional chemotherapeutic agents or as adjuvants to enhance their effectiveness. In this context, ethanolic extracts from *Withania Somnifera* (WS) and *Arctium Lappa* (AL) have gained attention for their potential anticancer properties across various cancer types. Objective: This study aimed to investigate the in vitro anticancer efficacy of ethanolic extracts from both WS and AL in the MCF7 breast cancer cell line. **Methods:** The study encompassed assessments of total antioxidant activity, total flavonoids, total phenolic compounds, and cytotoxicity for each extract. Unraveling the molecular mechanisms underlying the action of these extracts involved Real-time PCR and flow cytometry to analyze apoptotic markers and cell cycle dynamics, respectively. **Results:** Results indicated that the WS ethanolic extract exhibited better efficacy, especially in terms of IC_{50}. To explore its potential as an adjuvant with doxorubicin (DOX) in breast cancer therapy, 16 non-constant combination ratios were tested. The combination of 1/2 WS to 1/4 DOX, with a combination index (CI) of -0.229 was selected for further downstream molecular analysis. Gene expression analysis revealed a significant upregulation ($p < 0.05$) of *P53, Cas3, Cas7*, and *Bax*. Flow cytometric analysis further highlighted cell cycle arrest at the S phase. Additionally, the study explored the synergistic inhibitory effects of WS and DOX on key enzymes in the glycolytic pathway, namely, pyruvate kinase and aldolase. **Conclusion:** In conclusion, the ethanolic extract of WS demonstrated a synergistic effect as an adjuvant with DOX, showing promise as a combination therapy for breast cancer.

Keywords: *Withania Somnifera*- Breast Cancer- Cell Cycle Arrest- Adjuvant

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Introduction

Breast cancer is one of the most aggressive and life-threatening diseases. According to the GLOBOCAN 2020 report on cancer incidence and mortality estimates, it was identified as the second most prevalent malignancy, accounting for a substantial 2.3 million new cases (11.7%) [1]. It is the fifth leading cause of cancer-related death, contributing to 6.6% of global cancer deaths. Globally, the age-standardized incidence rate is significantly higher in countries with higher human development indices (HDIs), registering 54.5 per 100,000 in the female population, versus 31.3 in countries with lower HDIs to moderate contrast [1]. Furthermore, its impact on the female population is profound; In 2018 alone, 2,088,849 new cases of breast cancer were diagnosed, and 626,679 people died. The age-adjusted global rate was 46.3 per 100,000 population, reflecting a nearly four-fold difference worldwide [1]. Especially in Egypt, breast cancer constitutes a large proportion, representing 38.85% of all female cancer cases, and stands out as the primary cancer among Egyptian women [2].

The approved chemotherapeutic agent for breast cancer is Doxorubicin (DOX), classified among the first-line drugs for breast cancer chemotherapy [3]. This antibiotic, with antineoplastic activity, originates from the Streptomyces peucetius bacterium and has been extensively employed in chemotherapy since the 1960s. As a member of the anthracycline group of chemotherapeutic agents, DOX may be used in the treatment of soft tissue and bone sarcomas, as well as cancers affecting the breast, ovary, bladder, and thyroid [4]. Despite the demonstrated clinical efficacy of DOX in the treatment of breast cancer, its significant side effects pose a challenge in terms of

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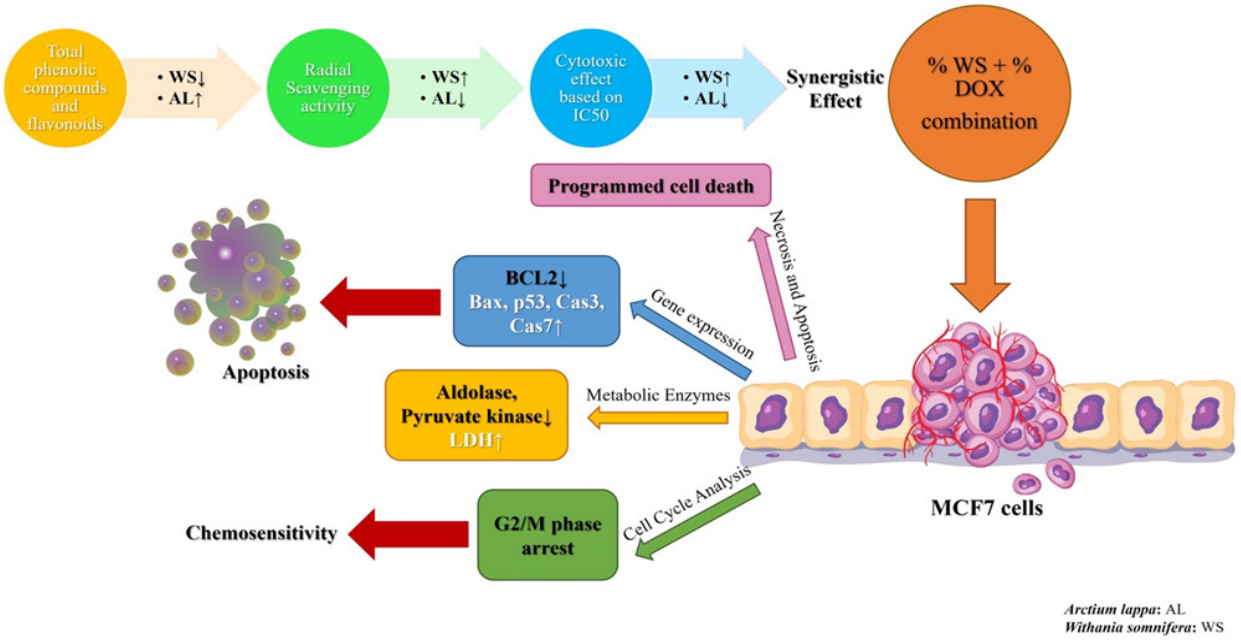

Graphical Abstract. The Potential of Natural Adjuvant Therapy, Specifically Withania somnifera (WS) and Arctium Lappa (AL), in amplifying the effectiveness of doxorubicin (DOX) against MCF7 cancer cells.

tolerability. The intravenous administration of DOX leads to severe tissue ulceration and necrosis, a condition that exacerbates over time. Furthermore, the drug is associated with cardiotoxicity, limiting the long-term use of therapies. The mechanism of DOX-induced cardiotoxicity is distinct from its antitumor activity, which includes increased oxidative stress, downregulation of cardiac genes, and induced cardiac myocyte cell death [5]. Therefore, many natural products have been extensively studied for their potential as anticancer agents in cancer therapy, including plant extracts and marine extracts [6, 7]. *Withania somnifera* (WS), commonly known as Ashwagandha, belongs to the Solanaceae family [8]. Several research articles have highlighted its anti-cancer properties. In Ayurvedic medicine, an ancient system of holistic medicine that originated in India, parts of the plant have been used in prescriptions for many years, and their anti-cancer properties were first demonstrated in 1992 [9]. WS exhibits the inhibition of cancer cell proliferation by inducing apoptosis through the production of reactive oxygen species (ROS) and induces programmed cell death in various cancers [10, 11]. The most bioactive element of WS, named withanolides, exhibits very potent anticancer activity [12]. Moreover, WS exhibits a spectrum of beneficial biological activities, including anti-inflammatory [12], anti-stress [13], antioxidant [14], and anti-angiogenic effects [15]. Notably, WS has been found to enhance the synthesis of BCL-2, a protein that is crucial in regulating programmed cell death through the mitochondria-dependent pathway, helping in the reduction of breast cancer progression and improved patient survival rates [16, 17]. Another study delved into the induction of apoptosis by WS, investigating reactive oxygen species (ROS) formation associated with mitochondrial dysfunction [18]. *Arctium Lappa* (AL), commonly known as Burdock and belonging to the Asteraceae family, has been demonstrated to possess antioxidant, anti-inflammatory, and anticancer activities [19]. Recent research specifically explored burdock's cytotoxicity on MCF7 cell lines, yielding significant results [20].

In this context, both WS and AL can be considered potential anti-cancer agents or adjuvants for cancer therapy. Previous studies have individually highlighted the anti-cancer activities of WS and AL. This present study aims to uncover a potential combinational therapy for breast cancer by delving into the cytotoxic effects of WS and AL. The goal is to identify a promising candidate that can be synergistically combined with DOX as an adjuvant. The comprehensive investigation of this selected therapeutic approach depends on assessing the gene expression of key apoptotic genes, the enzymatic activity of metabolic proteins crucial in cancer metabolism, and the analysis of the cell cycle.

Materials and Methods

Materials

*Arctium lappa (*AL) and *Withania somnifera* (WS), cultivated in Sinai, Egypt, were generously provided by Prof. Nassar Abdelateef from the Botany Department, Faculty of Science, Mansoura University, Egypt. Doxorubicin (DOX) (Hui Chem, China; Cat. no. 284461–73-0) was used as the chemotherapeutic agent. Human breast cancer (MCF7), which is an invasive breast ductal carcinoma with luminal epithelial phenotype and normal human skin melanocyte (HFB4) cell lines; a model to study the complexity of cellular aging were acquired from the National Cancer Institute, Egypt (Source: ATCC). The cell culture was maintained using DMEM medium (Gibco, USA; Cat. no. 11995073), supplemented with Fetal Bovine Serum (FBS) (Gibco, USA; Cat. no. 10099133), L-glutamine (Gibco, USA; Cat. no. 25030024), Penicillin/

Streptomycin (Gibco, USA; Cat. no. 10378016), and Phosphate Buffer Saline (PBS), pH 7.4 (GIBCO, USA; Cat. no. 10010023). The dissociation of cells was performed using 0.25% Trypsin-EDTA (GIBCO, USA; Cat. no. 25200056). MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was conducted using reagents from Invitrogen, USA (Cat. no. M6494), and the solvent used for dissolving formazan crystals was Dimethyl sulfoxide (Sigma Aldrich, USA, Cat. no. 673439).

Ethanolic extraction of Withania Sominfera (WS) and Arctium Lappa (AL)

WS and AL were obtained in the form of ground plants. The crude material was extracted by immersing 100 gm of each plant separately in 250 ml of 95% ethanol and then incubated at room temperature for 48 hours with intermittent shaking. The resulting crude extracts were collected and filtered through the Whatman No.1 filter paper. Subsequently, the extracts were concentrated at 50°C using a rotary evaporator. The resulting oily extracts from each plant were collected and stored at 4°C until utilized.

Cytotoxicity assay

MCF7 and HFB4 cells were cultured in DMEM medium supplemented with 10% Fetal Bovine Serum, 2% L-glutamine, and 1% Penicillin/Streptomycin within a humidified 5% CO2 atmosphere at 37°C. Regular mycoplasma testing was routinely conducted using PCR to ensure the absence of contamination, maintaining the integrity and reliability of cell cultures. Periodic rinsing and washing of the cells with PBS, pH 7.4, was performed, and cells were detached using 0.25% Trypsin-EDTA. Subculturing in fresh complete DMEM medium occurred when cells reached 80-90% confluence, typically after 48 hours. The MTT-based cytotoxicity assay previously described [21] involved applying serial dilutions of single WS and AL (25, 50, 100, 200, and 400 µg/ml) and DOX (1.25, 2.5, 5, 10, and 20 µg/ml) to both MCF7 and HFB4 cells, incubating for 48 hours. For MCF7 cells, 16 combinations of WS and DOX at non-constant ratios were applied and incubated for 48 hours. The inhibitory concentration at 50% (IC_{50}) of WS and DOX, along with the combination index (CI), were calculated as described previously [21].

Gene expression analysis

MCF7 cells were subjected to the most effective combination ratio of WS and DOX. Total RNA was extracted from both untreated (negative control) and treated MCF7 cells using the RNeasy microextraction Kit (Qiagen, Germany, Cat no. 74004). The concentration and purity of RNA were assessed using Nanodrop2000, and RNA integrity was verified by 1.5% agarose gel electrophoresis. Subsequently, 500 ng of RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific, USA, Cat no. 4368814). The synthesized cDNA was utilized for determining the relative gene expression of p53, Bax, BCl2, Cas3, and Cas7 genes, normalized against GAPDH, using 2X Maxima SYBR Green/ ROX qPCR Master Mix (Thermo Scientific, USA, cat no. K0221) with the primers listed in Mansour et al. 2021 [21]. The qPCR was conducted in a 25 µl reaction mixture, comprising 1 µl cDNA, 1 µl of each primer (10 pmol), and 12.5 µl of Maxima SYBR Green Master Mix. The amplification protocol involved an initial denaturation step of 10 min at 95°C, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, utilizing the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument. A melting curve was generated by raising the temperature from 72 to 95°C at the end of the last cycle. GAPDH served as the housekeeping gene for normalizing relative expression differences, and the data were analyzed using the $2-\Delta\Delta Ct$ method [22].

Cell cycle analysis

The impact of combined DOX and WS on the cell cycle of MCF7 cells was scrutinized through flow cytometry. Cells, both untreated and treated, were suspended in warm PBS, fixed by ice-cold 96% ethanol, and then incubated at -20°C for 24 hours. Following washing the cells with PBS, cells were immersed in propidium iodide (PI) solution [100 µl (0.02 mg/ml) PI, 50 µl (0.2 mg/ml) RNase A, and 0.1% v/v Triton X-100 in PBS] and kept in the dark for 40-50 min at room temperature. Subsequently, the samples were analyzed using the Attune flow cytometer (Applied Biosystems, USA), with the forward scatter (FSC) and side scatter (SSC) measured to identify single cells.

Measuring total phenolics and flavonoids of WS and AL

For the quantification of total phenolic compounds, a stock solution of Gallic acid (1 mg/ml in methanol) was prepared, and seven serial dilutions ranging from 1000 to 50 µg/ml were created. Samples, prepared in concentrations of 5 mg/mL in DMSO, were assessed at 630 nm. Likewise, for total flavonoid determination, a Rutin stock solution (1 mg/ml in methanol) was prepared, and serial dilutions were made in concentrations of 1000, 600, 400, 200, 100, 50, and 10 µg/ml. The samples, also prepared in concentrations of 5 mg/mL in DMSO, were measured at 420 nm [23, 24].

Antioxidant effect of WS and AL

According to the DPPH protocol [25], a 0.1mM DPPH solution was prepared by dissolving 4 mg of DPPH in 100 ml of methanol. Various concentrations of WS and AL extracts (10, 20, 30, 40, 50, and 60 mg/ml) were prepared, and DPPH (0.1 mM) solution was added to each tube. DPPH only served as the control, and methanol was employed as the blank. The reaction mixture was incubated in the dark at room temperature for 20 minutes. Absorbance was read at 517 nm, and the percentage of radical scavenging activity was calculated using the formula: %RSA = (Abs control - Abs sample/ Abs control) × 100.

Measuring metabolic enzymes in WS/DOX-treated MCF7 cells

The levels of LDH, Aldolase, and Pyruvate kinase were measured using the Lactate Dehydrogenase B (LDH-B)

Human SimpleStep ELISA® Kit (Abcam, UK, cat no. ab183367), Aldolase Activity Assay Kit (Colorimetric) (Abcam, UK, cat no. ab196994), and Pyruvate Assay Kit (Colorimetric/Fluorometric) (Abcam, UK, Cat no. Ab65342) respectively. Measurements were taken at 450 nm using the ROBONIK P2000 ELISA READER.

Statistical Analysis

The experimental data was statistically analyzed by student-independent T-test using GraphPad Prism version 10.2.1. Data were accepted as significantly different when $p < 0.05$.

Results

Total phenolic compounds and flavonoids as well as radical scavenging activities of crude ethanolic extracts of Arctium lappa (AL) and Withania somnifera (WS)

In the context of managing the side effects of chemotherapeutic administrations, diverse approaches are under investigation to enhance the therapeutic process. Recent studies emphasize the use of natural products as adjuvants [26], given their abundance and cost-effectiveness. As illustrated in Tables (1 and 2), it is apparent that the crude ethanolic extract of AL leaves exhibits higher total phenolics and total flavonoids compared to WS. Conversely, WS demonstrates superior radical scavenging activity compared to AL and the standard Ascorbic acid.

Effect of WS, AL, and DOX on MCF7 and HFB4 cell viability

The present study showed the least cytotoxic effect of ethanolic extract AL on MCF7 with IC_{50}=131.2 ug/ml, even though it was moderately toxic on human skin normal cells IC_{50}=62.33 ug/ml, indicating lower safety on normal cells (Figure 1). On the other hand, ethanolic extract of WS showed higher toxicity against MCF7 with IC_{50}=47.13 ug/ml with moderate safety on normal HFB4 cells with IC_{50}=149.8 ug/ml (317% of the IC_{50} on MCF7). DOX showed the highest toxicity on MCF7 with IC_{50} =4.73 ug/ml with great cytotoxic effect and anticancer activity as measured by IC_{50} of crude ethanolic extracts of *Arctium lappa* (AL) and *Withania somnifera* (WS) leaves against human breast cancer cell line MCF7 and normal human skin cell line HFB4 as compared to Doxorubicin (DOX) (Table 3). Subsequently, one extract between AL and WS was selected to be combined with DOX as an adjuvant according to the best IC_{50} value on both breast cancer and normal cells, which is WS. Recent studies revealed that the IC_{50} of AL is 97 ug/ml after 48 hour-treatment [27], ~32 ug/ml of WS [28], and ~ 1.8 μM of DOX [29].

Effect of combination of WS/DOX on MCF7 cell viability

Sixteen different non-constant combination ratios were investigated between WS and DOX to elucidate their synergistic and antagonistic relationship (Table 4). Each ratio exhibited a distinct cytotoxic effect on MCF7 compared to individual treatments. The calculated combination index (CI < 1) indicates a synergistic effect between WS and DOX, as represented in Table 1. Notably, the ½ WS: ¼ DOX combination demonstrated a CI of 0.229, signifying a synergistic effect. This combination was chosen for further analysis due to its potential advantages, notably the ability to reduce the dosage of DOX, which could help preserve normal cells. This study marks the demonstration of the potential of WS to be used as an adjuvant with DOX in breast cancer treatment for the first time, supported by a CI < 1. Previous studies primarily focused on the singular administration of WS [30]. The ½ WS: ¼ DOX combination can potentially be used to enhance the efficacy of breast cancer treatment with reduced side effects.

Gene expression levels of pro-apoptotic and anti-apoptotic target genes in treated MCF7 cells with ½ WS: ¼ DOX combination ratio compared to untreated MCF7 cells

The expression of the pro-apoptotic gene Bax was significantly increased in WS/DOX-treated MCF7 cells compared to untreated ones with fold changes of 1.53. Bax is known for promoting apoptosis, and its upregulation suggests induction of programmed cell death (Figure 2) [31]. The genes Cas3 and Cas7, both associated with the execution phase of apoptosis, exhibited substantial upregulation [32]. Their fold changes were 2.81 and 1.82, respectively. This indicates that the combined treatment led to a significant activation of processes involved in the

Table 1. Total Phenolic and Total Flavonoid Content of Crude Ethanolic Extract of *Arctium lappa* (AL) and *Withania somiefra* (WS) Leaves

Extract	Total Phenolics content (mg/g) extract	Total flavonoid content (mg/g) extract
AL	40.62 ± 3.92	10.26 ± 0.96
WS	20.99 ± 1.36	3.95

Table 2. Percentage of Radical Scavenging Activity (RSA) of Crude Ethanolic Extract of AL and WS Leaves as Compared to Standard Ascorbic Acid.

Concentrations mg/ml	% RSA of AL	%RSA of WS	% RSA of Ascorbic Acid
60	84.6%	94%	88.5%
50	10%	94.5%	85.6%
40	5%	62%	88.8%
30	6%	61%	88%
20	0%	75%	83%
10	2.4%	54%	88%

Table 3. IC_{50} and Selectivity Index of Crude Ethanolic Extracts of AL and WS Leaves against Human Breast Cancer Cell Line MCF7 and normal human skin cell line HFB4 as compared to Doxorubicin.

	AL	WS	DOX
IC_{50} on normal human cell line (HFB4) μg/ml	62.33	131.2	
IC_{50} on MCF7 cancer cell line μg/ml	149.8	47.13	4.378
Selectivity Index	0.475	3.17	

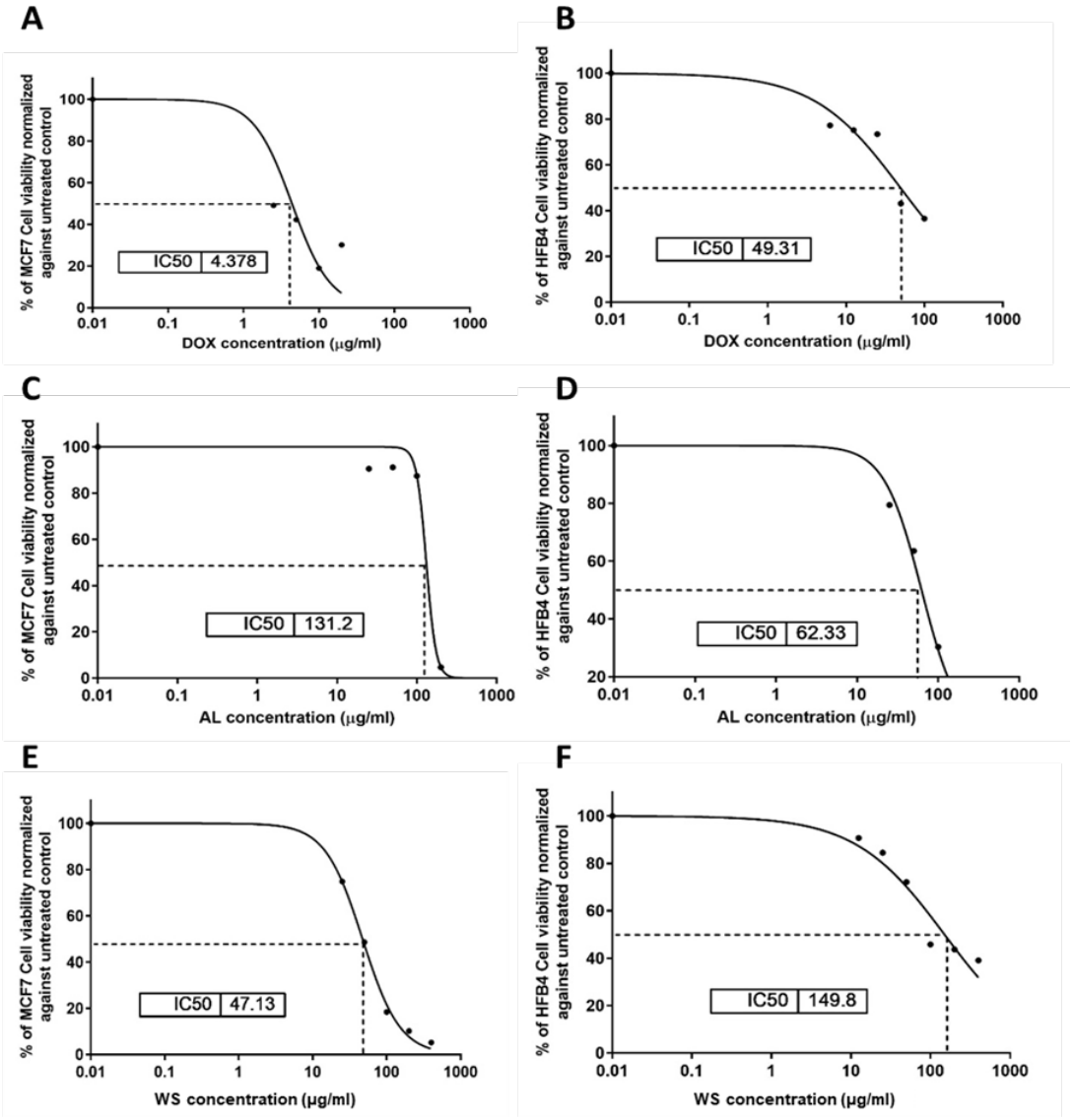

Figure 1. The Sigmoidal Dose-Response Curve of WS, AL, and DOX on % viability of MCF7 and HFB4 Cells and their IC_{50} after 48 hours.

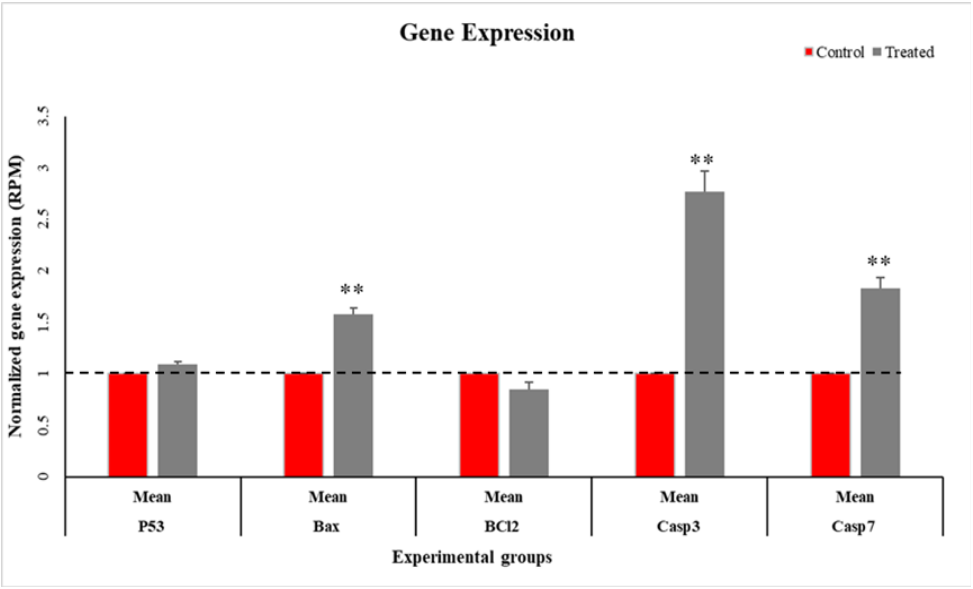

Figure 2. Graphical Representation of Real-Time PCR Results Showed the Relative Expression Level of Bax, caspase 3 (Cas3), caspase 7 (Cas7), BCl2, and P53 genes in MCF7 cells after administration of combined WS to DOX, for 48 hours. The experiment was conducted in triplicate (n = 3), $p < 0.05$.

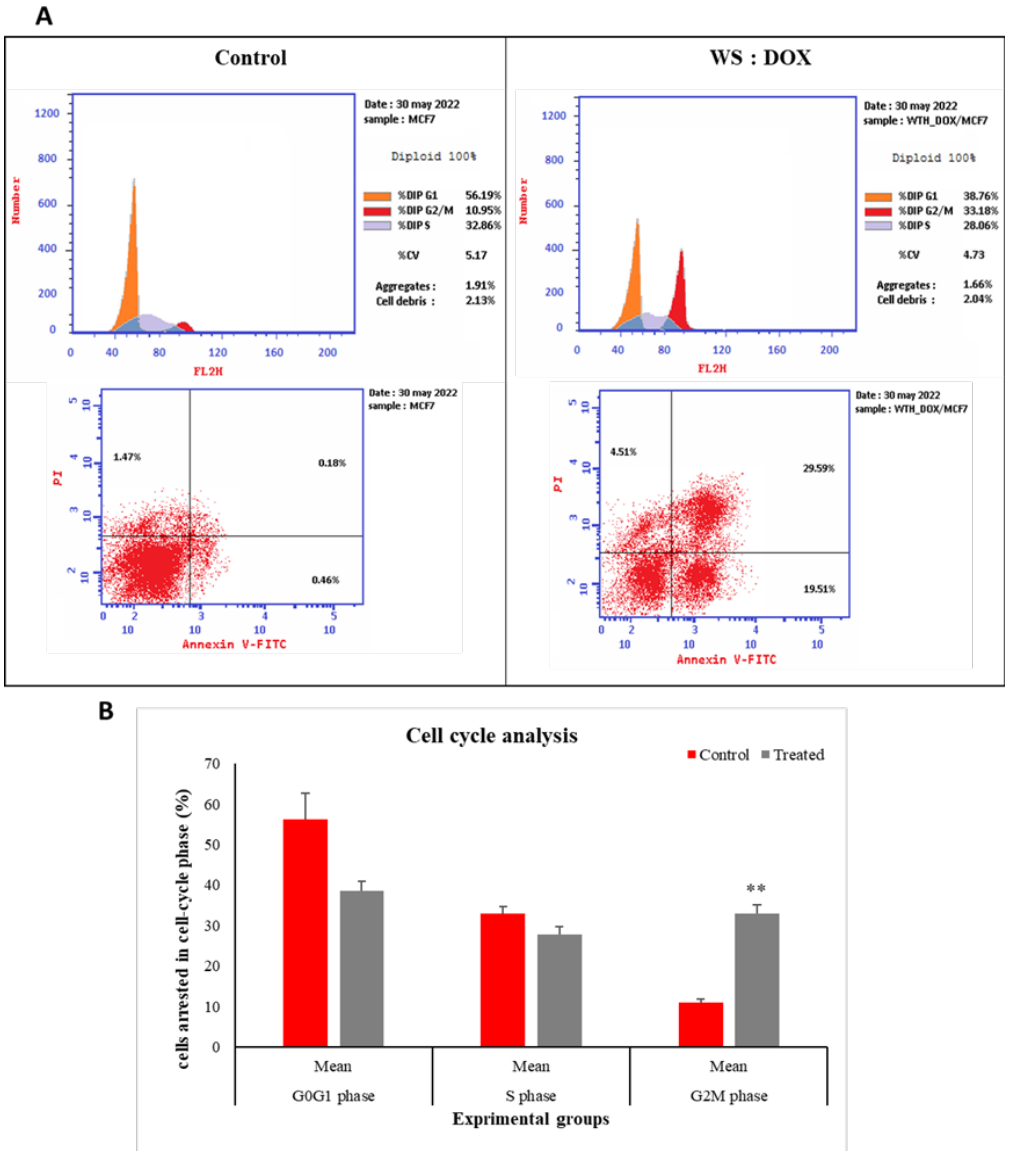

Figure 3. Cell Cycle Analysis. (A) The impact of WS/DOX on the cell cycle of MCF7 cells was assessed using flow cytometry in comparison to the control. (B) Data were presented as the mean percentage of cells ± standard error (SE). The experiment was conducted in triplicate (n = 3), $p < 0.05$.

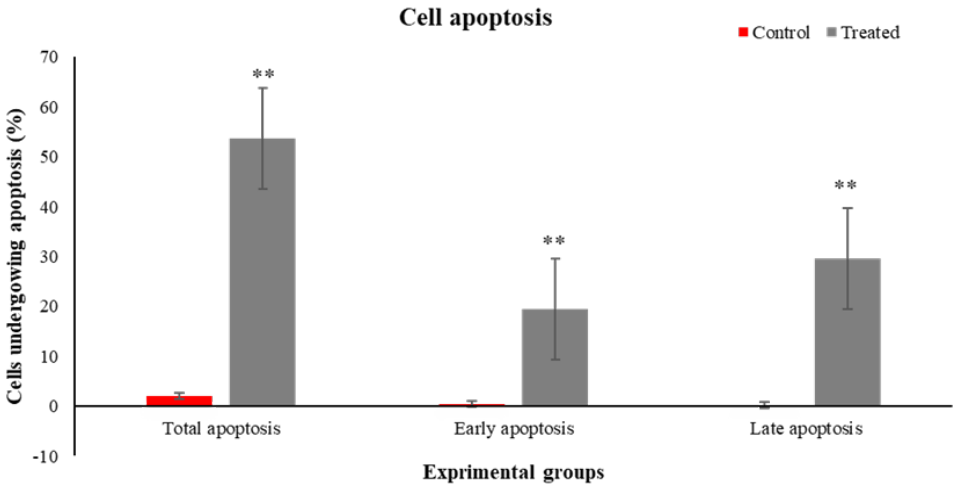

Figure 4. Effect of WS/DOX Treatment on Apoptosis of MCF7 Cells. Apoptosis was estimated by using Annexin-V stain detected by flow cytometry. The results are presented as mean ± standard error (SE), and all samples were measured independently in triplicate (n=3), $p < 0.05$.

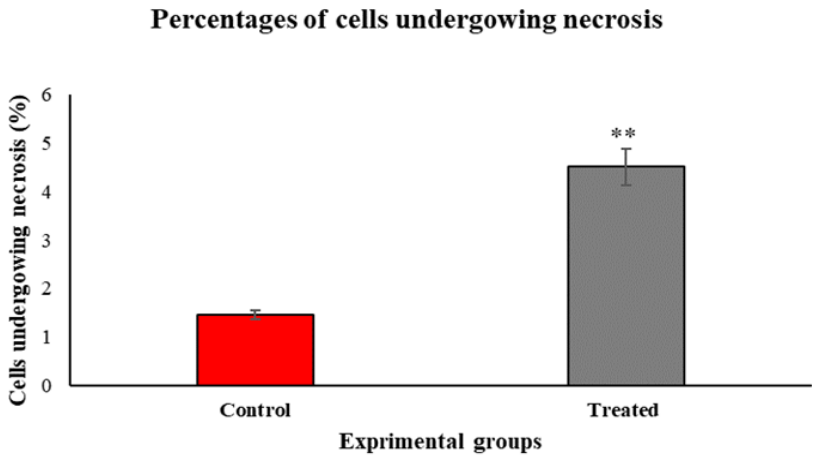

Figure 5. Effect of WS/DOX Treatment on Necrosis of MCF7 Cells. The results are presented as mean ± standard error (SE), and all samples were measured independently in triplicate (n=3), $p < 0.05$.

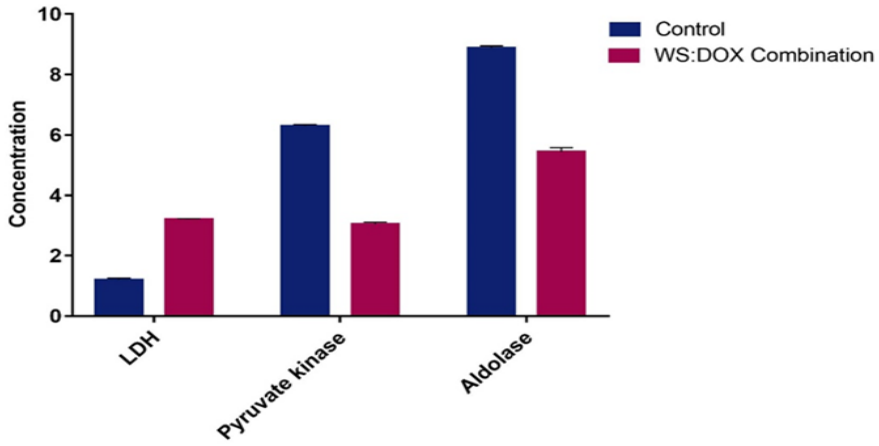

Figure 6. The Levels of Metabolic Enzymes. The graph represents LDH conc (ng/ml), Pyruvate Kinase (U/ml), and Aldolase (nmol) levels before and after treating MCF7 cells with WS and DOX.

Table 4. Cytotoxic Effect and Combination Index (CI) of 16 Different Concentration Ratios between WS and DOX. The second lowest CI With the lowest DOX concentration (*), shows a high synergistic effect.

Ratio WS: DOX	WS (µg/ml)	DOX (µg/ml)	Inhibitory Effect percentage	Viability percentage	Combination index (CI)	Interaction type
½: ½	23.57	2.19	80.8	19.2	0.219	synergism
½: ¼	23.57	1.09	78.8	21.2	0.229*	synergism
½: ¾	23.57	3.28	80.9	19.1	0.245	synergism
½: 1	23.57	4.38	81	19	0.271	synergism
¾: ¼	35.35	1.09	72	28	0.544	synergism
¾: ½	35.35	2.19	72.5	27.5	0.59	synergism
¾: ¾	35.35	3.28	79.6	20.4	0.366	synergism
¾ :1	35.35	4.38	81.1	18.9	0.347	synergism
1: ¼	47.13	1.09	72.4	27.6	0.708	synergism
1: ½	47.13	2.19	74.5	25.5	0.649	synergism
1: ¾	47.13	3.28	79.3	20.7	0.469	synergism
1:01	47.13	4.38	80	20	0.471	synergism
2: ¼	94.26	1.09	66.2	33.8	2.104	antagnoism
2: ½	94.26	2.19	67	33	2.064	antagnoism
2: ¼	94.26	3.28	69.2	30.8	1.839	antagnoism
2: ¾	94.26	4.38	77	23	1.065	antagnoism

execution of apoptosis (Figure 2). In contrast, the anti-apoptotic gene BCl2 showed a significant downregulation with a fold change of 0.843. BCl2 is known for inhibiting apoptosis, and its reduced expression suggests suppression of anti-apoptotic signals (Figure 2) [33]. This gene expression pattern strongly indicates that the combined treatment of WS and DOX is promoting apoptosis in MCF7 cells.

Effect of combined treatment of WS/DOX on the cell cycle of MCF7 cells

The combined treatment of WS/DOX has led to a notable cell cycle arrest primarily at the G2/M phase, coupled with minor changes observed in the S phase, in contrast to the control cells, with statistical significance at $p < 0.05$ with a noticeable decrease in the number of cells in the G0/G1 phase (Figure 3). The G2/M phase arrest indicates that the combinational treatment has interfered with the normal progression of the cell cycle, potentially impeding cell division and proliferation [34]. The reduction in the G0/G1 phase suggests a decreased proportion of cells in the resting phase, further supporting the idea that the combinational treatment is influencing the dynamics of the cell cycle [35]. These findings provide valuable insights into the mechanism of action of the WS/DOX combination, highlighting its potential impact on cell cycle regulation.

Effect of combined treatment of WS/DOX on the apoptosis and necrosis of MCF7 cells

Combination Treatment (WS/DOX): Refers to the simultaneous administration of WS and DOX. Knowing that the use of a combination of treatments is common in cancer therapy to exploit potential synergistic effects. The Combined treatment (WS/DOX) used in this study has led to programmed cell death (apoptosis) and cellular damage resulting in cell death (necrosis) (Figures 4 and 5). These results indicate that the combination treatment of WS and DOX is having a significant impact on cell death processes, as evidenced by the induction of apoptosis and necrosis when compared to untreated cells. This is a positive outcome, indicating the potential effectiveness of the combined treatment in combating cancer cells.

Effect of combined treatment of WS/DOX on the metabolic enzymes of MCF7 cells

MCF7 cells subjected to the combined treatment of WS and DOX exhibited a considerable elevation in lactate dehydrogenase (LDH) levels, as illustrated in Figure 6.

Discussion

Concurrently, there was a noteworthy reduction in the levels of aldolase and pyruvate kinase. Notably, DOX is known to elevate oxidation levels, as indicated in previous studies [36, 37]. These outcomes suggest that WS possesses potent antioxidant properties, suggesting its potential to mitigate oxidative stress when combined with DOX. Intriguingly, the selected combination demonstrated a significant decrease in LDH, a metabolic enzyme that typically sees an increase in breast cancer scenarios [38]. This dual impact on reducing LDH and enhancing antioxidant activity suggests a potential therapeutic synergy in the WS/DOX combination, emphasizing its promise in addressing aspects linked to breast cancer metabolism and oxidative stress.

In Conclusion, this study strategically explored the potential of natural adjuvant therapy, represented by *Withania somnifera* (WS), to enhance the efficacy of doxorubicin (DOX) against MCF7 breast cancer cells. The main goal was to achieve an enhanced therapeutic impact with minimal dosage, thereby optimizing treatment outcomes while concurrently minimizing the risk of side effects on normal cells. The combination of WS with DOX demonstrated synergistic effects, as evidenced by decreased LDH levels, increased aldolase and pyruvate kinase levels, and notable apoptosis induction. While the results hold promise for an enhanced treatment strategy for breast cancer, certain limitations should be addressed. The study was limited to in vitro experiments using a single cell line, which may not fully represent the complexity of breast cancer subtypes or in vivo conditions. Variability in natural extracts due to differences in plant growth conditions and extraction methods also poses challenges for reproducibility. Furthermore, while key apoptotic markers and glycolytic enzymes were analyzed, deeper mechanistic insights and validation in vivo are necessary for translation into clinical practice. Nonetheless, these findings underscore the potential of natural adjuvants like WS to refine the landscape of cancer treatment standards, paving the way for more targeted and resource-efficient therapeutic interventions. Future research addressing the limitations will strengthen the evidence for incorporating WS as a reliable adjuvant therapy in breast cancer treatment.

Author Contribution Statement

All authors contributed equally in this study.

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None.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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