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Effect of aloin on some gene biomarkers for epithelial-mesenchymal transition in breast cancer *in vitro* compared to an anthracycline analog (Doxorubicin)

Fayrouz A. Muhammad[1], Mahmoud N. El-Rouby[2], Mahmoud M. Said[1], Amr Y. Esmat[1*]

[1] *Biochemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt.*
[2] *Cancer Biology Department, National Cancer Institute (NCI), Cairo University, Egypt.*

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ABSTRACT

Epithelial-mesenchymal transition (EMT) is a biologic process that permits an epithelial cell to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype with enhanced migratory capacity and invasiveness. In the present study, the cytotoxic activity of aloin, a natural anthraquinone glycoside, was assessed against a breast cancer cell line (T47D) using MTT and clonogenic assays, compared to doxorubicin, an anthracycline analog. The effects of exposure of T47D tumor cells to IC_{50} values of aloin and doxorubicin (181.5 and 0.17 µM, respectively) for 72 h on the mRNA expression levels of a non-receptor tyrosine kinase (JAK2), a transcription factor (STAT5a) and some biomarkers for EMT were evaluated to provide new prognostic and therapeutic markers for estrogen receptor-positive breast cancer progression. Exposure to aloin down-regulated the expression levels of JAK2, STAT5a and vimentin mRNA in T47D cells, while the expression levels of E-cadherin and ZO-1 mRNA were not significantly changed. On the other hand, exposure of breast tumor cells to doxorubicin up-regulated the expression of E-cadherin mRNA, whereas the expression levels of JAK2, STAT5a, ZO-1 and vimentin mRNA were not affected. In conclusion, the chemosensitivity of aloin towards breast tumor cells is due to inhibition of JAK2/STAT5a signaling, which results in the inhibition of vimentin expression as a mesenchymal marker of EMT.

Introduction

Breast cancer is the leading cause of cancer death globally. It is the second most common cause of cancer deaths among women and represents 14.3 % of all deaths in the developing countries [1], and represents 35.1 % of all female cancers in Egypt [2]. The primary cause of breast cancer death is metastasis, which is regulated by several factors and signaling pathways, such as epithelial-mesenchymal transition (EMT), which is defined as the loss of epithelial characteristics and acquiring a mesenchymal phenotype. However, two recent studies highlighted an unexpected role of EMT in cancer drug resistance, while challenging the role of EMT in cancer metastasis [3, 4]. The mechanism by which an epithelial cell is able to acquire a mesenchymal phenotype underlies a loss of specialized epithelial cell adhesion molecules like E-cadherin, ZO-1 and cytokeratin and acquisition of mesenchymal-associated molecules like N-cadherin, vimentin and fibronectin. These changes allow the epithelial cancer cells to gain more invasiveness and metastatic capabilities [5-7]. Understanding the molecular and cellular basis of EMT provides fundamental insights into the etiology of cancer and can lead to new therapeutic strategies [8].

Cadherins are a family of Ca^{2+}-dependent cell-cell adhesion receptors that play a major role during embryonic development and in the maintenance of adult tissue architecture [9]. The cadherin family includes epithelial (E), neural (N) and placental (P) cadherins. E-cadherin, which is present in most normal epithelial cells, is related to the differentiation of epithelial cells. Loss of adhesive function of E-cadherin promotes the epithelial cells to a dedifferentiated and invasive malignant stage [10]. Clinical studies in patients with various human malignancies have also shown that E-cadherin expression is associated with dedifferentiated

* Corresponding author.
E-mail address: amryesmat@yahoo.com

and lymphogenous spread of tumors [11, 12]. Cell-cell-interactions have been recognized as one of the vital regulators of apoptosis, and that the apoptotic cell death occurring during invasion may be influenced by E-cadherin-mediated cell-cell interaction between tumor cells [13] .

Zonula occludens-1 (ZO-1) is a tight junction protein that is found at cell-cell adhesion membrane complexes in normal epithelial cells. Depending on the degree of cell differentiation and migration, ZO-1 shuffles during EMT from the adhesion membrane complexes to the cytoplasm and then to the nucleus [14]. During EMT, the dissolution of tight junctions is accompanied by decreased occludens and claudin expressions, along with ZO-1 diffusion from cell-cell contacts [15].

Vimentin, an intermediate filament protein, is used as a marker of mesenchymal cells to distinguish them from epithelial cells [16]. It regulates cell migration and controls recycling of endocytosed cell adhesion receptors as integrins to the plasma membrane [17]. Increased expression of vimentin is used as an EMT marker in cancer [18], and correlates with tumor growth, invasion and poor prognosis [19].

The mammalian Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway comprises four JAK domain-containing proteins, including JAK1,2,3 and tyrosine kinase 2 (TYK2), as well as seven Signal Transducers and Activators of Transcription (STATs), STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 [20]. Deregulation of JAK/STAT pathway has been involved in the promotion of oncogenic phenotypes, including tumorigenesis, invasion, metastasis, angiogenesis and anti-apoptosis [21, 22]. In breast cancer, the JAK/STAT signaling has been reported to be altered through several mechanisms, including down-regulation of phosphotyrosine specific phosphatases and STAT3 negative regulators [23, 24], as well as elevation in the activating ligand IL-6 [25]. Thus, JAK2 inhibitors are being evaluated in patients with breast cancer [26].

Aloin, the major anthraquinone glycoside of the *Aloe Vera* juice, is characterized as C-glycoside of aloe-emodin. The empirical formula of aloin is $C_{21}H_{22}O_9$, which is supported by the formulation 10β-D-glucopyranosyl-1,8-dihydroxy-3-hydroxymethyl anthracene-9-one [27]. In our previous studies, we reported on the antitumor activity of aloin against experimental murine tumors (ascites and solid Ehrlich carcinoma) [28, 29], with no detrimental side effects on the host metabolism [30]. Further studies in our lab have shown the cytotoxicity of aloin against different types of human cancer cell lines, such as breast and ovarian adenocarcinoma cell lines [31-33]. We have also demonstrated that repeated treatment of normal rats with the maximum tolerated dose of aloin (50 mg/kg bw) shows no cardiotoxicity due to its strong antioxidant and scavenging activities for free radicals and reactive oxygen species [34], as well as its strong iron chelating activity [35] .

We proposed this study to investigate the cytotoxic effect of aloin against breast cancer cell line (T47D), compared to an anthracycline analog, doxorubicin, that is frequently used as a chemotherapeutic drug in the treatment of many malignancies. Based on the roles for JAK/STAT signaling, we suggested that targeting JAK2 and STAT5a proteins may represent a valuable therapeutic strategy for breast cancer therapy. Also, the design of this study was extended to elucidate the mechanistic role of some genes involved in EMT in the cytotoxic activities of aloin and doxorubicin against T47D cells progression, thus providing novel prognostic and therapeutic markers.

Materials and Methods

Chemicals

Aloin was obtained in a pure powder (MW 418.4) form from Macfarlan Smith LTD, (Edinburgh, UK). Doxorubicin hydrochloride (Adriblastina®) (MW 579.5) was provided as a lyophilized red powder (10 mg/vial) from Pharmacia S.P.A (Milan, Italy). RPMI-1640 enriched with L-glutamine and fetal bovine serum (FBS) were provided from Gibco® (Thermo Fisher Scientific, Scotland). Penicillin streptomycin mixture (1×) and trypsin/EDTA (1×) were purchased from Biowest® (South Africa). Phosphate-buffered saline (PBS; pH 7.2), crystal violet, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and methanol were provided from Sigma-Aldrich (Germany). Sterile tissue culture flasks (75 cm^2), Petri dishes (58 cm^2) and 96-well microtiter plates were obtained from Greiner® (USA).

Cell line and cell culture

The human breast cancer cell line (T47D), ER-α+, PR+ and HER2- (luminal A) was obtained from ATCC, Manassas, VA. It exhibits an aneuploid karyotype with a mode of 65 chromosomes (2-3 % of the cells have a chromosome number close to 100) Catalog No: HTB-133™). Cells were grown in RPMI-1640/L-glutamine medium enriched with 10 % FBS and 1 % penicillin-streptomycin mixture in a humidified 5% CO2 incubator (Thermo Scientific, USA) at 37°C. Subculturing was routinely carried out twice a week using trypsin/EDTA.

Compounds preparation

Aloin solution (3.33 % in distilled water) was freshly prepared and sterilized before use by filtration through 0.22 µm filter (Millipore®, Merck, Germany). Five increasing aloin concentrations (20, 40, 60, 80 and 100 µg/ml) were prepared by diluting the stock solution in complete RPMI-1640 cell culture medium. As for doxorubicin hydrochloride, 2 mg/ml solution was prepared in sterile distilled water, aliquoted and stored at -70°C. Four increasing doxorubicin concentrations (0.05, 0.1, 0.15 and 0.2 µg/ml) were prepared by diluting the stock solution in complete RPMI-1640 cell culture medium.

***In vitro* proliferation assay**

Exponentially growing cells were enzymatically detached and a single tumor cell suspension in complete growth medium (150×10^3 cells/ml) was prepared. Cells were seeded in a 96-well microtiter plate (200µl/well) and allowed to attach for 24 h in a humidified 5 % CO_2 incubator at 37°C. After cell attachment, the culture medium in each well was aspirated and replaced with 200µl of fresh complete growth medium containing the different aloin or doxorubicin concentrations (3 wells per each dose), and then allowed to grow for 24 and 72 h, respectively. At the end of the exposure periods, 100µl of MTT (2 mg/ml in PBS) were added to each well and the plate was incubated at 37°C for further 2h. After careful aspiration of the culture medium, 150 µl DMSO were added to each well and the plate was left to stand for 1h at room temperature, and then read in an ELISA reader at 595 nm against blank (DMSO). The percentage of cell viability was calculated by multiplying the ratio absorbance of the sample versus the control by 100. Drugs IC_{50} values of aloin and doxorubicin were determined as the concentration that showed 50% cell growth inhibition as compared with the control cell growth [36].

Clonogenic assay

A single tumor cell suspension in complete growth medium was prepared at a density of 6000 cells/ml. Aliquots of cell suspension (5 ml) were then transferred to 75 cm^2 tissue culture flasks and incubated in a humidified CO_2 incubator at 37˚C for 24 h. Three culture flasks were set up for untreated cells (control) and for each compound concentration at each exposure period (24 and 72 h). After cell attachment, the culture medium was decanted and replaced with 5 ml of fresh complete growth medium in the control flasks and with 5 ml of fresh complete growth medium containing the different concentrations of aloin or doxorubicin, and then re-incubated at 37˚C for 24 and 72 h, respectively. After incubation, the media were aspirated and the cells were trypsinized, and then collected in 15 ml falcon tubes containing fresh complete growth medium. The tubes were centrifuged, and then the cell pellets were re-suspended in 4 ml of fresh complete growth medium. The viable cell numbers were counted by trypan blue exclusion method, and then the cells were diluted with the complete growth medium to 1000 cells/ml. A volume of 1 ml cell suspension was transferred to three Petri dishes for control and for each compound concentration followed by the addition of 3 ml complete growth medium. The dishes were incubated in a humidified 5 % CO_2 incubator at 37˚C for 10 days, during which the drug-free growth medium was replaced every 72h. At the end of the incubation period, the growth medium was decanted and the colonies were fixed in absolute methanol for 20 min, then stained with 2 % crystal violet and counted using a stereomicroscope (Olympus, Japan).

The number of colonies was scored by counting cell aggregates consisting of at least 50 cells (> 5 generations). At least 200 tumor cell colonies per flask were required in the control Petri dish to assure an adequate range for measurement of the compound effect. The mean colony count for control was taken as 100 % survival (0 % inhibition) and the percent inhibition of colony formation (ICF %) in compound-treated Petri dishes was calculated [31, 37].

Quantitative real-time polymerase chain reaction

The mRNA expression of Janus kinase-2 (JAK2) gene and PR-target gene signal transducer and activator of transcription-5a (STAT5a) were studied in T47D cells exposed to aloin or doxorubicin for 72h using quantitative real-time polymerase chain reaction (qRT-PCR). Also, epithelial markers including E-cadherin and zonula occludens-1 (ZO-1), and a mesenchymal marker, vimentin gene were examined. Briefly, counted T47D cells were distributed equally into three 75 cm^2 tissue culture flasks containing fresh cell culture medium, incubated for 48 h in a humidified atmosphere with 5 % CO_2 at 37˚C to allow monolayer formation. The growth medium was exchanged with a fresh one containing either no compound (control) or IC_{50} value of aloin or doxorubicin, and re-incubated in a humidified 5 % CO_2 incubator at 37˚C for 72 h. After incubation, the cell culture medium was aspirated and the cells were washed with PBS and trypsinized, followed by the addition of fresh complete growth medium. The cell suspension was centrifuged, and the supernatant discarded, and then the cells were immediately processed for RNA extraction. Total cellular RNA was extracted from T47D cells using RNeasy® Mini kit (Qiagen, Germany), quantified by NanoDrop One (Thermo Fisher Scientific, WI, USA), and then reversely transcribed by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Real-time quantitative polymerase chain reaction (RT-PCR) amplification of the candidate genes was done using QuantiTect SYBR Green PCR Kit and QuantiTect Primer Assays (Qiagen, Germany) in Applied Biosystems StepOne™ Analyzer (Applied Biosystems, Waltham, Massachusetts, USA) **Table 1**. The relative expression of each gene was determined by the $2^{\Delta\Delta CT}$ value method, where $\Delta\Delta CT = \Delta CT$ of treatment- ΔCT of control [38]. The data of mRNA expressions were normalized to the amount of GAPDH transcript.

Statistical analysis

The Shapiro-Wilks test for normality ($p > 0.05$) showed that all data were normally distributed [39]. Statistical analysis of the difference between means was carried out using one-way analysis of variance (ANOVA). In case of a significant F-ratio, post hoc Dunnett's and Bonferroni's tests for multiple comparisons were used to evaluate the statistical significance between treatment groups at $p < 0.05$ level of significance. All the statistical analysis was done using Statistical Package for Social Science (SPSS) version 20.0

(SPSS Inc., Chicago, IL, USA). GraphPad Prism (Version 5) software was used to calculate IC_{50} values. The expression ratio for each gene was calculated for aloin-treated or doxorubicin-treated cells relative to untreated cells. More than a two-fold increase or decrease was regarded as significant change (> 2 as up-regulated and < 0.5 as down-regulated) [40].

Results

Effect of 24 h treatment with aloin and doxorubicin on tumor cell proliferation

Fig. 1 illustrates that addition of increasing concentrations of aloin (20-100 µg/ml) or doxorubicin (0.05-0.2 µg/ml) in the culture medium of T47D cells for 24 h reduced the MTT absorbance (A and B, respectively) and the percentage of viable cancer cells (C and D, respectively) in a dose-dependent manner. Dunnett's test for multiple comparisons of parametric data revealed that the dose-dependent reduction in MTT absorbance started to be significant at 60 µg/ml for aloin, and significant at all used doxorubicin doses, compared to control T47D cells. A dose-dependent slight reduction in the percentage of cell viability was also obtained, attaining a maximum inhibition in growth of T47D cells (11.58 and 19.92 %) at the highest aloin (100 µg/ml) and doxorubicin (0.2 µg/ml) concentrations, respectively. Intergroup comparison between used doses of aloin using Bonferroni's test for parametric data demonstrated a non-significant change in the percentage of cell viability of T47D cells treated with aloin at doses 20-60 µg/ml, whereas a significant reduction in the percentage of cell viability was recorded at doses 80-100 µg/ml. As for doxorubicin, a non-significant change in the percentage of cell viability among all doxorubicin doses was noticed. However, a 24-h incubation of T47D cells with the different aloin and doxorubicin concentrations was insufficient to induce a 50 % inhibition in cellular growth.

Effect of continuous treatment (72 h) with aloin and doxorubicin on tumor cell proliferation

Fig. 2 shows that continuous exposure (72 h) of T47D cells to increasing concentrations of aloin (20-100 µg/ml) or doxorubicin (0.05-0.2 µg/ml) significantly reduced the MTT absorbance (A and B, respectively) and the percentage of viable cancer cells (C and D, respectively) in a dose-dependent manner. A significantly dose-dependent reduction in the MTT absorbance was demonstrated at all aloin and doxorubicin doses, compared to control, recording a 53.40 and 58.88 % inhibition in the viable T47D cells at the highest aloin (100 µg/ml) and doxorubicin (0.2 µg/ml) concentrations, respectively. Intergroup comparison between the aloin doses using Bonferroni's test for parametric data revealed a non-significant change in the percentage of cell viability treated with 20 and 40 µg/ml aloin, and by contrast, a significant reduction in the percentage of cell viability at doses of aloin (60-100 µg/ml) was recorded. Also, a significant reduction in the percentage of cell viability treated with doxorubicin at doses 0.1-0.2 µg/ ml was recorded, compared to the lowest dose (0.05 µg/ml).

Effect of aloin and doxorubicin treatments on the cell colony formation

Table 2 shows the effect of treatment of T47D cells with different increments of aloin (20-100 µg/ml) or doxorubicin (0.05-0.2 µg/ml) for 24 or 72 h on the number of colonies formed. Exposure of T47D cells to aloin for 24 h caused a significantly gradual increase in the percentage of inhibition of colony formation, which reached 10.67 % at the lowest dose (20 µg/ml) and 38.67 % at the highest dose (100 µg/ml), compared to controls. The inhibitory effect of aloin on colony formation was augmented in the continuous exposure regimen, amounting 27.17 % at the lowest dose and 83.04 % at the highest dose, compared to controls. Likewise, doxorubicin treatment for 24 h or continuous exposure showed a significantly gradual increase in the percentage of inhibition of colony formation, which amounted to 18.89 & 23.70 %, respectively, at the lowest dose (0.05 µg/ml) and 91.33 & 96.09 %, respectively, at the highest dose (0.2 µg/ml), compared to controls.

Calculation of IC_{50} values of aloin and doxorubicin

Fig.3 represents the plotting of % viability of T47D cells against log concentrations of aloin and doxorubicin (72 h exposure), which was used to calculate the concentrations of aloin and doxorubicin that kill 50 % of cells (IC_{50}), and were found to be 75.93 (181.5 µM) and 0.099 µg/ml (0.17 µM), respectively.

Effect of aloin and doxorubicin on mRNA expression of some candidate genes

Table 3 show that treatment of T47D cells with the calculated IC_{50} value of aloin at 72 h (75.35 µg/ml) displayed down-regulation of mRNA expression levels of JAK2, STAT5a and vimentin genes (fold change 2.86, 2.56 and 2.56, respectively), whereas the expression levels of E-cadherin and ZO-1 were insignificantly changed, compared to control cells. On the other hand, T47D cells treated with the calculated IC_{50} value of doxorubicin at 72 h (0.099 µg/ml) showed a significant up-regulation of E-cadherin gene expression (fold change 2.82), while the expression levels of JAK2, STAT5a, vimentin and ZO-1 did not appear to be significantly changed, compared to control cells.

Discussion

Cell lines are used as *in vitro* tools to mimic certain types of *in vivo* system, and T47D cells have been widely used in breast cancer studies. The cancer cell lines are important for understanding the mechanisms of action, the resistance and the sensitivity pattern of chemotherapeutics already in use in cancer treatment and the development of new targeted anticancer drugs. Also, cell lines are exceptional for the fundamental study of the cellular pathways and for disclosing critical genes involved in cancer.

Table 1: The catalogue numbers of the candidate gene primers (Qiagen, Germany)

Gene	Assay Name	Cat. No.	Lot. No.
JAK-2	Hs_JAK2_1_SG	QT00062650	183633557
STAT-5a	Hs_STAT5A_1_SG	QT00066101	183633576
E-cadherin	Hs_CDH1_1_SG	QT00080143	183633578
ZO-1	Hs_TJP1_1_SG	QT00077308	183633577
Vimentin	Hs_VIM_1_SG	QT00095795	183633558
Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) (House keeping gene)	Hs_GAPDH_1_SG	QT00079247	183633579

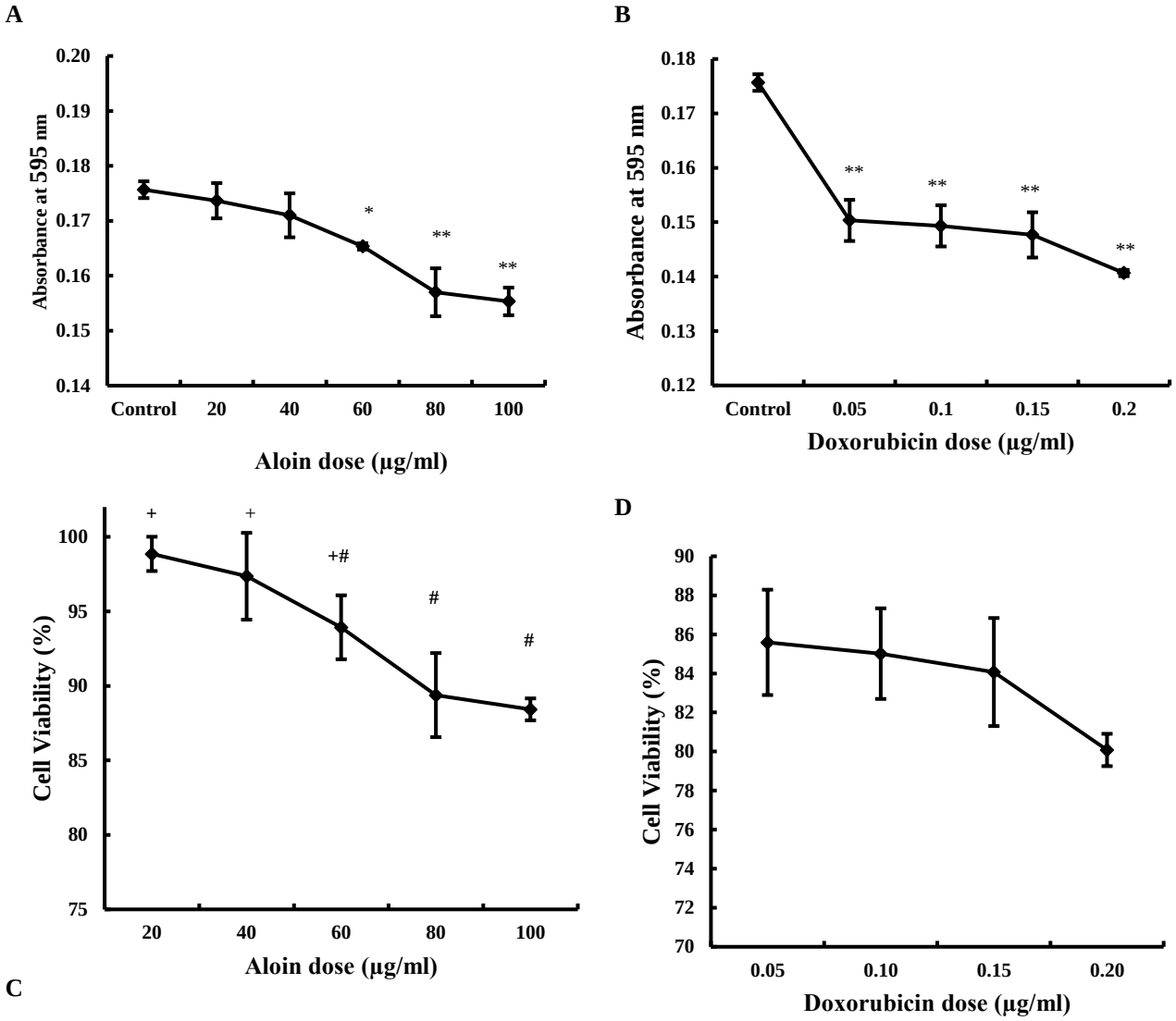

Fig. 1: Effect of 24 h exposure to increasing concentrations of aloin or doxorubicin on the growth of T47D cells. (A) MTT growth inhibition assay following exposure to increasing concentrations of aloin. (B) MTT growth inhibition assay following exposure to increasing concentrations of doxorubicin. (C) Percentage of cell viability in aloin-treated T47D cells, compared to control (100 %). (D) Percentage of cell viability in doxorubicin-treated T47D cells, compared to control (100 %). Significant versus control at * $p < 0.01$ or ** $p < 0.001$ (A&B); Different symbols denote significance (C&D).

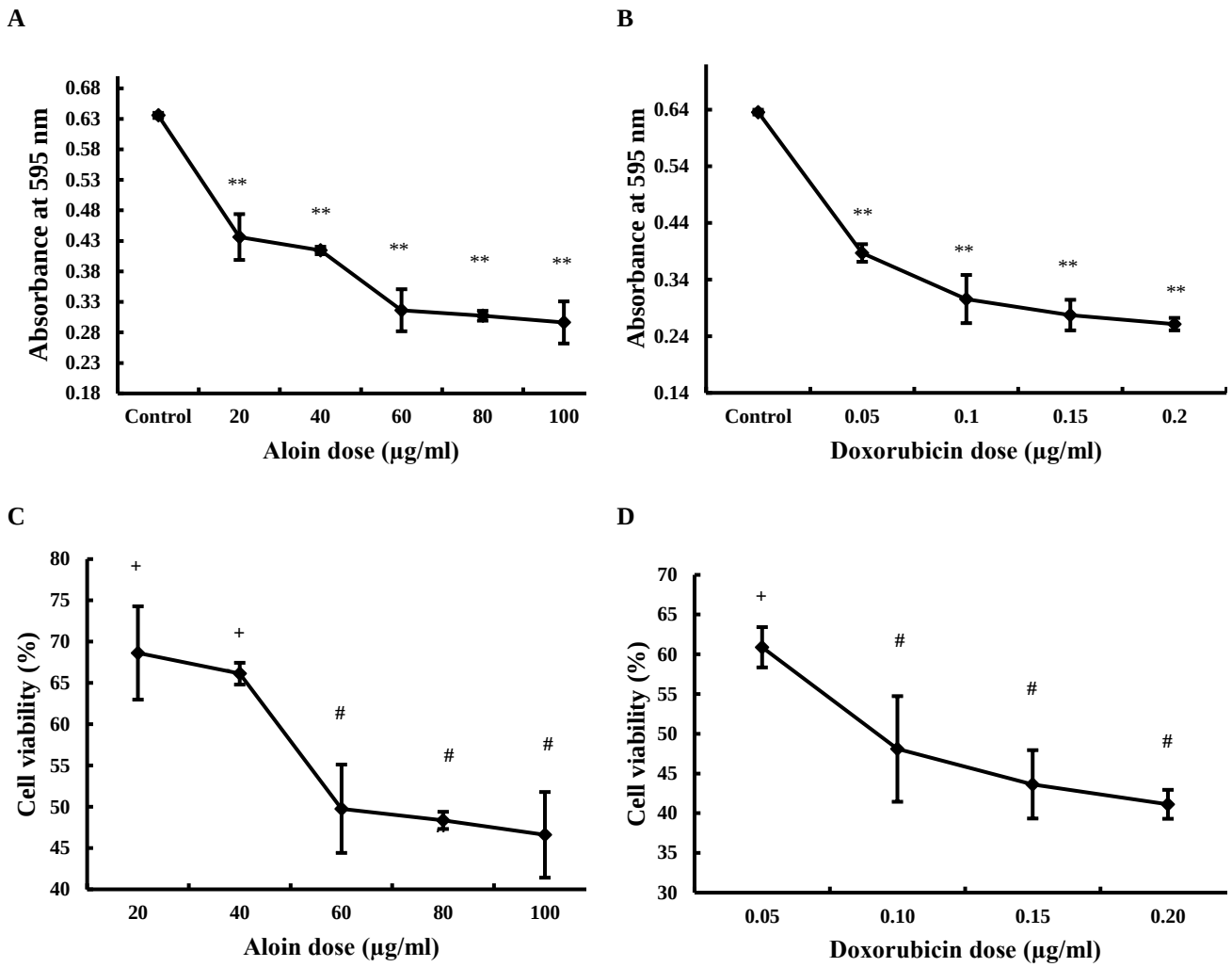

Fig. 2: Effect of 72 h exposure to increasing concentrations of aloin or doxorubicin on the growth of T47D cells. (A) MTT growth inhibition assay following exposure to increasing concentrations of aloin. (B) MTT growth inhibition assay following exposure to increasing concentrations of doxorubicin. (C) Percentage of cell viability in aloin-treated T47D cells, compared to control (100 %). (D) Percentage of cell viability in doxorubicin-treated T47D cells, compared to control (100 %). Significant versus control at $^{**}p < 0.001$ (A&B); Different symbols denote significance (C&D).

Table 2: *In vitro* sensitivity of T47D cells to aloin and doxorubicin

Dose of drugs (µg/ml)		Duration of exposure to drugs			
		24 h		72 h	
		Number of colonies per flask (mean ±SD)	ICF %	Number of colonies per flask (mean ±SD)	ICF %
Control		450 ± 14.93	0	460 ± 11.79	0
Aloin	**20**	402 ± 15.71*	10.67	335 ± 12.49**	27.17
	40	376 ± 23.64**	16.44	305 ± 8.0**	33.70
	60	313 ± 17.35**	30.44	260 ± 11.36**	43.48
	80	294 ± 10.54**	34.67	112 ± 6.24**	75.65
	100	276 ± 9.17**	38.67	78 ± 12.49**	83.04
Control		450 ± 14.93	0	460 ± 11.79	0
Doxorubicin	**0.05**	365 ± 18.0**	18.89	351 ± 8.0**	23.70
	0.1	285 ± 16.37**	36.67	235 ± 7.81**	48.91
	0.15	103 ± 10.0**	77.11	46 ± 6.08**	90.00
	0.2	39 ± 2.65**	91.33	18 ± 2.64**	96.09

ICF%=Percentage of inhibition of colony formation
Results are mean ± SD of triplicate plates
*Significant versus control at $p < 0.01$
**Significant versus control at $p < 0.001$

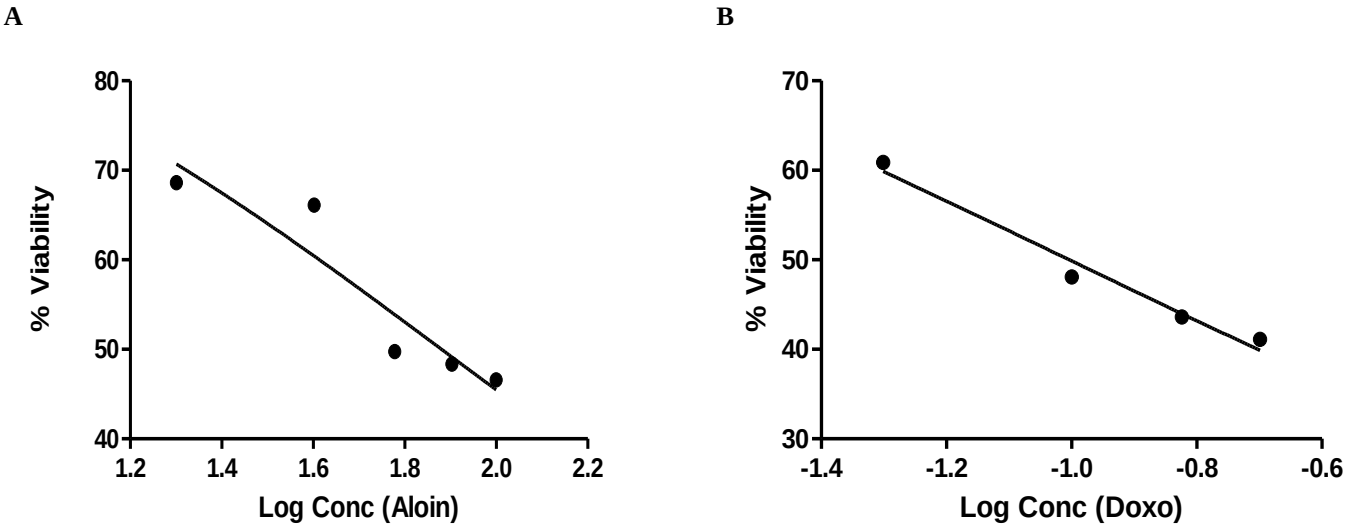

Fig. 3: Plotting of % cell viability of T47D cells against log concentrations of aloin or doxorubicin after 72h exposure. (A) IC_{50} value of aloin. (B) IC_{50} value of doxorubicin.

Table 3: Changes in the mRNA expression level of selected genes after treatment of T47D cells with aloin or doxorubicin for 72 h.

Groups	JAK2	STAT5a	E-cadherin	Vimentin	ZO-1
Aloin					
Mean ±SE (RQ)	0.35*± 0.14	0.39*± 0.19	1.55 ± 0.30	0.39*± 0.13	0.72 ± 0.16
Fold change	-2.86	-2.56	1.55	-2.56	-1.39
Doxorubicin					
Mean ±SE (RQ)	1.53 ± 0.14	0.99 ± 0.23	2.82**± 0.25	1.92 ± 0.09	1.04 ± 0.27
Fold change	1.53	-1.01	2.82	1.92	1.04

Results are mean ± SE of four replicate independent experiments and represent the relative mRNA expression of selected genes, compared to GAPDH.
* Significant down-regulation; ** Significant up-regulation.
In case of down-regulation, the fold change was calculated as follows: -1/RQ, where RQ = relative quantification.

In the present study, we have demonstrated that the exposure of T47D cells to aloin for 24 and 72 h shows a significantly dose-dependent reduction in the percentage of cell viability at higher doses (60, 80 and 100 µg/ml) in the 24 h exposure regimen, and at all aloin doses (20-100 µg/ml) in the continuous exposure regimen, compared to controls **Fig. 1 & 2.** However, aloin at all tested doses significantly inhibited the colony formation of T47D cells amounting to 38.6 7% at the highest dose (100 µg/ml). This effect was augmented in the continuous exposure assay showing ICF of 83 % at the highest dose, compared to control **Table 2**. However, doxorubicin treatment, which was used as an anthracycline analog, showed a significantly dose-dependent reduction in the percentage of cell viability and a significant inhibition of colony formation at all implemented doses in the 24 and 72 h exposure regimens **Fig. 1 & 2 and Table 2**. In our previous studies, we have emphasized that treatment of T47D tumor cells with aloin shows an increase in the fraction of tumor cells at the S-phase, and the appearance of cells cycling at a higher ploidy level (> G2M) (i.e aneuploid DNA pattern). This strongly suggests that aloin does not inhibit initiation of DNA synthesis, nor causes a cell cycle blockade at G2M, and that cells continue to cycle at higher ploidy levels, but have difficulty in mitosis [31, 32]. Further studies in our lab showed that treatment of human breast cancer cell lines, with (SKBR-3) and without (MCF-7) erbB-2/topoisomerase IIα co-amplification, with aloin caused a decrease in the fraction of cells undergoing mitosis, inhibition of topoisomerase IIα protein expression, and down-regulation of cyclin B1 protein expression in MCF-7 cell line. In SKBR-3 cell line, erbB-2 protein expression was not affected and topoisomerase IIα protein expression was mildly down-regulated at higher concentrations of aloin only [33]. On the other hand, doxorubicin (7.5 nM) was previously shown to induce cell accumulation at G2M phase in T47D cell line [41].

In the current study, results obtained from the MTT assay of aloin and doxorubicin in the 72h exposure regimen were used to calculate their IC_{50} values, which were found to be 75.93µg/ml and 0.099µg/ml, respectively to be used in the subsequent studies **Fig. 3**. A comparable result was previously obtained by Lee *et al*. [42], who reported on the cytotoxic activity of aloin against adenocarcinomic human alveolar basal epithelial cell line (A549) at IC50 value of 150 µM.

The foregoing findings strived studying of the effect of continuous exposure of T47D cells to IC50 values of aloin and doxorubicin on the expression of JAK2 and STAT5a genes, as well as some gene markers for EMT. JAK2 has been associated with important downstream proteins, including STATs. STAT5 regulates genes expression that promote cell survival and proliferation in breast cancer cells. Therefore, STATs have emerged as pharmacological targets for cancer therapy, and the development of novel STATs inhibitors is of considerable clinical interest for treating breast cancer [43]. Despite the homology in the structure of aloin and doxorubicin, they exerted different actions on the expression levels of JAK2 and STAT5a mRNA. The exposure of T47D cells to IC50 value of aloin (181.5µM) for 72h significantly down-regulated the relative expression levels of JAK2 and STAT5a mRNA, whereas doxorubicin did not affect them **Table 3**. Thus, aloin is recognized as a potent inhibitor of JAK2/STAT5a signaling. The inhibition of JAK2/STAT5a signaling along with the sharp reduction in the percentage of cell viability provide insight into the important and suppressive role of aloin in breast cancer cells progression.

Previously, we reported on the overexpression of p53 protein in breast tumor cells (MCF-7) treated with aloin at doses 150-300 µg /ml, which was less strong at dose levels of 80-100 µg/ml and normal at 40-60 µg/ml [33]. The dose-dependent effect of aloin on activation of p53 expression along with our current results regarding the inhibition of JAK2/STAT5a genes at the adopted dose are in harmony with the previous findings of Mukhopadhyay *et al.* [44], who reported that the STAT5a transcription factor is a direct transcriptional target gene of p53, and that STAT5a is well expressed in p53 wild type cells but not in p53 null cells. Inhibition of p53 reduces STAT5a expression. Two p53 binding sites are identified in the STAT5a gene, one in the promoter region and one in the 5th intron.

In harmony with our results, aloin (100, 150, 200 µg/ml) has been shown to inhibit JAK1, STAT1 and STAT3 phosphorylation in a dose-dependent manner, and the nuclear translocation of STAT1 and STAT3 in lipopolysaccharide-induced inflammation in RAW 264.7 macrophages [45]. This suggests that aloin might act as a pan inhibitor of JAKs. Previously, Reiterer and Yen [46] studied the role of JAK signals in cell cycle transit and maintenance of genome stability in HL-60 myeloblastic leukemia cells, and found that all JAKs (JAK1, 2, 3 and tyrosine kinase 2), as well as JAK 2 or 3 inhibitors caused a drastic reduction in cell growth with a major G2M arrest evident 24 h after treatment. However, targeting all JAKs caused endoreduplication 48 and 72 h after treatment. The authors also recorded mitotic cells in both G2 (4N DNA) and G4 (8N DNA) subpopulations of cells treated with an inhibitor of all JAKs. Likewise, we previously studied the ploidy pattern of breast (T47D) cells treated with aloin (60 µg/ml) for 24 and 72 h [31]. We found that breast tumor cells treated with aloin showed 18 and 4 % of tumor cells with 2N DNA content,16 and 80 % of tumor cells contained > 4N DNA content in 24 and 72 h exposure assays, respectively. Taken altogether, it could be strongly suggested that aloin might act as an inhibitor of all JAKs.

Additionally, the inhibition of JAK2 gene in a human colorectal cancer cell line (SW1116) with the DNA methyltransferase inhibitor, 5-aza-deoxycytidine, induced G2 cell cycle arrest and apoptosis in SW116 cells through regulation of downstream targets of JAK2/STAT3/STAT5 signaling [47]. Britschgi *et al.* [48] reported that inhibition of JAK2 by genetic or pharmacological means bypasses resistance to PI3K/mTOR inhibition and decreases cancer cell number, tumor growth and metastasis, as well as increasing *in vivo* survival, and that combined inhibition of JAK2/STAT5 and PI3K/mTOR leads to activation of the pro-apoptotic protein Bim and degradation of the anti-apoptotic protein Mcl-1. This explanation warrants the investigation of the effect of aloin on PI3K/AKT/mTOR.

EMT has been implicated in carcinogenesis and confers metastatic properties upon cancer cells by enhancing mobility, invasion and resistance to apoptotic stimuli.Exploring of the effect of treating T47D cells with IC_{50} values of aloin on the mRNA expression of some EMT-related gene markers displayed a significant inhibition in the vimentin gene expression, whereas the expression of E-cadherin and ZO-1 genes were not changed **Table 3**. The positive correlation between increased expression of vimentin gene and tumor aggressiveness was previously documented [49, 50], and attributed to the lack of steroid receptors [17, 51]. The literature contains several studies on the regulatory effect of JAK2 on vimentin synthesis. Colomiere *et al.* [52] reported that the treatment of EGF-induced OVCA433 cells with a JAK2 inhibitor (AG490) results in the inhibition of STAT3 activation, as well as inhibition of the expression of N-cadherin and vimentin proteins. Additionally, Stewart *et al.* [19] found out that a pan JAK inhibitor (JAK inhibitor I) significantly inhibited the expression levels of vimentin mRNA and protein in EGF-mediated EMT induction in MDA-MB-468 breast cancer cells. Taken altogether, it is emphasized that aloin which acts as a JAK2/STAT5a inhibitor reduces EMT through inhibition of the expression of vimentin mRNA.

On the other hand, doxorubicin (0.17 µM) exposure for 72 h increases the expression of E-cadherin mRNA in T47D cells, while the expression of other candidate genes remains unchanged **Table 3**.

Our findings are in accordance with [53], who found out that cell-cell adhesion of YMB-S cells (which proliferate without aggregation) was increased on day 2 and day 4 after exposure to adriamycin (0.4 µM) associated with increased expression of E-cadherin and β-catenin mRNA and protein levels. The authors reported that cell-cell adhesion induced by adriamycin is induced by increased expression of E-cadherin and decreased MUC1 expression. E-cadherin mediates early cellular adhesion events that are necessary for the formation of junctional complexes including gap junction intercellular communication (GJIC) [54], which is also important for apoptosis to occur in solid tumors [55]. If intercellular communication is required for an apoptotic signal to be transferred to cells in solid tumors, E-cadherin-mediated cell-cell adhesion may be beneficial to apoptotic signal transduction elicited by adriamycin [53].

In conclusion, although aloin and doxorubicin are anthracyclines, yet they act in a different way. Aloin is recognized as a potent inhibitor of JAK2/STAT5a signaling, which could provide the first evidence for establishing a mechanistic link between inhibition of JAK2/STAT5a and its cytotoxic action against T47D breast tumor cells. Aloin also caused inhibition of the expression of the mesenchymal gene marker, vimentin mRNA, that could be a useful marker to explore as a potential prognostic and therapeutic target in ER+ breast cancer.

Conflict of Interest

The authors declare no conflict of interest with this article.

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