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Cytotoxicity Activity of Doxorubicin, Tamoxifen Drug, and n-Hexane *Annona squamosa* Extract Against Cancerous Cell Line (MCF-7 and SKOV-3) and Normal Cell Line NHF

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Abstract

Herbal medications are usually regarded as an alternative treatment option for cancer. Many novel cytotoxic chemicals have been identified from the plants that are used to fight cancer. The current research determined the cytotoxicity of *Annona squamosa* extract (ASE). Different cancer cell line (MCF-7 and SKOV-3) and normal cell line with different concentrations (1000, 500, 250, 125, 62.5 and 31.25) as fallow G1 (n-Hexane *Annona Squamosa* extract, HAS), G2 (doxorubicin, DOX), G3 (Tamoxifen, TAM), G4 (HAS+DOX, HDA), (HAS + TAM, HTA). Each inhibitory agent group was used to treat the cancer cell lines MCF-7, SKOV-3, and NHF to obtain IC_{50}% by MTT assay. In 1000 µg/ml from HAS showed significantly increased all three cell lines compared with other inhibitory concentrations. The recorded data demonstrated that the inhibitory ability of each of HTA and HDA towards the MCF-7 significantly increased compared with the SKOV-3 cell line. The interaction between different concentrations of DOX and TAM showed a high inhibitory effect on MCF-7 compared with SKOV-3 cancerous cell lines. The HAS and TAS showed a significant effect on MCF-7 compared with SKOV-3. Both HAD and HTA showed antagonistic activity on normal cell lines, while HAD and HTA showed synergistic activity towards MCF-7 and SKOV-3.

Keywords: Tamoxifen, doxorubicin, interaction index, selectivity index, Annona squamosa, NHF.

Introduction

Phytochemicals found in medicinal plants have good medicinal properties. Modern medicine employs certain bioactive compounds originating from medicinal plants [1,2]. Ethnopharmacological research has led to the development of effective medicinal drugs derived from herbal plants that have fewer side effects. These therapies can be replaced with more expensive contemporary medications at a cheaper cost for rural folks [3,4]. Recently, there has been interest in reducing drug toxicity [5-7]. The combination of chemotherapy medications and bioactive compounds from medicinal herbs may have anti-cancer efficacy and have fewer adverse effects [8,9]. The purpose of medicinal plants is to treat cancer cells that have been identified through clinical research. These can not only treat but also prevent cancer by interrupting the cell cycle, inducing apoptosis, regulating carcinogen metabolism, and that are required for cancer development. Curcumin, vinblastine, quercetin, vincristine, and resveratrol are plant-derived compounds with anticancer effects [10,11]. Doxorubicin is an antibiotic used as a chemotherapeutic drug to treatment of different cancers such as breast and ovarian cancers [12,13]. DOX effectively kills quickly proliferating cells and delays the growth of solid and liquid malignancies [14]. One common non-steroidal antiestrogen medication is tamoxifen. It exerts antiestrogenic

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actions on breast cancer cell receptors and tissue-stimulating-antagonistic effects on breast cancer tissue. [15,16]. Because it promotes breast tissue division and proliferation, which bears the risk of mutations that cause cancer, the estrogen receptor catalyzes the growth of cancer [17,18].

Material and methods

Collection and preparation of the plant

The Annona squamosa seed has been collected from the local Iraqi market. Following cleaning and oven drying at 50 °C, 100 mg of finely ground plant material was extracted and put into an extraction thimble. After extracting using 80 milliliters of n-hexane, leave it for a full day. Following filtration via gauze and filter paper (Whatman No. 1), the extraction process's end product is dried in an oven at 50 degrees Celsius by evaporating the supernatant.

cytotoxicity Assay

Experimental Design

The cell line was treated with the inhibitory agent type and concentration (1000, 500, 250, 125, 62.5, and 31.25 µg/ml). Annona Squamosa n-Hexane extract (HAS) was administered to group one (G1), doxorubicin drug (DOX) was administered to group two (G2), tamoxifen drug (TAM) was administered to group three (G3), tamoxifen with extract (HTA) was administered to group four (G4), and doxorubicin with extract (HDA) was administered to group five (G5). To determine the cytotoxicity, each inhibitory substance was applied to the normal cell line NHF and the malignant cell lines MCF-7 and SKOV-3.

Cell line culture procedure

This work employed a commercially acquired MCF-7. Both the ovarian and breast cancer cell lines received ethical approval. In an 85% humidified atmosphere at 37°C and 5% CO2, cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma Aldrich: D5796) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin (Sigma Aldrich; P4333), and 1% amphotericin B (Sigma Aldrich, A2942). T175 culture flasks were used to develop cells, and they were subcultured twice a week until confluence. In 35mm culture dishes, cells were cultivated at a density of 5 × 105 cells/mL of culture media. Before each experiment, they were incubated for four hours to facilitate attachment and homeostatic recovery.

Harvesting is a method that detaches and disaggregates adherent monolayer cells from the bottom of the culture flask using the proteolytic enzyme trypsin. It has been done anytime cells were to be collected for cell counting and cell line sub-culturing [19].

The Interaction Index (IAI) of the drug combination

The (HAS+TAM) and (HAS+DOX) Interaction Index (IAI). It was quantified using the following equation and utilized as an inhibiting agent for MCF-7 and SKOV-3:

IAI = D1/d1+D2/d1……. [< 1 synergistic. = 1, additive. > 1 antagonistic.]

Statistical Analysis:

The results of the cytotoxicity test with ANOVA p> 0.001 were assessed in one of two methods using the GenStat (version 12) software.

Results

Determination of IC50 and dose response curve by MTT Assay

The result showed that the inhibiting activity (IC50) of HDA and HTA high significant effect on MCF-7 compared with SKOV-3. IC50 calculated by MTT Assay for MCF-7, SKOV-3, and NHF. (Table 1) (Figure 1). The inhibitory activity of TAM toward MCF-7 was lower than SKOV-3. However, DOX's IC50 for MCF-7 was greater than SKOV-3's. When comparing MCF-7 to SKOV-3, the rate of efficacy of IC50 (HDA) and (HTA) was considerable. The findings showed that in MCF-7, HAS decreased TAM cytotoxicity more than DOX.

Table 1. Inhibition concentration (µg/ml) of the compound and IC50 of the cell lines by MTT Assay

Compounds (µg/ml)	Cancerous cell line		Normal cell line	Average b
	MCF-7	SKOV-3	NHF	
HAS	615.5	327.3	11837.4	4260.067
DOX	305.1	199.3	500.96	335.12
TAM	552.5	543.5	21094.2	7396.733
HTA	270.14	168.16	31744.74	10727.68
HDA	225.83	163.88	12995.56	4461.757
Average a	393.814	280.428	15634.57	
LSD. p>0.05	a=423.1 b=327.8 ab=732.9			

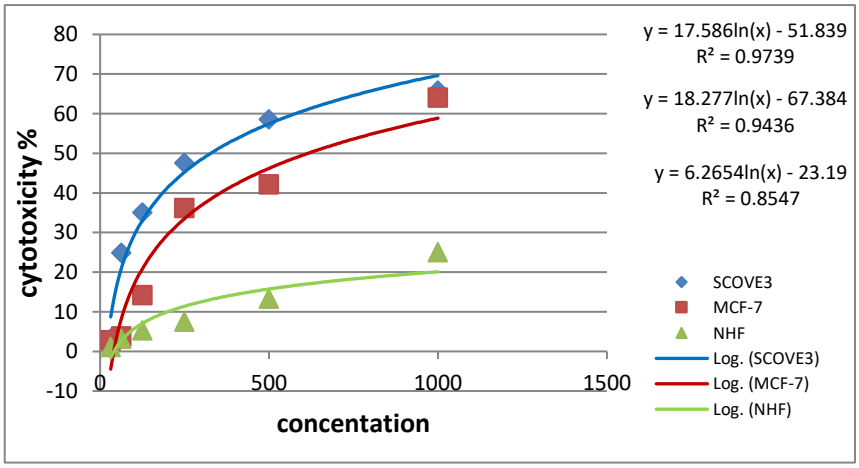

Figure (1): Showing the curve growth inhibition activity of SAH on MCF-7 and SKOV-3, and the NHF cell lines

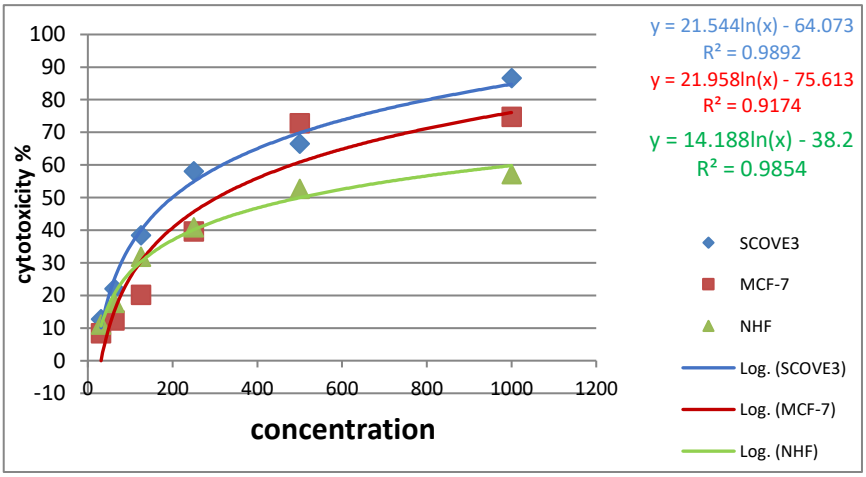

Figure (2): Showing the curve growth inhibition activity of doxorubicin on MCF-7 and SKOV-3, and NHF cell lines

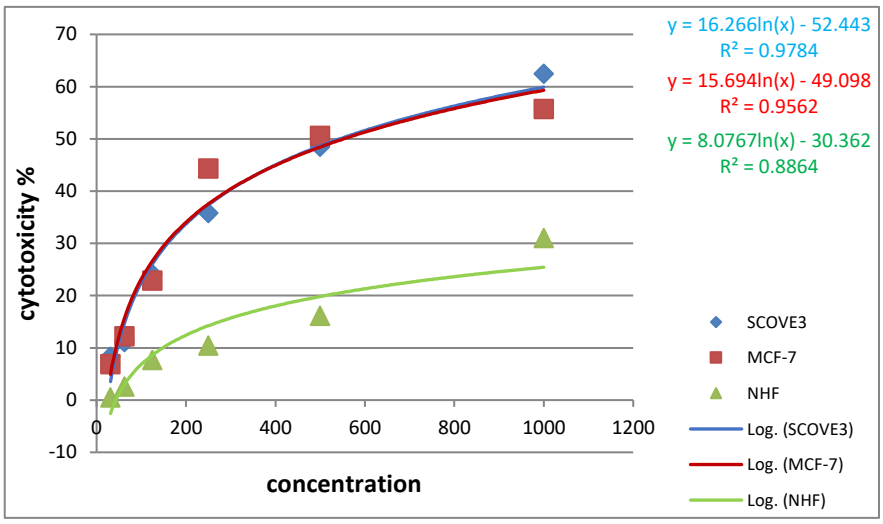

Figure (3): Showing the curve growth inhibition activity of tamoxifen on MCF-7 and SKOV-3 and NHF cell line

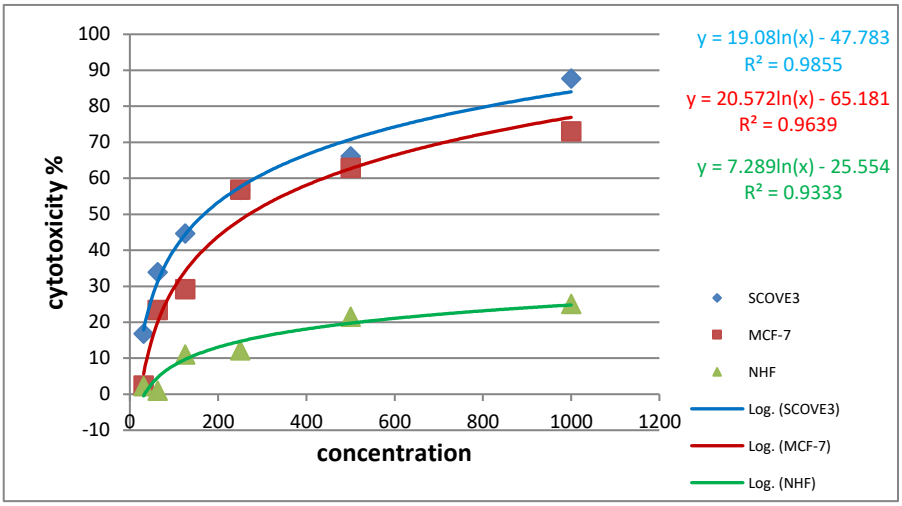

Figure (4): Showing the curve growth inhibition activity of tamoxifen and ASH on MCF-7 and SKOV-3, and NHF cell lines

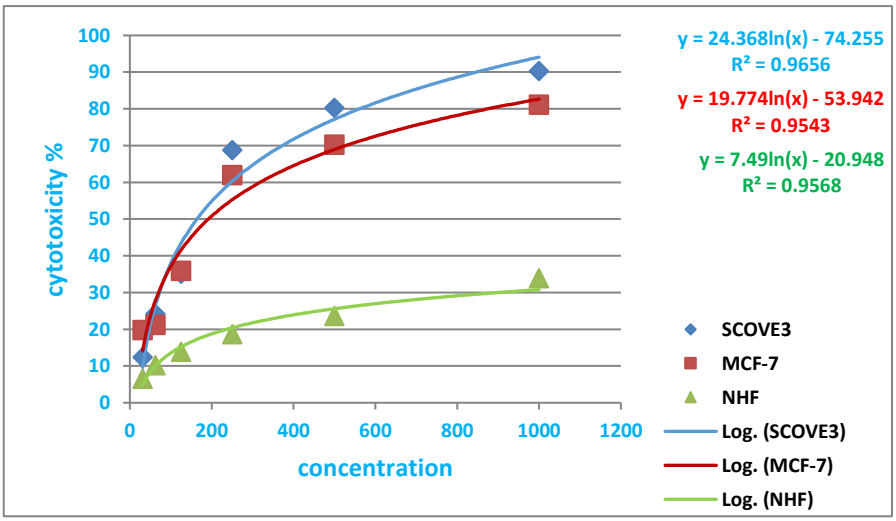

Figure (5): Showing the curve growth inhibition activity of doxorubicin and ASH on MCF-7 and SKOV-3, and NHF cell lines

3.3. The interaction index (IAI) of drug combinations

The IAI of (HTA) and (HDA) combined on MCF-7, SKOV-3, and NHF. The IAI is calculated according to the following equation:

$$\textbf{IAI} = \frac{d1}{D1} + \frac{d2}{D2} \quad \begin{cases} < 1, \text{ synergistic} \\ = 1, \text{ additive} \\ > 1, \text{ antagonistic} \end{cases}$$

The stock solution of the mixture was prepared according to a (1:1) partition.

Ovarian cancer cell line (SKOV-3) combined with HAS tamoxifen

The stock solution of the (HTA) mixture was prepared according to (1:1 partition.

TAM = 1/2 = 0.5 HAS = 1/2 = 0.5

$d_1 = d_{TAM} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 168.16 = 84.08 µg/ml

$d_2 = d_{HAS} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 168.16 = 84.08 µg/ml

$D_1 = D_{TAM} = 543.5$

$D_2 = D_{HAS} = 327.3$

IAI = (84.08/543.5) +(84.08/327.3)

IAI = 0.15 + 0.26

IAI = 0.41

0.41 < 1, so there is a very synergistic effect.

1. Ovarian cancer cell line (SKOV-3) combined with HAS and doxorubicin

DOX = 1/2 = 0.5

ASH = 1/2 = 0.5

$d_1 = d_{DOX} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 163.88 = 81.94 µg/ml

$d_2 = d_{ASH} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 163.88 = 81.94 µg/ml

$D_1 = D_{DOX} = 199.3$

$D_2 = D_{HAS} = 327.3$

IAI = (81.94/199.3) +(81.94/327.3)

IAI = 0.41 + 0.25

IAI = 0.66

0.66 < 1, so there is a very synergistic.

2. Breast cancer cell line (MCF-7) combined with HAS and doxorubicin

DOX = 1/2 = 0.5, ASH = 1/2 = 0.5

$d_1 = d_{DOX} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 225.83 = 112.92 µg/ml

$d_2 = d_{HAS} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 225.83 = 112.92 µg/ml

$D_1 = D_{DOX} = 305.1$

$D_2 = D_{HAS} = 615.5$

IAI = (112.92 /305.1) +(112.92 /615.5)

IAI = 0.37 + 0.18

IAI = 0.6

0.6 < 1, so there is a very synergistic effect.

3. Breast cancer cell line (MCF-7) combined with HAS and tamoxifen

TAM = 1/2 = 0.5, ASH = 1/2 = 0.5

$d_1 = d_{TAM} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 270.14 = 135.07 µg/ml

$d_2 = d_{HAS} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 270.14 = 135.07 µg/ml

$D_1 = D_{TAM} = 552.5$

$D_2 = D_{HAS} = 615.5$

IAI = (135.07/552.5) +(135.07/615.5)

IAI = 0.24 + 0.22

IAI = 0.46

0.46 < 1, so there is a very synergistic.

4. Normal human fibroblast (NHF), combined with HAS and Tamoxifen

DOX = 1/2 = 0.5, ASH = 1/2 = 0.5

$d_1 = d_{TAM} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 31744.74 = 15872.4 µg/ml

$d_2 = d_{HAS} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 31744.74 = 15872.4 µg/ml

$D_1 = D_{TAM} = 21094.2$

$D_2 = D_{HAS} = 11837.4$

IAI = (15872.4 /21094.2) +(15872.4 /11837.4)

IAI = 0.75+1.34

IAI = 2.09

2.09 > 1, so there is high antagonism.

5. Normal human fibroblast (NHF) combined with HAS and doxorubicin

TAM = 1/2 = 0.5, ASH = 1/2 = 0.5

$d_1 = d_{DOX} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 12995.56= 6497.78 µg/ml

$d_2 = d_{HAS} = 0.5 \times IC_{50}$ %(mixture) = 0.5 × 12995.56= = 6497.78 µg/ml

$D_1 = D_{DOX} = 500.96$

$D_2 = D_{HAS} = 11837.4$

IAI = (6497.78 /500.96) +(6497.78 /11837.4)

IAI = 12.97+0.54

IAI = 13.51

13.51 >1, so there is antagonism.

Table 2. Selectivity index (SI) and interaction index (IAI) of the drug combination of inhibitory concentration

Compounds (µg/ml)	Cancerous cell line				Normal cell line
	MCF-7		SKOV-3		NHF
	SI*	IAI**	SI	IAI	IAI
HAS	192.31	--	361.6	--	--
DOX	2.51	--	1.64	--	--
TAM	38.54	--	37.919	--	--
HDA	79.30	0.6	67.75	0.66	13.51
HTA	187.98	0.46	117.51	0.41	2.09

* SI < 2 means absent selectivity index; SI > 2 means present selectivity index.

** IAI > 1 means antagonistic; IAI < 1 means synergistic; IAI =1 means additive interaction.

Discussion

The plants used in alternative medicine are one of the main resources used in pharmaceutical sciences. Had cytotoxic and cytocidal effects on cell lines at various doses [20,21]. This study showed that MCF-7 significantly differed from SKOV-3, as shown in Table 1 and Figure 1. The number of cell viability in the breast cancer cell line reduced as the extract dose increased, which results in agreement with the study reported by [22,23].

A. Squamosa anti-proliferative activity has been documented for seed extract, with notable results against human tumor cell lines (like breast and ovarian cancer) [24-26]. According to a different research, annonaceous acetogenins, which are potent inhibitors of mitochondrial complex I with considerable cytotoxicity, are abundant in A. squamosa seeds [27]. The structure of bioactive chemicals influences their potential to cure and prevent cancer. The efficacy of cytotoxic action is determined by the large number of hydroxyl groups flanking the γ-lactone ring, as well as the ring's stereochemistry [28,29]. The results showed the high effect of doxorubicin on breast cancer (MCF-7) compared with ovarian cancer (SKOV-3). After doxorubicin therapy, cell invasion was reduced in both cancerous cell lines [30]. Despite its active efficacy against growing cancer cells, its usage has been limited due to the accompanying risk of cardiac damage and resistance acquisition [31].

DOX induces apoptosis by activating death-signaling pathways in target cancer cells. Apoptosis can be triggered by the simultaneous or significant activation of death receptor systems, mitochondrial malfunction, ROS damage, DNA processing, and proteolytic caspase [32]. impact of doxorubicin on cardiotoxicity enzymes (CK-MB, myoglobin, LDH, and cTnI) and the heart muscle [33].

The result of the current study disagrees with [34], which reported that doxorubicin was identified in cancer of the breast cell lines MCF-7, with the greatest levels reported in ovarian cancer. Tamoxifen inhibits the growth of ER-positive cancers by suppressing the transcriptional activity of the estrogen receptor alpha. Tamoxifen resistance is a complicated, multifaceted condition that involves the estrogen receptor (ER). Acquired mutations: When the ERα gene is mutated, the receptor's structure changes, making it less susceptible to tamoxifen binding. As a result, the medication's ability to block estrogen signaling is diminished, enabling cancer cells to proliferate. [35]. Through its effects on cell cycle and apoptosis-related genes, TAM may cause apoptosis and cycle arrest in ER-positive ovarian cancer. The limitations of TAM monotherapy, which might lead to recurrence and drug resistance, were examined in our study [36].

The results showed that a mixture of TAM + n-Hexane extract had a synergistic cytotoxic effect in both cancerous cell lines. In this work, we examined the synergistic effect of hexanoic and TAM on MCF-7 and SKOV-3 cells. Tamoxifen was found to promote apoptosis and autophagy in MCF-7 in a dose-dependent manner. Also, hexanoic acid had a synergistic impact with TAM on MCF-7, which results in agreement with [37]. The ASH reduced the toxicity and increased the activity of tamoxifen in the treatment of both cancerous cell lines and potential to reduce the development and growth the tumors, then causing apoptosis [38]. Another study reported that adjuvant acetogenins (extracted from *Annona Squamosa*) treatment with anticancer medicines can be useful in treating [39].

The results showed that a mixture of DOX + ASH has a synergistic cytotoxic effect in both cancerous cell lines. In this work, we examined the synergistic effect of ASEE and DOX on MCF-7 and SKOV-3 cells.

In MCF-7 and ovarian cancer, n-Hexane extracts caused apoptosis. Hexanoic extract treatment of MCF-7 and ovarian cells led to lower intracellular glutathione levels, nuclear condensation, DNA

fragmentation, and the development of reactive oxygen species (ROS). Additionally, Annexin V staining, which suppressed Bcl-2 and PS externalization, showed that both extracts caused oxidative stress-induced apoptosis in MCF-7. These results suggest that extracts from A. squamosa may specifically cause apoptosis in specific types of cancerous cells [40,41].

Conflict of interest: NIL

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