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A comparative study of the use of indomethacin with doxorubicin and their pharmacological effects on the growth and reproduction of breast cancer cells (AMJ13) in vitro study

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ABSTRACT

Breast cancer (BC) is the second most frequent cause of cancer-related deaths in women worldwide. in Iraq it ranks the first among the population and the leading cause of cancer related female mortality.

The purpose of this study is to assess the cytotoxicity of indomethacin and doxorubicin on the breast cancer cell line AMJ13.Additionally, they support how these drugs either promote or discourage cell development or death. Lastly, to research the combined impact of both drugs on cell activity.

The investigation of study made use AMJ13 cell line. The median inhibitory concentration (IC50) was calculated using the methyl thiazolyltetrazolium test and ranged between 1000, 500, 250, 125, 62.5, and 31.2 µg /ml. The same method was applied to the combination study. The combination index was calculated using the compuSyn software to determine the inhibitor doses that were most effective methods. In treated and untreated breast cancer cell lines, crystal violate morphological alterations and Acridine orange / Propidium iodide apoptosis were used.

AMJ13showed reduction in the proliferation, growth, cell viability, and induced morphological changes and apoptosis. Through apoptosis induction, there were cytotoxic effects of Indomethacin, Doxorubicin and the combination as well. The percentages of AMJ13 cell growth inhibition by Indomethacin concentrations (1000, 500, 250, 125, 62.5, and 31.2 µg/ml) were (41.3%, 33.3%, 19.5%, 7.3%, 4.3%, and 2.3%) respectively. The Median Inhibitory Concentration (IC50) value of Indomethacin ranged (314 to 959.8 µg/ml is 549 µg/ml). The percentages of AMJ13 cell growth inhibition by Doxorubicin (GI %) were (58.8%, 46.4%, 32.3%, 23.8%, 11.3%, and 0.896%) at each concentration, respectively. The Median Inhibitory Concentration (IC50) value of Doxorubicin ranged (162.2 to 308.3µg/ml is 223.6µg/ml) At each of the aforementioned concentrations. the percentages of co-treatment-induced AMJ13 cell growth inhibition (GI) are (62.2%,37%,28.2%,18.8%,8.7%, and 1.6%), respectively. The CompuSyn Isobologram was employed, and the co-treatment IC50 value.

Introduction

The second biggest cause of mortality worldwide, cancer is a significant issue for public health. A group of disorders known as cancer are defined by the unchecked growth and division of aberrant cells. Death may result if the spread is not prevented[1] .

Cancer treatment aims to eliminate cancer cells with least amount of damage to healthy cells [2]. To minimize side effects, localized, systemic, and/or supportive medicines are employed in the treatment of cancer [3].

One of the most common cancers in women is breast cancer [4]. Moreover, breast cancer is the most common form of malignant tumor in Iraqi women and the main reason why women die from malignant neoplasms [5]. After substantial research, the illness is still incurable and has a two-year survival rate[6] .

Surgery, chemotherapy, adjuvant hormone therapy, and radiotherapy are all options for treating breast cancer [7]. Age, parity, family history of breast cancer, particularly in first-degree relatives, radiation exposure, smoking, and genetics of BRCA1 and BRCA2 gene mutations are all associated risk factors for breast cancer in females [8] .

Indole-3-acetic acid derivative indomethacin is a nonsteroidal anti-inflammatory medication (NSAID). The medication is generally used to treat painful inflammatory disorders like osteoarthritis and gout [9]. The fact that indomethacin is a nonselective inhibitor of the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isozymes has been used to demonstrate the mechanistic function of the drug in the reduction of pain [10].

With regard to a wide range of cancer cell types, both in vitro and in vivo, it was discovered that The NSAIDs indomethacin and the others have potent anticancer properties [11].

Doxorubicin (Dox) is an anthracycline that is produced by the mutant strain of Streptomyces peucetius var. caesius. Dox is an efficient antineoplastic drug for many different types of cancer. Yet numerous systemic side effects limit its widespread usage in cancer treatment. It is one of the well-known and frequently applied antineoplastic drugs used to treat a variety of diseases, such as breast cancer, leukemia, and pediatric cancer. Dox damages DNA because it inhibits the DNA topoisomerase II enzyme[12] .

The objective of the study:

To investigate the cytotoxicity of Indomethacin and Doxorubicin on the AMJ13 cell line (breast cancer) and explain how these medications either hinder or encourage cell growth or death. to investigate the combination effect of both medicines on cell activity.

Material and Methods

Maintenance of Cell Cultures

The Iraq Biotech Cell Bank Unit provided AMJ13, which was kept alive 10% Fetal bovine supplemented RPMI-1640, 100 units/ml penicillin, and 100 g/ml streptomycin were used. Trypsin-EDTA was used to passage the cells, and they were reseeded twice a week at 50% confluence, and 37 °C was used for culture.

Cytotoxicity Assays

Viability of MTT cells experiment was performed on a 96- microplate wall (96WMP) to assess the cytotoxic effect. 104 cells per well were used to seed the cell lines. Cells that had formed a confluent monolayer after 24 hours were treated with the experimental substance After cell lines received treatments with Indomethacin, Doxorubicin, and combination therapy (full dose), the concentration of inhibition that kills 50% of cells (IC50) was determined.. Utilizing GraphPad Prism (version 7), the IC50 for Indomethacin, doxorubicin, and combination treatment was determined. Depending on the IC50 values, the cells were treated with various concentrations of Indomethacin, Doxorubicin, and combination therapy (1000, 500, 250, 125, 62.5, 31.2 µg /ml). For each concentration of each treatment technique, three duplicates were used [13].

On several cell lines, the CompuSyn computer program compared the IC50 of Indomethacin, Doxorubicin, and each of them alone and the IC50 of them together[14] .

After 72 hours of treatment, cell viability was assessed by removing the medium, adding 28 µ L of an MTT solution containing 2 mg/ml, and incubating the cells for 1.5 hours at 37 °C. The residual MTT-Formosan crystals in the wells were solubilized after the MTT solution was removed by adding 130 µ L of DMSO (Dimethyl Sulphoxide), which was then incubated at 37 °C for 15 min while being shaken [13].

The assay was carried out in triplicate, and the absorbency was measured using a microplate reader at 492 nm (the test wavelength). The following equation was used to calculate the percentage of cytotoxicity, or the rate at which cell growth was inhibited. Cell viability is calculated as follows: 100% cytotoxicity = 100% cell vitality (cell absorbance compared to

untreated cell absorbance). GI% is calculated as (mean of controls - mean of treated / mean of controls) * 100[15].where the average optical density of untreated wells is OD control, and the optical density of treated wells is OD sample .

MTT was used to assess the effects of Indomethacin, Doxorubicin, and their combination in a study on tumor cell viability. The experiment is based on how mitochondrial enzyme activity reduces colorless tetrazolium salt through metabolism in live cells. It is especially useful for assessing cell suspensions because of its selectivity for living cells [16].

Propidium Iodide/Acridine Orange Assay for Estimating Apoptosis

Using (AO/PI), the apoptotic rates in cell lines (infected and control) were assessed. For classic dual staining, 5000 cells/well were planted in a plate and then infected for 24 hours in a 37 °C incubator. Exact 50µl of the AO/PI stain mixture (at room temperature) were applied to the test wells for 30 seconds. The stain was then eliminated. Leica fluorescence microscope was used to capture the photographs.

Statistical analysis

GraphPad Prism 6's unpaired t-test was used to statistically assess the data acquired [17].

The results were provided as the mean± SD of measurements made in triplicate [18].

To compare the variations between groups under various circumstances, isobologram version 1 was used. P values greater than 0.05 were regarded as significant**.** The combination index CI was evaluated by the CompuSyn software program algorithm. On Chou-Talalay lines, combined dose-response curves were fitted. Antagonism is indicated by CI > 1.1, and synergism by CI < 1 1. A cumulative impact (CI) of between 1 and CI = 1 to 1.1 is implied [19].

Results and Discussion

In vitro Indomethacin, Doxorubicin, and Co-Treatment Effects on the Morphology of AMJ13

The cultivated AMJ13 Cells exhibited an elongated multipolar epithelial-like cell shape, nuclear polymorphisin, and numerous in most of the cells, which expressed the characteristics of cell morphology, as well as showing many cells with mitotic figures (4-1).

The morphological images for AMJ13 in vitro were full of cells and had a monolayer cell form. Indomethacin, Doxorubicin, and co-treatment for used concentrations were each (1000,500,250,125,62.5,31.2) turn after drug exposure The number of cells started to drop as they went into single cell suspension.When the dosage of endomethacine, doxycycline, and cotreatment is increased, there is a graduate drop in cell quantity and lethal effect on the graduate.

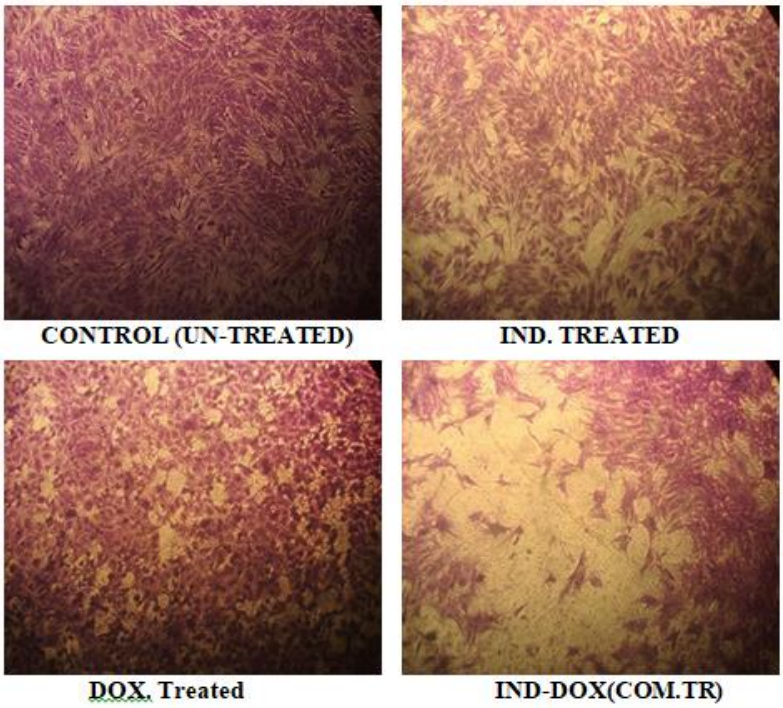

Figure (4.1) Morphological pictures for AMJ13 in vitro un-treated (as control cells) and Cytotoxicity under an inverted microscope (10x),when concentration is 1000 µg/ml.

Traditional theories explain how NSAIDs fight breast cancer by inhibiting the COX-2 enzyme, which is overexpressed in several types of breast tumors [20]. and is possibly connected to a poor prognosis [21] . Indomethacin therapy has been shown to decrease invation in a variety of cancer cell types and tumor model organisms, not just breast cancer cells [22] . As an anthracycline antibiotic, doxorubicin is effective against a variety of tumors; just a few cancer types are resistant to the medication. The list of cancers treated with doxorubicin includes Hodgkin's and non-Hodgkin's lymphoma, breast, ovarian, testicular, acute leukemia, soft tissue sarcoma, lung, bladder, gastric (stomach), thyroid, hepatoma, Wilm's tumor, and neuroblastoma [23]. DNA damage is the main mechanism by which topoisomerase II inhibitors, such as DOX, cause cell death[24] . Additionally, they are known to cause lipid peroxidation and free-radical DNA damage [25].

The Inhibiting Effect of Indomethacin, Doxorubicin, and Co treatment on AMJ13 Growth Rate *in Vitro* .

Different Indomethacin, Doxorubicin, and co-treatment concentrations were used to measure the cytotoxicity. (1000, 500, 250, 125, 62.5, and 31.2 µg/ml) using the MTT cytotoxicity test. These results suggest that cytotoxicity or growth inhibition increase with increasing inhibitor concentration. As shown in Figures (4- 2, 3, 4), statistically, there is a substantial distinction between inhibition by Indomethacin, Doxorubicin, and co-treatment for breast cancer cell lines. The effects of Indomethacin, Doxorubicin, and co-treatment were compared to those of the RPMI-1640 medium, which served as a positive command.

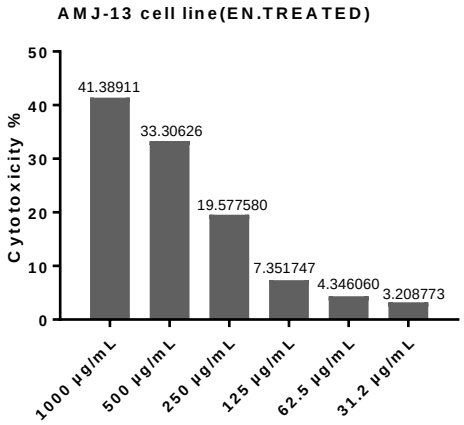

Figure (4-2): Cytotoxicity effect (CT %) after treatment of cell lines with Indomethacin to AMJ13 cells .

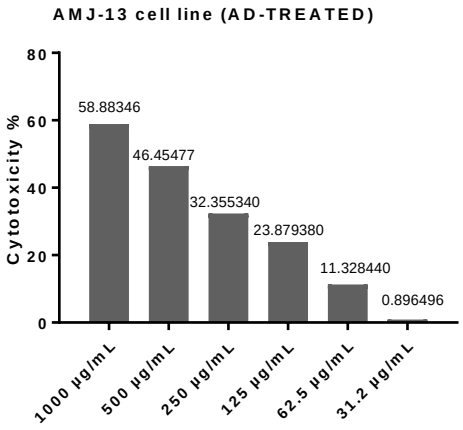

Figure (4-3): Cytotoxicity effect (CT %) after treatment of cell lines with Doxorubicin to AMJ13 cells .

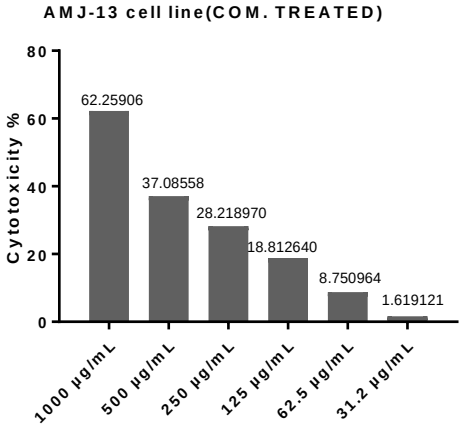

Figure (4-4): Cytotoxicity effect (CT %) after treatment of cell lines with combination to AMJ13 cells.

The combination's cytotoxicity activity (IND and DOX) on cell lines showed the inhibitory rates. These results showed that the six dose combination concentrations used on the AMJ13 cell lines had a synergistic effect.

In the current investigation, it was discovered that combining IND and DOX on AMJ13 resulted in a high percentage of inhibition rates at six doses (1000, 500, 250, 125, 62.5, and 31.2 µg /ml).

By combining a synthetic, potent microtubule-targeting anticancer drug with a cytotoxic anticancer agent (Indomethacin, Doxorubicin), this method is utilized to examine the in vitro pharmacodynamics interactions. To automatically determine the synergistic and antagonistic interactions between all doses or effect levels, CI 1 suggests synergism, CI = 1 to 1.1 shows an additive effect, and CI > 1.1 indicates antagonism.

Step-by-step illustrations are provided of the pharmacologic interactions between

indomethacin and doxorubicin that prevent the growth of the breast cancer cell line AMJ13, from the design of the experiment to the analysis of actual results. The chemopreventive and chemotherapeutic actions of NSAIDs have been attributed to a number of COX-independent mechanisms [26] .

The anti-tumor effects of indomethacin observed here are primarily due to COX-2 inhibition, as the selective COX-2 inhibitor SC-236 was as effective in this study for all parameters evaluated as the non-selective inhibitor indomethacin at the doses analyzed. This implies that COX-2 selection might have a similar therapeutic impact to non-selective NSAIDs while also having a lower toxicity profile [27].

Even while earlier research has demonstrated that indomethacin treatment can reduce invasion in a dose-dependent manner at doses more than 10µ [28].

Indomethacin, Doxorubicin, (IC50) Calculation in In Vitro for AMJ13

The impact of each treatment on cell growth was assessed using the half maximum inhibitory concentration (IC50) value in breast cancer cell lines. The results demonstrated that AMJ13 is efficiently exploited by breast cancer cells, and after 72 hours of infection, the infected cell lines experienced a strong cytopathic effect with a surprising impact on the AMJ13 cells' IC50 of Indomethacin treatment range.(314 to 959.8 was 549) , IC50 of Doxorubicin treatment range (162.2 to 308.3 was 223.6) for AMJ13.

It identifies the drug concentration at which 50% of cells become inactive, It explains why the IC50 value in AMJ13 was present. The results showed that after medication treatment, there are significant differences in IC50 among AMJ13 cell lines (Figures 4–5and 6).

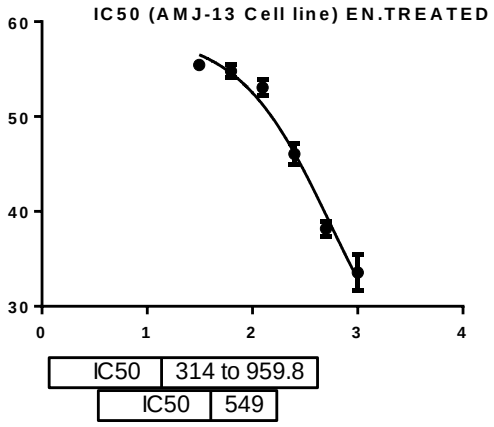

Figure 4-5 : After indomethacin was administered to AMJ13 cell lines, the IC50 values were calculated using GraphPad Prism software.

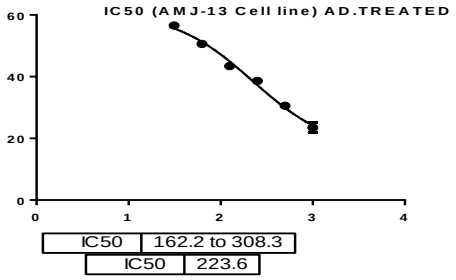

Figure 4-6: After Doxorubicin was administered to AMJ13 cell lines, the IC50 values were calculated using GraphPad Prism software.

An indicator of a pharmaceutical inhibitor's capacity to inhibit AMJ13 is the half maximal inhibitory concentration (IC50). A quantitative method for estimating the concentration of an inhibiting drug is the IC50 value. To stop the spread of breast cancer, indomethacin, doxorubicin, and co-treatment are necessary. Indomethacin, Doxorubicin, and co-treatment had a highly noticeable impact on breast cancer cell lines. High dosages of the drugs prevent growth, and the best results are achieved when they are combined. The combination of IND and DOX was a noticeably better promoter and enhancer of growth inhibition in AMJ13 when compared to either drug alone. In terms of cytotoxicity, IND and DOX together were more potent than IND or DOX alone.

Combination therapy has been demonstrated to be more effective against cancer than monotherapy. Chemotherapy has severe toxicity and immunosuppression whereas monotherapy non-selectively targets quickly growing cells. Lower doses of individual medications are given as a result of combination therapy's additive or synergistic effects, which may help to decrease drug toxicity for healthy cells and tumor cells' problems with drug resistance. [29-30].

The Effect of Indomethacin ,Doxorubicin and In vitro effects of co-treatment on Apoptosis in AMJ13

In this study, morphological changes as well as the ratios of apoptotic, necrotic, and normal viable cells were identified using a fluorometric cell viability assay with acridine orange and propidium iodide (AO/PI). All nucleated cells will be stained by AO, which will cause green fluorescence. PI can only be taken up by dead cells with inadequate membrane integration. As a result, PI will be used to stain all dead nucleated cells, which will cause red fluorescence. Due to quenching, cells labeled with AO and PI fluoresced red.

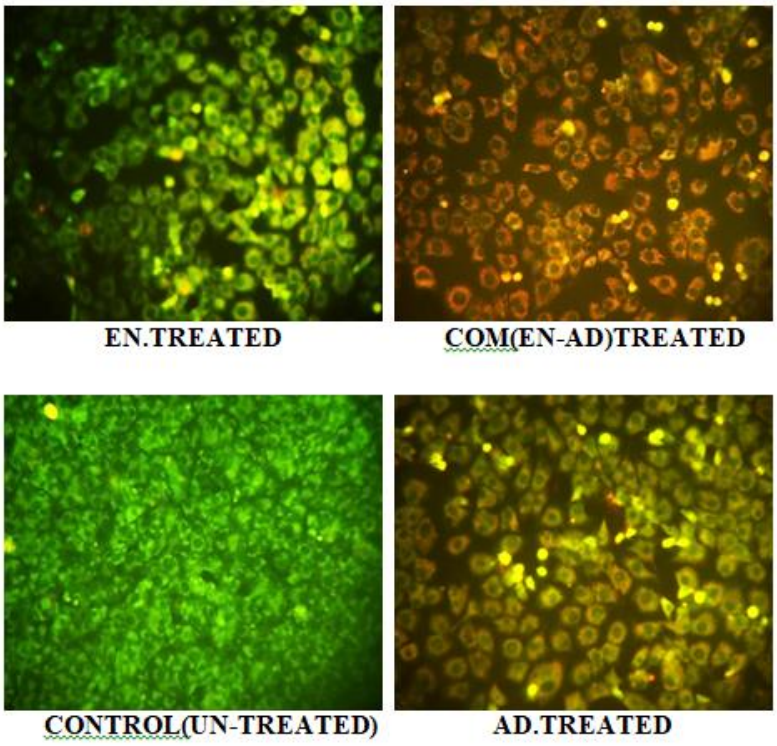

Figure 4-7 Analysis of the effects of Indomethacin and Doxorubicin, as well as their combination, on apoptosis in AMJ13 cell lines after treatment with high concentrations (1000 µg/ml)every of treated cells as well as untreated cells (control) cells. examined under a fluorescence microscope (10X). Following treatment with a dose of Indomethacin and Doxorubicin for 72 hours, the green hue represents viable cells and the red color displays dead cells.

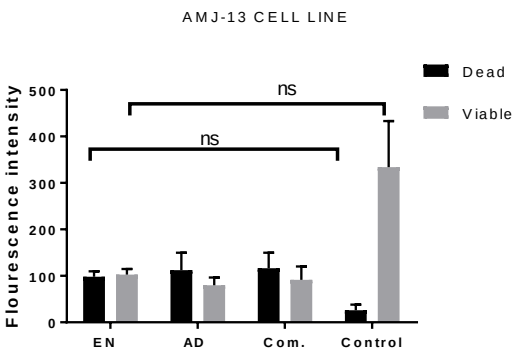

Figure 4-8 Red-stained cells (treated cells) and green fluorescence in untreated control cells illustrate that apoptosis is guaranteed by the fluorescent intensity used to quantify apoptosis in treated cells.

Apoptosis is a normal, designed cell death process that can be caused by a variety of physical and chemical factors and is carefully regulated by the organism. Despite the fact that apoptosis involves three main signaling pathways (mitochondrion, death receptor, and endoplasmic reticulum signaling pathways).At the mitochondrial level, signaling is frequently amplified and integrated [31].

It has been noted that indomethacin does not generate ROS or RNS, and when it is enclosed in nanoparticles, its current antioxidant potential lowers signaling pathways and causes cell death by apoptosis, making it an antineoplastic agent [32].

Conclusion

The results obtained from this study, the effect of Indomethacin and Doxorubicin and co-treatment therapy in addition to morphological changes and apoptosis was significantly enhancer of growth inhibition. Combination index analysis (CI) showed the presence of synergistic inhibitory effect between Indomethacin , Doxorubicin and co-treatments against human breast cancer cells type AMJ13 in vitro.

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دراسة مقارنة لاستخدام الاندوميثاسين مع الدوكسوروبسين وتاثيراتهما الدوائية على نمو وتكاثر الخلايا السرطانية (AMJ13) ، دراسة مختبرية

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الملخص

سرطان الثدي(BC) هو السبب الرئيسي الثاني للوفيات المرتبطة بالسرطان بين النساء على مستوى العالم . الغرض من هذه الدراسه هو تقييم السمية الخلوية للاندوميثاسين والدوكسوروبيسين على خط خلايا سرطان الثدي AMJ13 ,بالاضافة الى كيفة اعاقة هذه الأدوية او تشجيعها على نمو الخلايا او الموت.

أخيرا لبحث التأثير المشترك لكلا العقارين على نشا في هذه الدراسة تم استخدام خط الخلايا AMJ13 .لتحديد متوسط تركيز المثبطAMJ13 تم استخدام مقاييس ميثيل ثيازوليلتيترازوليوم (1000 , 500 , 250, 125 , 62.5, 31.2 ميكروغرام \مل). نفس الاجراء تم استخدامة للدراسة المركبة. مؤشر المجموعة تم قياسه باستخدام برنامج compuSyn لتحديد الجرعات الفعاله و المثبطة.في خط خلايا سرطان الثدي المعالجه وتحت العلاج تم استخدام الكرستال المنتهك/ التغيرات المورفولوجية, اكردين برتقال/ بروبيديوم يوديد /موت الخلايا المبرمج.

أظهر استخدام الأدوية الاندوميثاسين و الدوكسوروبسين على الخط الخلوي AMJ13 انخفاضًا في نموها وتكاثرها وحيويتها الخلوية ,التغيرات المورفولوجية المستحثة وموت الخلايا المبرمج. من خلال تحريض موت الخلايا المبرمج، كان هناك تأثير سام للخلايا لكل من الاندوميثاسين والدوكسوروبسين والمركب. كانت النسب المئوية المثبطه لنمو خلايا AMJ13، بتراكيز الاندوميثاسي (62.5 ,31.2 , 125 , 250 , 500, 1000 ميكروغرام \مل) (2.3% ,4.3% ,7.3% ,19.5% ,33.3% ,41.3%) في كل منها التركيز المذكور على التوالي. التركيز المثبط المتوسط للاندوميثاسين (IC50) كان 549 مايكروغرام \ مل تتراوح قيمته من314 الى 959 مايكروغرام \مل .كانت النسب الئوية للدوكسوروبسين (GI%) التي تبطأ نمو الخلايا AMJ13(58.8% ,46.4% ,32.3% ,23.8% ,11.3% ,0.896). عند كل تركيز مذكور ع التوالي. متوسط التركيز المثبط للدوكسوروبسين كان 223.6 مايكروغرام \مل تتراوح قيمته من162.2 الى 308.3 مايكروغرام \مل. نسب تثبيط نمو الخلاياAMJ13 الناجم عن المعالجه المشتركه هي (62.2% ,37% ,28.2% ,18.8% ,8.7% ,1.6%). تم استخدام CompuSyn Isobologram و قيمة IC50 للعلاج المركب.