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THE ANTI- CANCER SYNERGISTIC EFFECT OF COLCHICINE AND ON CANCER CELLS PROLIFERATION *(IN VITRO* STUDY) CHOLOROQUINE

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Abstract:

Abstract Objective:
This study aimed to investigate the possible in vitro cytotoxicity of Colchicine and Chloroquine.
Methods:
The powder was drugs from Colchicines, Chloroquine and combnation drugs. evaluated by MTT assay for cytotoxicity against AMJ13, breast cancer cell lines.
Results:
The results showed synergistic cytotoxicity against breast cancer cells by Colchicine, Chloroquine and combnation drugs has potent cytotoxicity against AMJ13 cell where IC50 of Colchicine treated range(2.351 to 20.73 was 6.982) for AMJ13, IC50 of Chloroquin treated range (2.696 to 10.9 was 5.42) for AMJ13 , IC50 of combnation drugs used CompuSyn Isobologram when it was for AMJ13.The cytotoxicity of drugs with Colchicine, Chloroquine and combnation drugs showed a synergistic activity on AMJ13 cell lines).
Conclusion:
Colchicine , Chloroquine and combnation drugs has potent cytotoxicity against AMJ13 cell lines

Keywords:

INTRODUCTION

Cancer

Cancer is a major public health issue around the world, and it is the second leading cause of death. Cancer is a category of diseases characterized by the uncontrollable growth and spread of abnormal cells. If the spread is not stopped, it can lead to death (Siegel et al., 2019). Cancer therapy tries to destroy cancer cells while causing the least amount of harm to normal cells (Fournier & Schirrmacher, 2013). Localized and / or systemic therapies, as well as supportive therapies, are used in cancer treatment to reduce side effects (Miller et al., 2019).

Europe accounts for 23.4% of all cancer cases and 20.3% of all cancer deaths worldwide. America has 13.3% of the global population and is responsible for 21.0% of global incidence and 14.4% of global mortality.

Cancer mortality rates in Asia and Africa (57.3% and 7.3%, respectively). Breast cancer is a global health problem and the most common cancer affecting women around the world and the second leading cause of cancer related death after lung cancer. Breast cancer in women (6.6 percent), Breast cancer is the most prevalent cancer diagnosed in women (24.2 percent). Breast cancer is the most frequent female tumor in Iraq, with the most new cases in 2018 (Bray et al., 2018).

In general, breast cancer affects more women than any other type of cancer, and it is especially prevalent in Iraq, where 23% of all female cancer cases worldwide were caused by breast cancer (Coleman et al., 2008). It is the most frequent type of malignancy in the Iraqi population in general, accounting for almost one-third of all recorded female malignancies and nearly one-quarter of all female cancer deaths (Al-Hashimi& Wang, 2014).

Breast cancer has become one of the biggest hazards to Iraqi female health during the previous two decades, with a clear increase in incidence rates (Mualla& Al-Alwan, 2014).

Cell lines are an important experimental tool in cancer research because they provide an infinite supply of a relatively homogeneous cell population capable of self-replication that can be widely distributed to facilitate comparative studies (Pandrangi et al., 2014).

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The development of drug resistance in tumors counteracts the therapeutic effects of chemotherapeutic drugs, resulting in more aggressive tumor recurrence and worse prognoses for cancer patients (Liu et al., 2020). Colchicine is a well-known and potent microtubule targeting agent; however, colchicine's therapeutic value against cancer is limited by its toxicity to normal cells. However, there is no evidence that it has cytotoxic potential against lung cancer cells at clinically acceptable or lower concentrations that are minimally toxic to non-cancerous cells (Bhattacharya et al .,2016).

Autophagy is an effective intracellular catabolic process that uses lysosomal degradation to degrade aberrant cellular protein aggregates and damaged organelles. However, it is required for cellular homeostasis and renovation (Wang et al., 2019). The autophagy process begins with the formation of the phagophore and concludes with the death of the autophagosome. However, cell biologists have been interested in the cellular and molecular mechanisms of this pathway since the late 1950s (Y) (Yu et al., 2017). Under some conditions, autophagy is regarded a cytoprotective process in cancer therapy, and it is implicated in the development of therapy resistance as a clinical barrier to successful cancer treatment, leading to a poor prognosis in cancer patients (Liu et al., 2020)

METHODS

Maintenance of cell cultures

AMJ13: Ahmed Mortada jabria 2013 was obtained from the Iraq biotech Cell Bank Unit and maintained in RPMI-1640 supplemented with 10% Fetal bovine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were passaged using Trypsin-EDTA reseeded at 50% confluence twice a week, and incubated at 37 °C .

Cytotoxicity Assays

To determine the cytotoxic effect, the MTT cell viability assay was conducted on 96-well plates. Cell lines were seeded at 1 $\times 10^4$cells/well. After 24 hrs. or a confluent monolayer was achieved, cells were treated with tested compound. Cell viability was measured after 72 hrs of treatment by removing the medium, adding 28 µL of 2 mg/ml solution of MTT (and incubating the cells for 1.5 h at 37 °C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 130 µL of DMSO (Dimethyl Sulphoxide) followed by 37 °C incubation for 15 min with shaking (Al-Shammari et al., 2016). The absorbency was determined on a microplate reader at 492 nm (test wavelength); the assay was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as the following equation:- (Al-Shammari et al., 2020).

Cell viability % =(absorbance of treated cell / absorbance of non- treated cell)*100

cytotoxicity % =100- cell viability

GI % = mean of control – mean of treted / mean of control *100

Apoptosis Estimation (Propidium Iodide/Acridine Orange Assay):

The apoptotic attentions in cell lines (infected and control) were measured using (AO/PI). 5000 cells/well were seeded in plate, next infected with (gold N.P) for 24 hours in a 37 °C incubator. For traditional dual staining. The tested wells received exactly 50µl of the AO/PI stain mixture (at room temperature) for 30 seconds. After then, the stain was removed. The images were taken using a Leica fluorescent microscope (Al-Shammari et al., 2020).

Statistical analysis:

The obtained data were statically analyzed using an unpaired t-test with Graph Pad Prism 6 (Mohammed et al., 2019). The values were presented as the mean ± SD of triplicate measurements (Al-Ziaydi et al 2020). Isobologram version 1 were performed to compare the difference between groups under different conditions, P values >0.05 were considered significant. CompuSyn software program algorithm assessed the combination index CI. Combined dose-response curves were fitted on Chou-Talalay lines. CI > 1.1 indicates antagonism and CI < 1 shows synergism CI between 1 and CI = 1 to 1.1 implies an additive impact (Chou, 2010).

Cell Lines Breast cancer

Cell lines (AMJ13) cell line were supplied by tissue culture unit / ICCMGR (Iraqi Centre for Cancer and Medical Genetic Researches), Baghdad, Iraq (Al-Shammari et al., 2015). These cells were maintained in RPMI-1640 media (Roswell Park Memorial Institute - 1640 medium) with fetal bovine serum (FBS), 100 unit's/ml penicillin, and 100 unit's/ml streptomycin, and incubated at 37°C for 24 hrs to allow cell attachment, proliferation, and confluent monolayer achievement. These cells were regularly assessed for standard growth characteristics and regularly authenticated. in the humid atmosphere of 5% CO2 . 3-(4, 5-dimethylthiazol-2-yl)- 2, 5- diphenyltetrazolium Bromide (MTT) Assay.

cell lines AMJ13 were detached from their flasks when they reached the subconfluent monolayer by trypsinization. Culture medium (20 ml) and 10% serum were added to the falcons and mixed gently with cells to prepare cell suspension. The cell suspension in the culture flask was poured aseptically into a sterile

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beaker. Using a multi micropipette, 200µ1 of the cell suspension was transferred into each well in a 96-well microplate, and the plate was covered with a sterile adhesive film, The absorbance reading was taken at 492 nm by using micro-plate reader (Freshney, 2015) . The absorbance of cells cultured in control media was taken to represent 100% viability. The viability of treated cells was determined as a percentage of that for the untreated control. Each concentration was tested in triplicate, and the experiment was repeated twice.

The concentration of the cells in each well was 1x104 . The percentage of cell line inhibition was determined as the mean ± SD using the following equation.

RESULTS:

The Effect of Colchicine , Chloroquine, and Co-treatment on the Cell Number AMJ13 in Vitro

To study the potential interaction between Colchicine and Chloroquine chemotherapy in vitro, the effectiveness of combined treatment of several concentrations of Colchicine and Chloroquine was evaluated, in the AMJ13 cell lines. Cells were treated with Colchicine and Chloroquine or with combination of Colchicine and Chloroquine, and the cell viability was determined after 72 hrs by MTT assay. Enhanced cytotoxicity observed in the combination treatment of Colchicine and Chloroquine, which refer to synergistic effect.

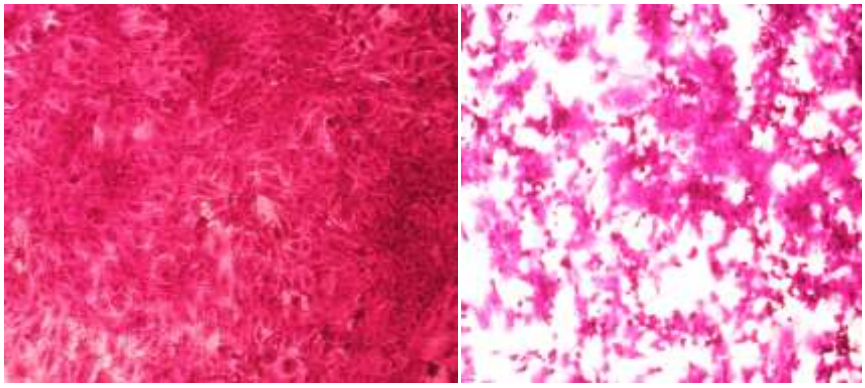
The Colchicine extract concentrations used were (100, 50, 25, 12.5, 6.25, and 3.125µg/ml), with each concentration in triplicate and the experiments were repeated twice. The data is represented as the mean ±SD. The percentages of AMJ13 cell growth inhibition of colchicine (GI %) were (59.1 % ,48.8 % ,44.7 % ,39.9 %, 36.4 % , and 21.6 %) at each mentioned concentration respectively. The IC50 value of colchicine extract was range (2.351 to 20.73µg/ml is 6.982 µg/ml)

The data is represented as the mean ±SD. The percentages of AMJ13 cell growth inhibition were Chloroquine (GI%)were (72.7 % ,69.9 %, 66.1 % ,52.6 % , 24 % , and 16.7 %). at each mentioned concentration respectively. The IC50 value of Chloroquin extract was range (2.696 t0 10.9 µg/ml is 5.42 µg/ml).

The percentages of AMJ13 cell Growth inhibition of Co-treatment (GI%) were (91.4 % ,84.7 %,78.5 % ,64.7 % ,61.6 % ,and 46.8%). at each mentioned concentration respectively. The IC50 value of Co-treatment was used CompuSyn Isobologram

The Effect of Colchicine ,Chloroquine, and Co-treatment on the morphology of AMJ13 *in Vitro*

The cultured AMJ13 cells had an elongated multipolar epithelial-like cell shape, with nuclear polymorphism and multiple nuclei in most of the cells, which expressed the characteristics of cell morphology ,as well as showing many cells with mitotic figures (1). The Morphological pictures for AMJ13 *in vitro* un-treated before was full number of cell, monolayer cell shape. After drugs exposure each of Colchicine ,Chloroquine,and Co-treatment for used concentrations were (100,50,25,12.5,6.25,13.2 µg/ml) turn into singale cell suspension, the number of cell began to decrease. the figures (1) refers to graduate decreased in cell number and killing effect of graduate when increase of conctration of Colchicine,Choloroquin and CO-treatment .



Un-treated COL.

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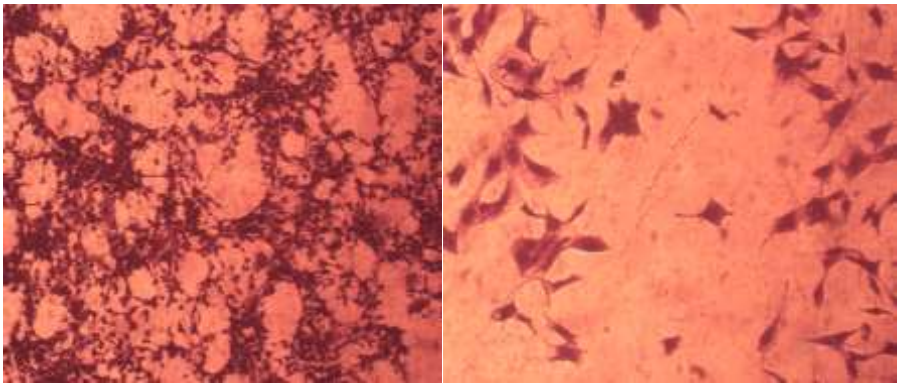

CLQ. COM.

Figure (1): Morphological pictures for AMJ13 in vitro un-treated (as control cells) and Cytotoxicity under an inverted microscope, (10x).

The Inhibiting Effect of Colchicine, Chloroquine, and Co-treatment on AMJ13 Growth Rate *in Vitro*

The cytotoxicity was assessed using different concentrations of colchicine, Chloroquine, and Co-treatment (100 ,50 ,25 ,12.5 ,6.25 ,3.12 µg/ml) Table (1), by MTT cytotoxicity assay. According to these findings, increasing the concentration of the inhibitor increases cytotoxicity or enhances growth inhibition . For breast cancer cell lines, there is a statistically significant difference between inhibition by Colchicine, Chloroquine, and Co-treatment as shown in Figures (2,3,4) the RPMI-1640 medium used as a positive control for comparing the effects of Colchicine, Chloroquin, and Co-treatment.

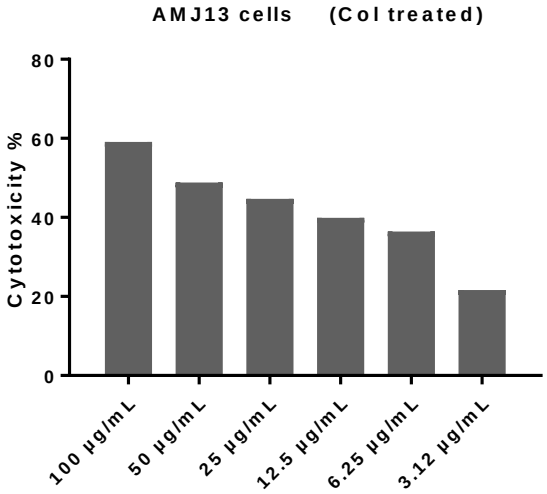

Figure (2): Cytotoxicity effect (CT %) after treatment of cell lines with Colchicine to AMJ13 cell.

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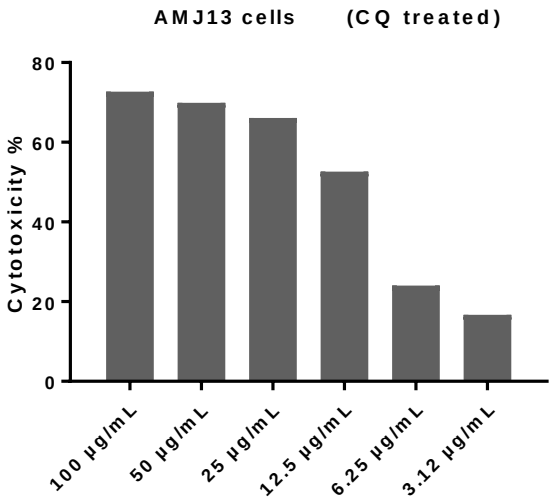

Figure (3): Cytotoxicity effect (CT %) after treatment of cell lines with Chloroquine to AMJ13 cells .

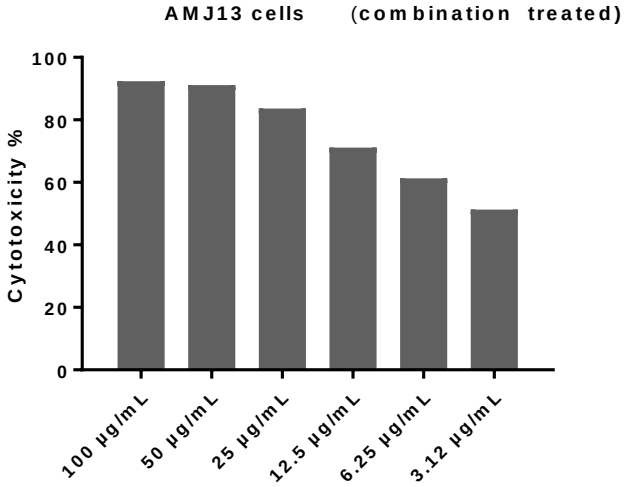

Figure (4): Cytotoxicity effect (CT %) after treatment of cell lines with combination treated to AMJ13 cells .

Estimation of Colchicine, Chloroquine and Co-treatment (IC50) in AMJ13 *in vitro*

The IC50 value in breast cancer cell lines was determined to evaluate the effect of each treatment on cell growth.The result shown that breast cancer cells are effectively infected and destroyed by live attenuated AMJ13 and it caused a significant cytopathic effect in the infected cell lines after 72 h of infection with remarkable effect on AMJ13 cells IC50 of Colchicine treated range(2.351 to 20.73 was 6.982) for AMJ13, IC50 of Chloroquin treated range (2.696 to 10.9 was 5.42) for AMJ13 , IC50 of combnation drugs used CompuSyn Isobologram when it was for AMJ13. Breast cancer treatment induces apoptosis significantly in breast cancer cell lines compared with control cells. Statistical data of IC50 for comparison of cell lines after treatment with of Colchicine , Chloroquine and Co-treatment.can be seen in Table (1). IC50 is a measure of a drug's and inhibitor's potency in inhibiting breast cancer cell lines. It is a quantitative measure that shows the amount of a certain inhibitory agent (Colchicine, Chloroquine, and Co-treatment) required to prevent breast cancer proliferation. It expresses the medication concentration at which 50% of cells are inhibited, which explains why the value of IC50 in AMJ13 appeared. The findings revealed that there are considerable changes in

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IC50 amongst AMJ13 cell lines following medication treatment Figure (5 and 6).

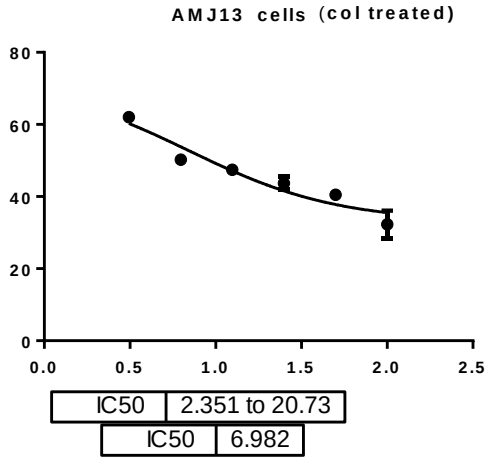

Figure (5): IC50 to exposure of Colchicine to cell lines: IC50 values after AMJ13 cell lines were treated with Colchicine by GraphPad Prism software.

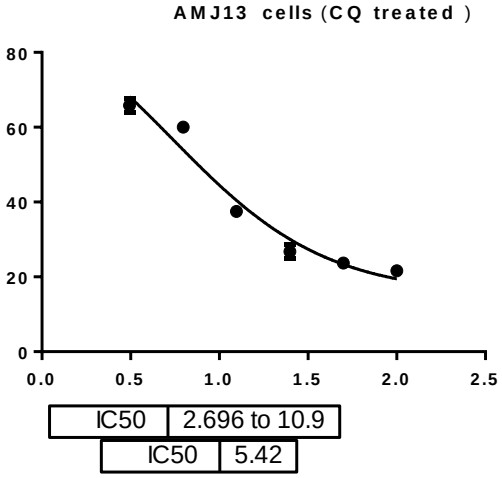

Figure (6): IC50 to exposure of Chloroquin to cell lines: IC50 values after AMJ13 cell lines were treated with Chloroquin by GraphPad Prism software.

The Inhibiting Effect of Co-treatment of Colchicine and Chloroquine on the Growth of AMJ13

The cytotoxicity ratios of Colchicin (6.982 µg/ml) and Chloroquin (5.42 µg/ml) were examined in order to investigate the effects of oncolytic of Colchicin, Chloroquin, and Co-treatment. All combined doses demonstrated synergism against breast cancer (AMJ13) cell lines Figure (2,3,4) Table (1).
The CI was calculated using the CompuSyn Isobologram from dose-effect data from single and combined therapies. CI < 1 shows synergism; CI = 1 to 1.1 implies an additive impact; and CI > 1.1 indicates antagonism (showing a synergistic effect or interaction between Colchicine and Chloroquine).
Colchicin and Chloroquin had a synergistic effect or interaction at all combination points for the combined treatment of the AMJ13 cell line Table (1) and Figure (7A and B).
The Chou-Talalay equation and dose-oriented isobologram technique analysis revealed synergism between Colchicine and Chloroquin in six combinations. 1(CI: 0.29558), 2 (CI: 0.34309), 3 (CI: 0.29807), 4 (CI: 0.38965),5 (CI: 0.23687), 6(CI: 0.30118) at 50% growth inhibition doses, as represented in figure (2 ,3 ,4).
Dose-Effect Curve

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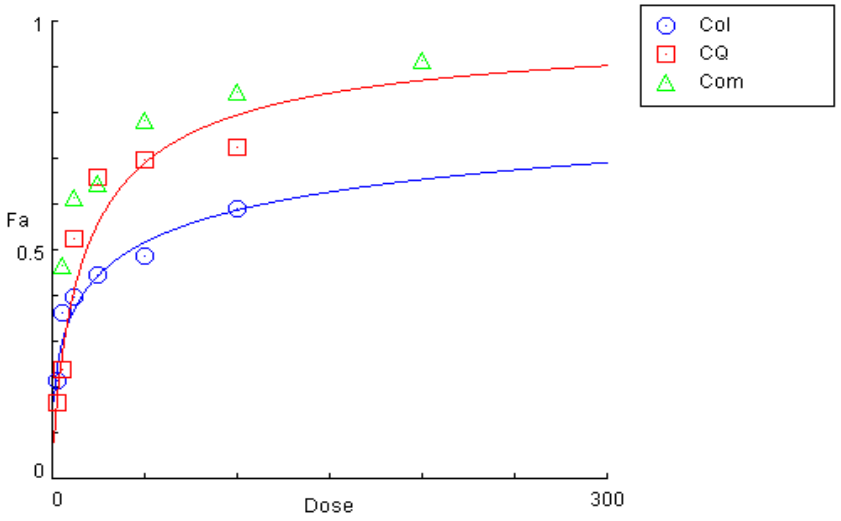
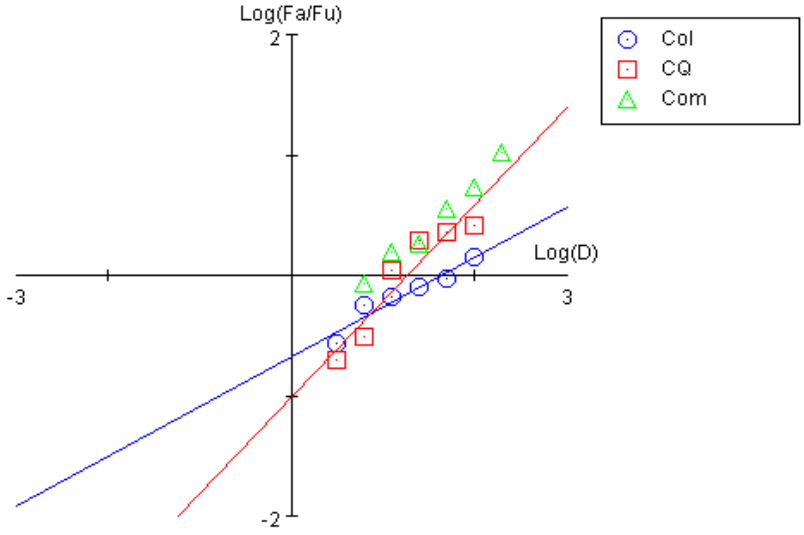


Figure(7A)
Median-Effect Plot



Figure(7 B)

Figure(7A and B) :combination of (Colchicine and Chloroquine) showed superior anticancer activity in AMJ13 breast cancer cells. then cell viability was measured by MTT assay.

Table (1) : The cytotoxic effect of (Colchicine and Chloroquine) combination or alone against AMJ13 cells. Combination index (CI) was measured by CompuSyn software. CI data for non constant Co-treatment (Colchicine and Chloroquine) of cell lines showing synergism effect between (Colchicine and Chloroquine) in AMJ13.

CI Data for Non-Constant Combo: Com (COL+CQ)

COL Dose	CQ Dose	Effect	CI
100.0	100.0	0.914	0.29558
50.0	50.0	0.847	0.34309
25.0	25.0	0.785	0.29807
12.5	12.5	0.647	0.38965
6.25	6.25	0.616	0.23687
3.12	3.12	0.468	0.30118

Normalized Isobologram for Combo: Com (COL+CQ)

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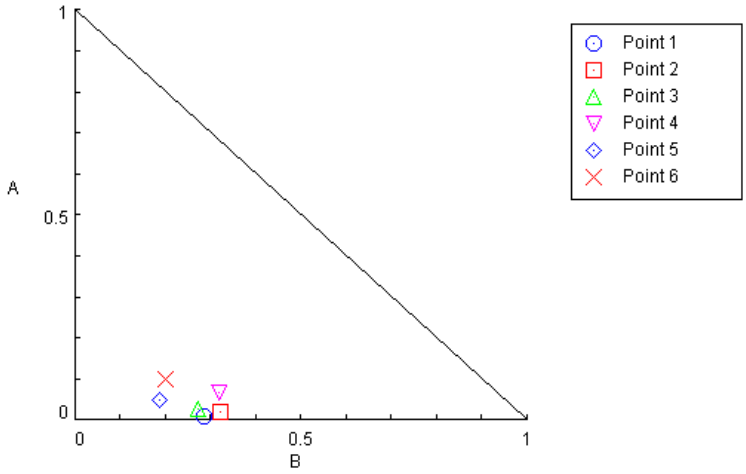

Figure(8): CI was measured by CompuSyn software. CI values from the treatment of AMJ13 cancer cells

The Effect of Colchicines, Choloroquine, and Co-treatment on AMJ13 Optical Density

Optical Density is reflected of cell density, decreased in cell density having decreased optical density , refers to graduate decreased in cell number and killing effect of graduate when increase of conctration. The Optical Density was assessed using different concentrations of Colchicine, Chloroquine, and CO-treatment (100 ,50 ,25 ,12.5 ,6.25 ,3.12 µg/ml).

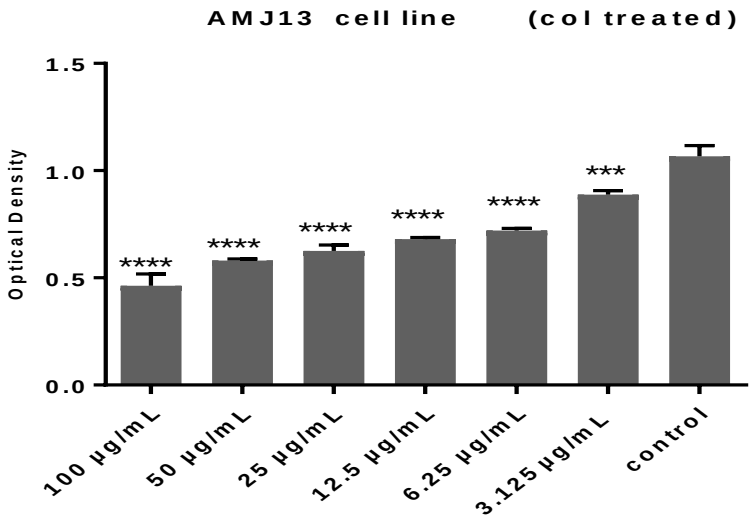

Figure (9): Optical Density is reflected of cell density, this figure showing Optical Density of Colchicine.

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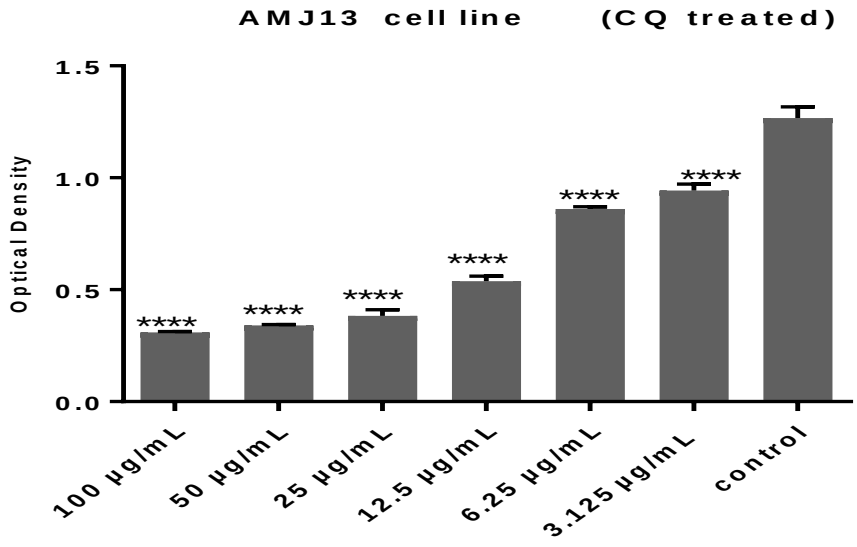

Figure(10): Optical Density is reflected of cell density, this figure showing Optical Density of Chloroquin.

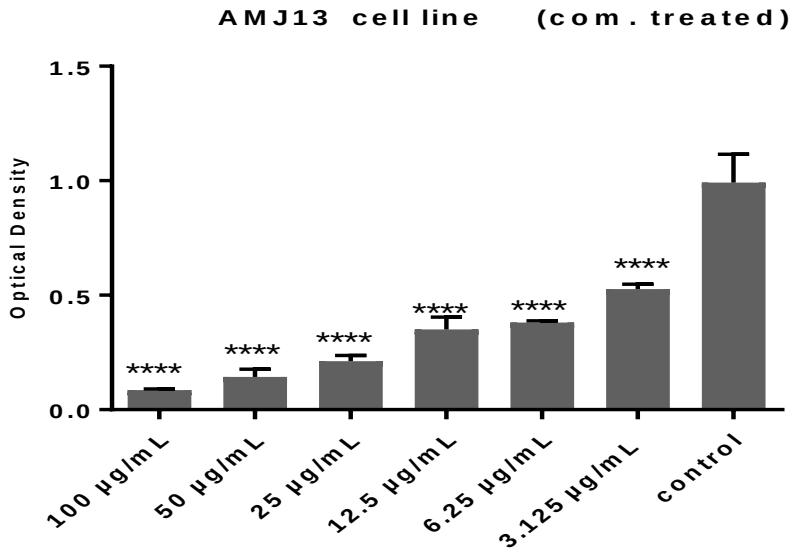

Figure (11): Optical Density is reflected of cell density, this figure showing Optical Density of CO-treatment (COL and CQ).

The Effect of Colchicine, Choloroquine and co-treatment on Apoptosis in AMJ13 *in Vitro*

In this research, a fluorometric cell viability assay with acridine orange and propidium iodide AO/PI was used to detect morphological alterations as well as the proportions of apoptotic, necrotic, and normal viable cells. AO will stain all nucleated cells to produce green fluorescence. Only dead cells with poor membrane integration can accept PI. As a result, all dead nucleated cells will be stained with PI, resulting in red fluorescence. Cells stained with AO and PI fluoresced red due to quenching. All live nucleated cells fluoresced green, while all dead nucleated cells fluoresced red. Viable cells fluoresced green under fluorescence microscopy because they were heavily stained with AO and did not integrate PI, whereas nonviable cells fluoresced orange. Apoptotic cells were observed in cell lines treated with the IC50 of AMJ13-attenuated breast cancer disease 72 hours after inoculation, exhibiting reduced size, integrated membrane, and condensation of chromatin visible as apoptotic cells with bright red/orange areas of condensed chromatin in the nuclei rather than uniform color as seen in necrotic cells. The combination of Colchicine and Chloroquine has more synergistic activity than either Colchicine or Chloroquine alone. in AMJ13 cell lines. Colchicine and Chloroquine

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was the anti-tumor activity of AMJ13 by increasing the cellular sensitivity to phytotherapy agent (inhibitor) and this enhanced sensitivity is partially caused by the induction of apoptosis. In (Figuer12), the amounts of non-apoptotic cells (green color) and apoptotic cells (red color) in the images were statically analyzed to histograms using Image J software.

Stained cells- AO/PI results

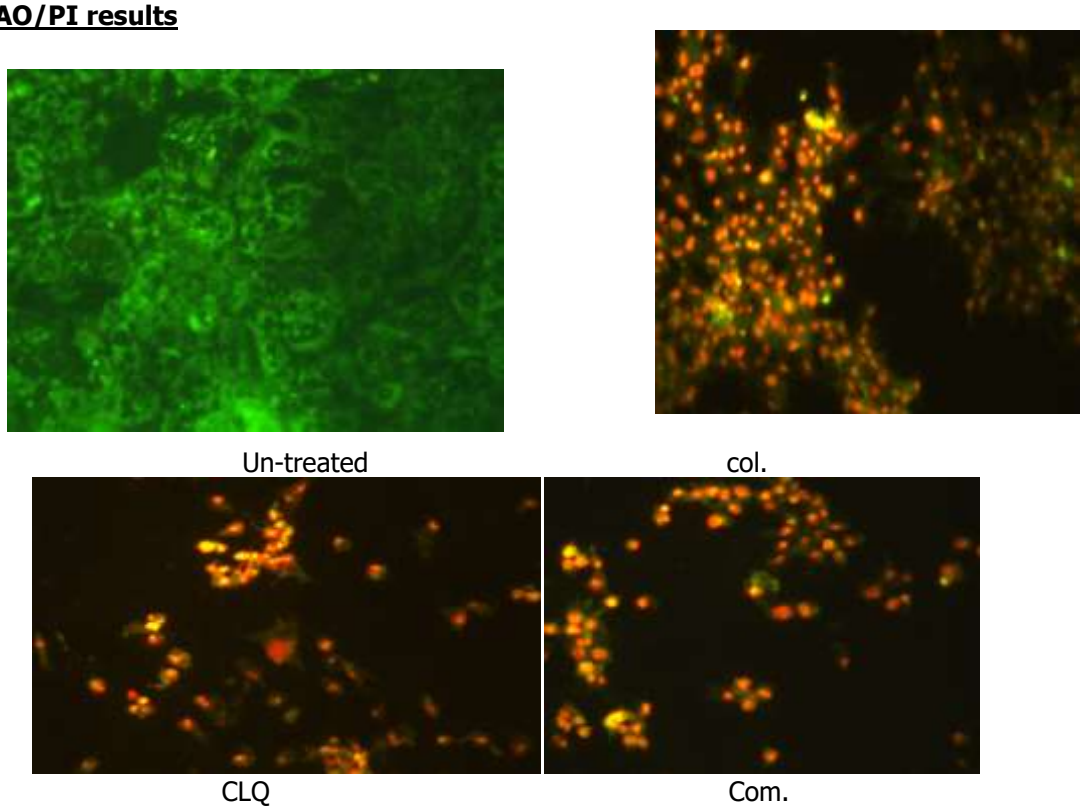

Figure (12): Analysis of apoptosis effect in AMJ13 cell lines after treatment with Colchicine and Chloroquine ,and their combination treated cells and untreated cells (control) in AMJ13. All cells were stained by AO/PI and used fluorescent microscope (10X) to explain apoptosis effects. The green color represent viable cells and red color show dead cells after treatment with a dose of Colchicine and Chloroquine for 72 hrs.

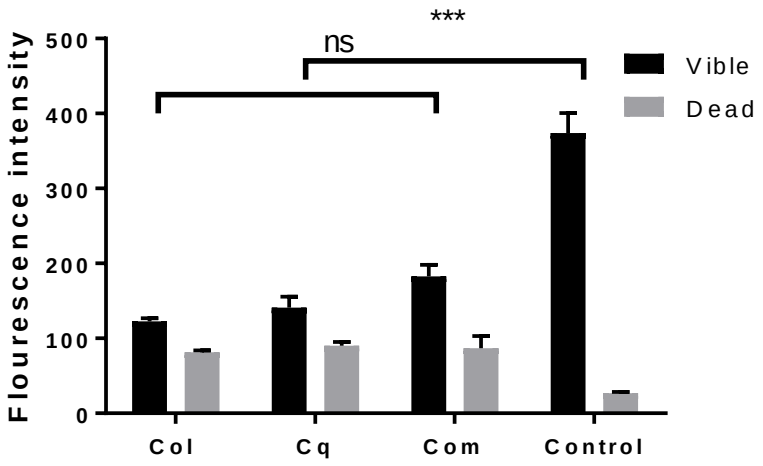

Figure (13) : Fluorescent intensity to calculate apoptosis in treated cells confirms that ensure apoptosis as demonstrated by red stained cells (treated cells) and green fluorescence in untreated control cells.

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DISCUSSION

Over the last two decades, there has been a rise in interest in the pharmacological effects of bioactive compounds on cancer treatment and prevention. It has been shown to possess numerous anti-cancer activities in various cancer cells through different forms of cytotoxic effects without exhibiting considerable damage to normal cells (Katiyar et al., 2009; Mantena et al., 2006).

The half maximum inhibitory concentration (IC50) of a pharmacological inhibitor is a measure of its ability to inhibit AMJ13. The IC50 value is a quantitative measure used to determine how much of a given inhibitory medication is present. (Colchicines, Choloroquin, and Co-treatment) is required to prevent the spread of breast cancer. This is because the effect of chemotherapy on cells differs according to type, and this is related to changes in cancer cells After being treated with drugs. The effect of (Colchicines, Choloroquin, and Co-treatment) on breast cancer cell lines was shown to be very clear .

Other studies have employed various strategies, one by halving the provided dose of a chemotherapeutic drug (rituximab or doxorubicin) to lessen chemotherapy toxicity (Al shammari et al., 2016). Tamoxifen, an anti-estrogen medication, is currently used widely in the prevention and treatment of estrogen receptor positive breast cancer (Lazarus et al., 2009). However, many patients acquire tamoxifen resistance and suffer from severe adverse effects (Lazarus et al., 2009).

The results implied that synergism inhibitor had more effect in inhibition of proliferation, anticancer growth action, and caused increase in cytotoxicity and lead to induced morphological changes and apoptosis. The in vitro results of this study revealed that increasing the concentrations each of COL and CLQ in AMJ13 increases cytotoxicity and improves anti proliferation against AMJ13 (Al-Shammari, et al.,2019). COL and CLQ were discovered to have a significant effect on breast cancer cells.The combination of COL and CLQ had the strongest effect on cancer cell lines. CI values revealed high synergism between COL and CLQ (CI < 1) in AMJ13 cell lines. They have also considered cytotoxicity due to the absence of any death rate greater than 50%. AO/PI assay results demonstrated that combination therapy was the best inducer of apoptosis, which agreed with our previous studies. tumor development and cancer prevention Our findings support the study's goal (Arora, et al.,2015).

Isobologram analysis, or the Chou-Talalay equation (Combination index), demonstrating the synergistic effect of COL and CLQ in six different doses used of AMJ13. Isobologram analyses in AMJ13 showed synergistic effect between drugs. Combination medicines provide significant benefits in terms of improved efficacy, decreased cancer toxicity, and reduced drug resistance development. As a result, these benefits have become a standard for the treatment of various diseases and are a potential option in cases of unmet medical need (Foucquier and Gued, 2015). There are numerous methods for determining the synergy of two or more chemotherapy regimens combined to treat a variety of diseases and tumors; these methods were used by the CompuSyn program (Chou, 2010; Rodea-Palomares et al., 2010; W. Humphrey et al., 2011).

The inhibitory rates were seen in the cytotoxicity activity of the combination (COL and CLQ) on cell lines. These findings revealed a synergistic effect of six combination concentrations employed on AMJ13 cell lines. The current study's findings reflected great important of combination therapy in the treatment of breast cancer, and it was discovered that the high rates obtained from the combination of COL and CLQ in the cell lines tumors AMJ13 may also be effective in treating other types of cancer (AL-Shammari et al., 2016).

Morphological alterations and apoptosis observed following treatment with Colchicines and Chloroquin are dependent on the combination index determined for COL and CLQ for 72 hours. Apoptosis was visible as red cells in AO/PI stained cell by fluorescent microscopy and treated cells, while healthy cells were green. Apoptosis is a natural process of programmed cell death that can be triggered by a range of physical and chemical causes and is precisely managed by the organism. Although there are three major signaling channels in apoptosis (mitochondrion, death receptor, and endoplasmic reticulum signaling pathways), apoptotic signaling is frequently integrated and amplified at the mitochondrial level (Guo et. al, 2016).The current investigation discovered an alternate or supportive treatment for chemotherapy or other conventional treatments by employing the inhibitor (COL) or (CLQ) or their combination. In addition, anti-tumor effectiveness and cancer cell growth inhibition were obtained through apoptosis induction. This can be an alternate treatment as a combination therapy that can be used to reduce the dose of chemotherapy or other conventional treatments while keeping the same or greater anti-proliferative activity and overcoming chemotherapy resistance or other treatments.The objective of combination therapy is to assault tumor cells through many mechanisms of action in order to prevent cancer cells from acquiring resistance to therapy (Kumar et al., 2014). Furthermore, one of the goals of this study was to lessen the harmful side effects of chemotherapy or other conventional treatments in breast cancer cell lines by using combination therapy

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(virotherapy and phytotherapy). When using chemotherapy with combination therapy, this can be done by lowering the provided dose while keeping the same or stronger anti-tumor activity.

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