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Interaction Effect of Methotrexate and Aspirin on MCF7 cell line Proliferation: *In vitro* Study

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Abstract

Methotrexate, a folic acid molecular alternative inhibiting dihydrofolate reductase (DHFR), is employed for the treatment of various types of tumors combined with aspirin; acetylsalicylic acid is a nonsteroidal anti-inflammatory drug (NSAID). The present study aimed to detect the combined effects of both medications on MCF7 cell line activity. The drug combinations of aspirin and methotrexate were tested for cytotoxicity against the breast cancer cell line MCF7 using the MTT assay. The results showed that methotrexate, aspirin, and combination drugs have potent cytotoxicity against MCF7 cells. The mean IC50 of the methotrexate-treated group was 155.7 µg/ml (range, 77.89 to 311µg/ml. However, the IC50 of the aspirin-treated group was 465 µg/ml (range, 243.3 to 888.6 µg/ml). The IC50 of combination drugs used in the CompuSyn Isobologram on MCF7 cell lines, the cytotoxicity of medications methotrexate, aspirin, and combination demonstrated a synergistic action, and combination drugs have potent cytotoxicity against MCF7 cell lines. In conclusion, the combination of methotrexate and aspirin has a potent anticancer effect.

KEYWORDS
Methotrexate, Aspirin, Cell proliferation, MCF7, Apoptosis

INTRODUCTION

Cancer is a significant public health concern because it has been the second-highest cause of mortality in the world. It was characterized by uncontrolled cell proliferation and spread. Mortality could occur if the growth is not stopped (Siegel *et al*., 2019). Several studies have been done to examine the interaction effects of methotorxate and asprin in rats (Al-Abdaly *et al*., 2021, Stewart, *et al*., 1987). Moreover, in an animal model, the efficacy of variable dosage of aspirin in combating methotrexate-induced intestinal toxicity has been performed (Gupta *et al*., 2015).

Cancer treatment aims to eliminate cancer cells while causing the fewest adverse effects on healthy cells. A cancer therapy, localized and/or systemic treatments are used to lessen uncomfortable symptoms, combined with complementary therapies (Miller *et al*., 2019). The most frequent malignancy in women was breast cancer (24.2%), which affects 6.6 percent of them and is the most prevalent cancer in Iraqi women (Bray *et al*., 2018).

The prognosis for individuals with breast cancer had been significantly improved due to breakthroughs in cancer therapy, but it was still not completely adequate. The heterogeneity of breast cancer was influenced by genetic, epigenetic, and environmental variables. Significant development has occurred in recent years in understanding the mechanisms that govern how breast cancer develops and spreads. More study is still needed to fully comprehend the novel pathogenic genes and cancer-causing pathways (Chen *et al*., 2021).

Aspirin might be used in conjunction with conventional chemotherapy or radiotherapy treatment. On the other hand, researchers found that individuals on a constant dosage of MTX who were also taking nonsteroidal anti-inflammatory medicines (NSAIDs) had a lower rate of kidney elimination of the drug (Le Merdy *et al*., 2021; Wilsdon, 2020).

Aspirin is metabolized to salicylic acid via carboxylesterase, which has many therapeutic reactions with various medications such as charcoal, methotrexate (MTX), and antacids. Most of such reactions arise due to aspirin's capacity to move medicines from albumin binding. Because it is metabolized in various ways, it may interfere with the metabolism of many other medicines. Whenever a high dosage of aspirin is combined with additional medicine, the level of the other drug may raise or reduce. Whereas if the pharmacodynamics and pharmacokinetics of the medication are understood (Alegbeleye *et al*., 2011).

Methotrexate (MTX) is a widely employed pharmaceutical drug that is understood to affect both healthy and malignant cells by interacting with and inhibiting dihydrofolate reductase (DHFR) (Raimondi *et al*., 2019). MTX, a folate anti-metabolite, has been primarily used to treat rheumatoid arthritis. Besides, it is also used to treat plenty of malignancies such as lymphoma (Feng and Chien, 2003). It has also been used to treat breast cancer (Manjappa *et al*., 2019). Studies on the combination of aspirin and MTX on the MCF7 cell line are scarce. Therefore, the present investigation aimed to assess the single and combined effects of MTX and aspirin on MCF7 breast cancer cell proliferation.

MATERIALS AND METHODS

Maintenance of cell culture

MCF7 was obtained from the Iraqi Center for Cancer and

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Medical Genetics Research, Baghdad, Iraq. ICCMGR is breast cancer cell line, maintained in Roswell Park memorial institute, in RPMI 1640 medium supplemented with 10% Fetal bovine, 100 units/ml of penicillin, and 100 g/ml of streptomycin. Trypsin-EDTA was used to passage the cells, and they were then reseeded at 50% confluence twice weekly and cultured at 37°C (Al-Shammari *et al*., 2020a)

Cytotoxicity Assays

Methyl thiazolyl tetrazolium (MTT) cell viability assay was conducted on 96-well plates. Cell lines were seeded at 1×10^4cells/well. When a confluent monolayer was achieved, cells were treated with MTX, ASA and combination. Cell viability was measured after 72 h of treatment by removing the medium, adding 28 µL of 2 mg/ml 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 Dimethyl Sulphoxide (DMSO) followed by incubation at 37°C for 15 min with shaking (Al-Shammari *et al*., 2016). The absorbance was determined on a microplate reader at 492 nm (test wavelength); the assay was performed in triplicate. Cell viability was calculated by the following formula: absorbance of treated cell/absorbance of the untreated cell*100.

Growth inhibition (GI%) is calculated as follows: mean of controls *100 *mean of treated * 100 (Al-Shammari *et al*., 2020b).

Acridine orange Propidium iodide test (AO\PI)

The apoptotic rates on cell lines (treated and untreated) were assessed by (AO/PI) for 24 hin an incubator set at 37°C; 5000 cells per well were infected with (gold nanoparticles). For the classic dual staining. Exactly 50 µl of the AO/PI stain mixture (at room temperature) was applied to each test well for 30 sec. The stain was then eliminated. The Leica fluorescence microscope was used to capture the photographs showing the cell line's apoptosis (Al-Shammari *et al*., 2020b).

Statistical analysis

Graph Pad Prism statistical software program version 6 was used to statistically evaluate the data. An unpaired T-test was performed to assess the differences between groups (Mohammed *et al*., 2019). The results of triple measurements were presented as the mean ± SD (Al-Ziaydi *et al*., 2020). Isobologram version 1 was used to compare the difference between drugs under different concentrations. 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 is synergism CI between 1 and CI = 1 to 1.1, an additive impact (Chou, 2010).

RESULTS

The results of the present study are shown in Figures 1-7. Briefly, it is revealed that when the concentration of methotrexate increased, the cytotoxicity percentage (Growth inhibition %) was raised (Figure 1A). Similarly, the result of aspirin was documented (Figure 1B). The half-maximal inhibitory concentration (IC50) of both methotrexate (MTX) and aspirin (ASA) is shown in Figures 2.

The optical density (OD) of both MTX and ASA showed a non-significant (ns) effect in fewer concentration of MTX (31.25), and significant in other concentration. A similar result was found for ASA, but more evident for a combination of both drugs (Figure 3).

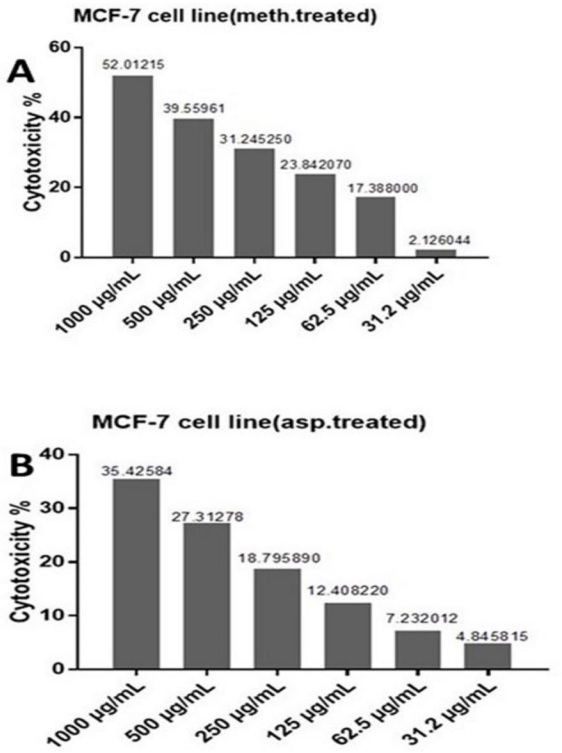

Figure 1. Cytotoxicity effect (CT %) of methotrexate (A) and aspirin (B) in different concentration on MCF7 cells.

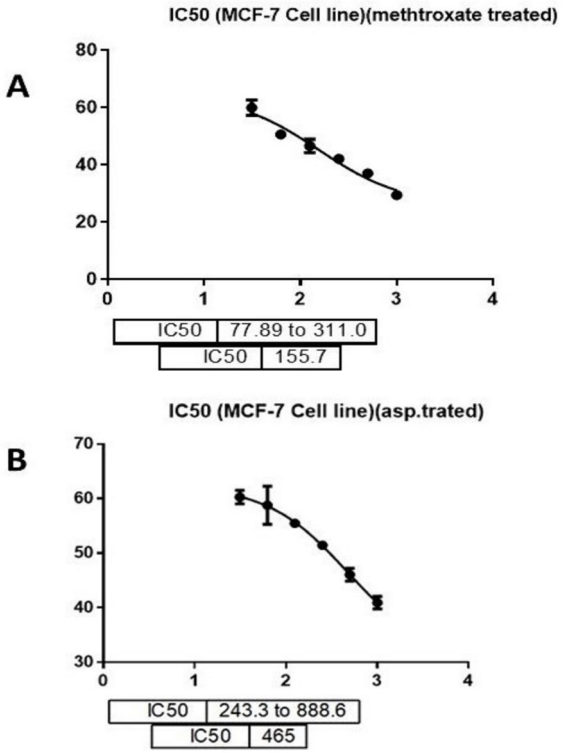

Figure 2. Half maximal inhibitory concentration (IC50) after exposure of the MCF7 cell line to methotrexate (A) and aspirin (B) in different concentrations using MTT assay and GraphPad Prism software.

The dose-Effect Curve (Figure 4A) and Median-Effect Plot (Figure 4B) of a combination of methotrexate and aspirin showed a higher anticancer effect in MCF7 breast cancer cells. Cell viability was measured by MTT assay. Designs of (Figure 4C) normalized isobologram of non-constant combination ratios, dose-effect curve, and median-effect plot for MCF7 were measured by the Chou-Talalay method where CI value quantitatively defines synergism. (CI < 1), additive effect (CI = 1-1.1) and antagonism

(CI > 1.1).

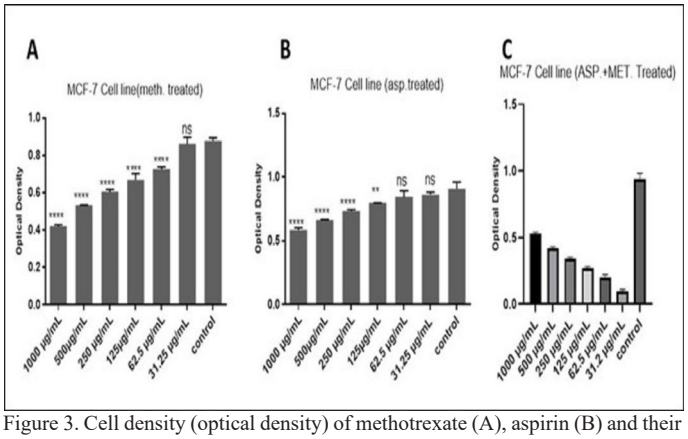

Figure 3. Cell density (optical density) of methotrexate (A), aspirin (B) and their combination (C) in different concentrations on MCF7 cell line using (GraphPad Prism software).

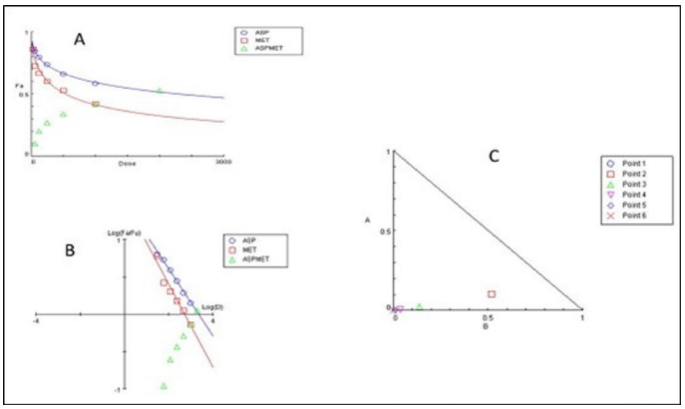

Figure 4. A. Dose-Effect Curve of methotrexate, aspirin, and combination (MTX, ASA) equal dose using CompuSyn Isobologram. Fraction affected (fa). 8B. Median-Effect Plot for an equal dose of methotrexate, aspirin, and their combination (MTX, ASA). The red line represents Methotrexate) single treatment, the blue line is the single treatment aspirin and green triangle is the combination treatment. 8C. Normalized Isobologram for Combination methotrexate and aspirin equally concentration measured by the Chou-Talalay method where CI value quantitatively defines synergism. (CI < 1).

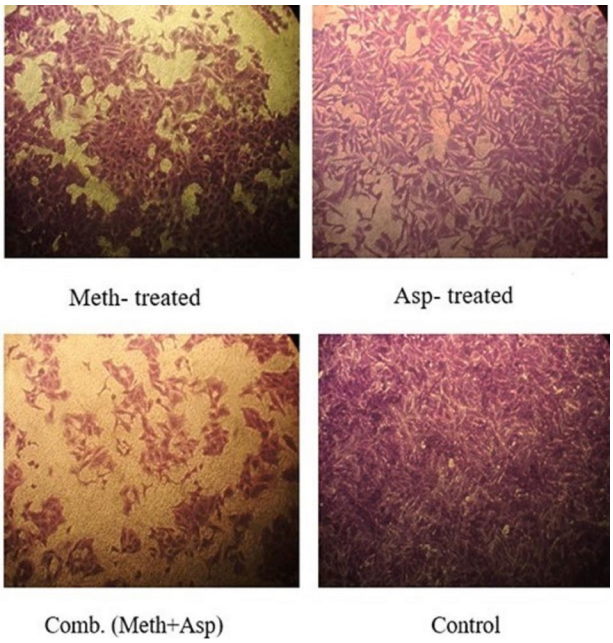

Figure 5. Morphological images for MCF7 *In vitro* before treatment were a full number of cells, monolayer cell shape. (An inverted microscope, 10x).

Following medication exposure, methotrexate and aspirin, and Co-treatment for utilized concentrations were 1000, 500, 250, 125, 62.5, 31.2 µg/ml turned into a single cell suspension, and the number of cells started to decline. That concentration of MTX, ASA, and combination depicts the reduction in cell number and killing impact of the graduate as the concentration of MTX, ASA, and combination increase (Figure 5).

To explain the consequences of apoptosis, all cells were dyed with AO/PI and examined under a fluorescence microscope. After receiving a dose of methotrexate plus aspirin for 72 hours, the green color represents live cells, and the red color displays dead cells (Figures 6,7).

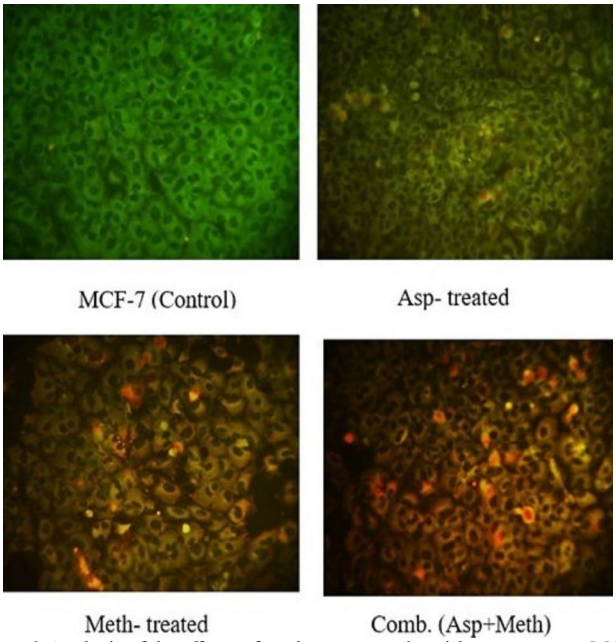

Figure 6. Analysis of the effects of methotrexate and aspirin treatment on MCF7 cell lines, as well as the combination of treated and control cells (fluorescence microscope 10X).

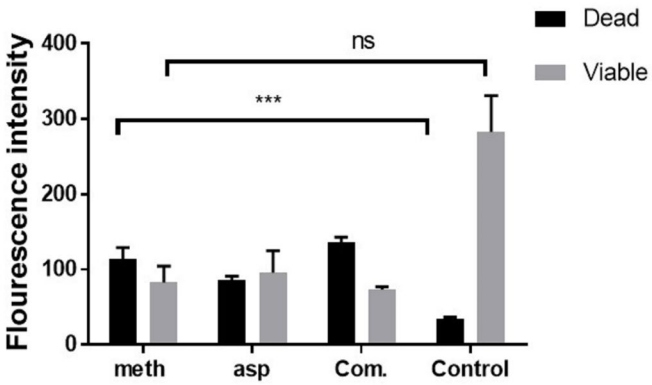

Figure 7. Fluorescent intensity to calculate apoptosis in treated cells confirms that ensure apoptosis as demonstrated by red stained cells (treated cells) and green fluorescence in untreated control cells.

DISCUSSION

Interest in the pharmacological effects of bioactive chemicals on the treatment and prevention of cancer has grown during the past two decades. It has been demonstrated to have several anti-cancer properties in a variety of cancer cells through distinct cytotoxic effects without significantly harming normal cells (Mantena *et al*., 2006; Katiyar *et al*., 2009).

A pharmacological inhibitor's capacity to inhibit MCF7 is quantified by the half-maximum inhibitory concentration (IC50). A quantitative method for estimating the concentration of an inhibiting drug is the IC50 value. To stop breast cancer from spreading, treatment must include methotrexate, aspirin, and co-treatment. This is because chemotherapy affects cells differently depending on their kind, and this is connected to alterations in cancer cells after medication treatment. It was demonstrated

that methotrexate, aspirin, and co-treatment strongly impacted breast cancer cell lines (Al-Shammari *et al*., 2016).

The findings suggested synergism inhibitors were more effective at suppressing proliferation, acting as an anticancer agent, increasing cytotoxicity, and inducing morphological alterations and apoptosis. According to this study's *In vitro* findings, raising the quantities of MTX and ASA in MCF7 increases cytotoxicity and enhances MCF7 resistance to antiproliferative agents (Al-Shammari *et al*., 2019). Breast cancer cells were found to be significantly affected by MTX and ASA. On cancer cell lines, MTX and ASA worked best as a combination. High synergism between MTX and ASA was indicated by CI values (CI) in MCF7 cell lines. Due to the lack of any death rate larger than 50%, they have also thought about cytotoxicity. According to the AO/PI assay results, the combined therapy was the most effective at inducing apoptosis, which was in line with our earlier research. Cancer prevention and tumor growth results support the study's objectives.

The Chou-Talalay equation (combined index) or isobologram analysis can show how MTX and ASA work together to increase the effectiveness of MCF7 at six different doses. Analyses of isobologram in MCF7 revealed a drug's synergistic effect. Combination medications offer notable advantages in increased efficacy, less cancer toxicity, and decreased emergence of drug resistance. As a result, these advantages are now considered a standard for treating many diseases and a viable choice in situations with an unmet medical need (Foucquier and Guedj, 2015). The CompuSyn program employed several methodologies for assessing the synergy of two or more chemotherapy regimens combined to treat a variety of diseases and malignancies (Chou, 2010; Rodea-Palomares *et al*., 2010; Humphrey *et al*., 2011).

The combination of MTX and ASA showed inhibitory rates in its cytotoxicity activity on cell lines. These results showed that the six combined concentrations used on MCF7 cell lines had a synergistic effect. The results of the current study demonstrated the critical role that combination therapy plays in the treatment of breast cancer, and it was found that the promising results obtained with the combination of MTX and ASA in the tumor cell line MCF7 may also be useful in the treatment of other cancers. (Al-Shammari *et al*., 2016).

The combined index found for MTX and ASA for 72 hours influences the morphological changes and apoptosis shown after treatment with methotrexate and aspirin. In AO/PI-dyed and treated cells, apoptosis was obvious as red cells, whereas healthy cells were green. The organism carefully regulates the natural process of planned cell death known as apoptosis and can be brought on by a variety of physical and chemical factors. Although apoptosis is regulated by three main signaling pathways (mitochondrion, death receptor, and endoplasmic reticulum signaling pathways), The mitochondrial level is typically where apoptotic signals are combined and intensified (Guo *et al*., 2016).

The current study used the inhibitors (MTX or ASA) or their combination to find a complementary or supportive treatment for chemotherapy or other traditional treatments. Additionally, apoptotic induction resulted in anti-tumor efficiency and the inhibition of cancer cell development. This alternative therapy can be used as a combination therapy to lower the dosage of chemotherapy or other traditional therapies while maintaining the same anti-proliferative activity or increasing it and overcoming chemotherapy resistance or other treatments. Combination therapy seeks to attack cancer cells using a variety of pathways to keep them from developing resistance to treatment. Using combination therapy, this study also aimed to reduce the negative side effects of chemotherapy or other traditional treatments in breast cancer cell lines. This can be accomplished by reducing the prescribed dose of chemotherapy while maintaining the same or stronger anti-tumor activity.

CONCLUSION

The *In vitro* study on MCF7 cell line, investigate the MTX and ASA decreased cell growth, reduced optical density, decreased cell number, increased killing impact, caused cytotoxicity in addition to morphological alterations and apoptosis in MCF7. The combination of the drugs considerably improves growth inhibition. Combination index analysis (CI) reveals that MTX and ASA had a combined inhibitory effect on the MCF7 cell line.

Recommendations: Examination of the signaling pathway is needed to further understand the mechanism by which methotrexate and aspirin have cytotoxicity effect on breast cancer cells. It is recommended to use other cancer cell line types is recommended. An in vivo study is required to confirm the impact of treatment on cancer cells on physiological parameters in laboratory animals.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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