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Metformin and Metronidazole as Combined Therapeutic Agents in Cervical Cancer: Modulation of Hsp60 and Hsp70 Chaperonins

Kawakeb N Abdulla [1], Aqeela Hayder Majeed [2], Nora Sabah Rasoul [3], Azal Hamoody Jumaa[4], Youssef Shakuri Yasin[*5]

[1,4]Iraqi National Cancer Research Center/University of Baghdad, Baghdad, Iraq

[2,3]College of Medicine -University of Karbala, Karbala, Iraq.

[5] Bilad Alrafidain University, Iraq.

[*]**Corresponding author's:** dryoussef@bauc14.edu.iq

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Abstract:

Objective: Our study aimed to investigate the anticancer effects of a metformin-metronidazole mixture on the HeLa cell line and evaluate its potential to target Hsp70 and Hsp60. **Methods**: over 24 and 72 hours of incubation, the HeLa cancer cell line and human fibroblast cell line were utilized to assess the cytotoxicity and selective toxicity of the mixture on cervical cancer proliferation, using cisplatin as a positive control. The concentrations of metformin, metronidazole, the mixture, and cisplatin ranged from 0.1 to 1,000 µg/ml. The combination index (CI) and selective toxicity index (SI) values were calculated to identify the synergistic behavior of the mixture's components and its selectivity for cancer cells. Furthermore, computational molecular docking simulations were conducted to evaluate the affinity of metformin and metronidazole for binding to heat shock proteins 70 and 60. **Results**: The cytotoxicity assay and SI results indicated that the mixture selectively inhibits cervical cancer growth, with a pattern dependent on concentration and incubation time, while exhibiting a reduced effect on the viability of the HFF cell line. The CI analysis revealed that the interaction between metformin and metronidazole showed a synergistic pattern, particularly after 72 hours of incubation. The computational molecular docking simulations demonstrated that metformin and metronidazole target Hsp70 and Hsp60 with docking scores of -6.2 kcal/mol and -6.7 kcal/mol, respectively. **Conclusion**: The findings from the study's MTT assay, SI, CI, and computational docking simulations suggest that the mixture offers a promising therapeutic option for cervical cancer, considering their established adverse effects and pharmacokinetic profiles.

Keywords: Metformin, Metronidazole, Combination index, Cisplatin, HeLa Cells, Molecular Docking Simulation.

1- Introduction:

Cervical cancer is one of the most common cancers in women and a major cause of cancer-related deaths **(1).** Traditional methods are not always effective in saving patients, because cancers are often diagnosed late. Therefore, it is important to develop new treatments that target cancer cells specifically and can stop their growth or kill them **(2)**. The treatment for this disease can lead to secondary complications such as infections, which can exacerbate the complications, including kidney and urinary problems, that affect the patient's well-being **(3)**. Recently, the anticancer effects of different groups of medications, alone or combined with other

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medicinal substances, have received a great deal of attention through significant preclinical investigations. The widely used commercial drugs Metformin, a member of the biguanide class of antihyperglycemic agents **(4)**, and Metronidazole, an antibiotic and antiprotozoal medication that is used alone or with other antibiotics, have demonstrated cytostatic effects that arrest the proliferation of cancer cells and show anti-invasive effects on breast cancer and melanoma cells. Many studies have focused on inhibiting cervical cancer malignant cell growth with either a synthetic drug or a natural one **(5)**. For a higher patient quality of life, synthetic drugs can be alternative agents to which the tumoral cells are responsive to obtain an efficient therapeutic scheme, offering intrinsic anti-inflammatory actions. Both metformin and metronidazole are oral drugs with proven efficiency in cervical cancer inhibition, and one of the appeals of metronidazole is the decrease in Hsp 60 and Hsp 70 protein expression **(6-8).**

Various treatment modalities for cervical cancer have been identified, including surgery, radiation, and chemotherapy. Radiation therapy is particularly effective as cervical malignancies are susceptible to radiation. It can be utilized in all stages of the disease when surgical interventions are not feasible. Surgery may deliver the best results compared with radiological treatment **(9, 10),** and the efficiency of chemotherapy is more than that of radiation therapy **(11)** Chemoradiotherapy implementation resulted in enhanced survival rates and reduced disease recurrence compared to the use of radiation alone **(12).**

Despite the availability of various treatment options for cervical cancer, there remains a need to find a safe and effective treatment that offers a better prognosis than standard cervical cancer therapies. Several attempts have been made to identify alternative treatment options for cervical cancer. Medicinal plants have been extensively documented for their anticancer properties, as they produce bioactive compounds capable of modulating immune responses and inhibiting tumor growth. **(13)** Recent developments in immunotherapy, including checkpoint inhibitors and CAR-T cell therapy, have significantly revolutionized the management of cancer by enhancing immune-mediated tumor response **(14)**. Another alternative approach involved using certain drugs already employed for other therapeutic purposes. One of these studies showed that esomeprazole can kill cervical cancer cells. The effectiveness of esomeprazole in killing these cells depends on the duration of exposure and the concentration used **(15)**. Another study has revealed that the potency of ciprofloxacin in suppressing the growth of carcinoma relies on its duration and concentration **(16)**.

Metformin is a drug with the potential to kill cancer cells. Multiple studies have been conducted on this topic, and they have found that metformin can effectively destroy breast cancer cells by impeding their proliferation rather than by enhancing insulin sensitivity in epithelial cells **(17)**. Another study exhibited that metformin has cytotoxic effects on ovarian cancer via targeting SPHK1 **(19).** Furthermore, metformin has shown the capacity to kill esophageal cancer cells by controlling SPHK1 and S1P expression **(18)**. Another study revealed that Metformin can impede melanoma cell growth by impacting the expression of microRNAs **(19).**

Likewise, metronidazole has also been included in investigating its potential as an anticancer agent. Several studies have been performed in this line, and one of these studies showed that metronidazole effectively diminishes the viability of MDA-MB-231 and MCF-7 breast cancer cell lines, particularly in lower concentrations, after 24 and 72 hours of incubation **(20).** Further studies have demonstrated that metronidazole can inhibit the growth of Chinese hamster ovary, cervical carcinoma, and human marrow cells **(21).** However, the metronidazole cytotoxic effect seems contingent upon the drug's concentration and the extent of hypoxia **(22-24).** Recent studies demonstrate that heat shock proteins

(HSPs) are frequently overexpressed in various cancer types (25).

Hsp60 and Hsp70 are molecular chaperones highly involved in the folding, unfolding, and translocation of proteins across intracellular membrane structures (26, 27). With an extensive number of functions in the modulation of the proteome and the establishment of chaperoning complexes with specific cochaperones, they orchestrate the protection and repair of misfolded proteins to sustain cellular homeostasis and avoid apoptosis (28). Depending on the client protein and the cellular conditions, the Hsp60/Hsp70 chaperone systems are activated in several cell compartments from the intermembrane space of mitochondria to the cytosol and the nucleus (29). The pathogenesis and etiology of various human cancers are linked to the involvement of Hsp60/Hsp70 in supporting oncogenic mechanisms through collaboration with other chaperones. Since Hsp60 appears overexpressed in cancer cells, it has been proposed as a diagnostic and prognostic marker for numerous neoplasms (30).

Hsp70 can act as an indirect tumor suppressor, and its crucial role in attenuating carcinogenesis, inducing apoptosis, and enhancing cancer-specific immune responses is widely known (31). Hsp70 and Hsp60 on tumor cell surfaces aid adhesion, promote metastasis and angiogenesis, and modulate immune responses to facilitate tumor immune evasion (32). Therefore, both Hsp60 and Hsp70 are considered important and rational targets for preventing cancer progression, improving cancer diagnosis, and as a new therapeutic strategy for most human cancer diseases (33). New anti-Hsp70/Hsp60 inhibitors targeting these chaperones could be incorporated into cancer therapy, in combination with standard treatment protocols, to engage multiple tumor targets and reduce the incidence of adverse effects associated with current chemotherapy treatments (34, 35). A member of the Hsp70 family, Hsp70, is markedly increased during spermatogenesis and the development of breast cancer, delaying cellular senescence (36). Hsp70 in the nucleus serves as a diagnostic marker for epithelial dysplasia, whereas hepatocellular carcinoma patients can be identified by their antibodies against Hsp70 (37).

The prognostic relationship between HSP60 and cervical cancer has recently become a significant focus of research. The study used Western Blot (WB) analysis, reverse transcriptase polymerase chain reaction (RT-PCR), a semi-quantitative method, and 2-dimensional electrophoresis (2-DE) to assess the prognostic importance of HSP60 in cervical cancer. The findings show that HSP60 is essential for the development of cervical cancer (38).

A significant relationship between HSP60 expression and tumor development was found in data analysis from individuals with advanced prostate cancer. In cases of locally advanced prostate cancer, there is a substantial association between HSP60 expression and androgen independence. The levels and distributions of HSP60 immunoreactivity in prostate cancer patients were indicators of biochemical recurrence. Patients with strong HSP60 staining in biopsy samples had a worse recurrence-free survival than those with mild HSP60 expression, according to the study. Prostate cancer tissues exhibit higher levels of HSP60 expression compared to normal prostatic tissue, as determined by an examination of individuals with the disease (39-41).

A non-anticancer drug combination strategy was employed to identify effective anti-cancer alternatives with reduced adverse effects. Multiple studies have been conducted in this area, including one that demonstrated the capacity of the esomeprazole–amygdaline combination to selectively eliminate cervical cancer cells in a manner that depends on both time and concentration (42, 43). Another study demonstrated the efficacy of combining ciprofloxacin with laetrile in inhibiting the growth of esophageal cancer cells (16). The studies reveal a research gap concerning the antiproliferative effects of metformin-metronidazole on cervical cancer cells. This study assessed the

antiproliferative efficacy of metformin-metronidazole on cervical cancer cells and their capacity to target heat shock proteins.

2- Materials and methods:

2-1- Medications:

Study medications were utilized as raw materials sourced from Samarra Pharmaceutical Factory. Medications were diluted with Roswell Park Memorial Institute medium (RPMI media), resulting in a diverse array of concentrations, ranging from 0.1 µg/ml to 1000 µg/ml.

2-2- Culture of human cervical cancer cells:

A HeLa cancer cell line was obtained from the Iraqi Centre for Cancer and Medical Genetics Research, a tissue culture section using normal human fibroblast cells, and a malignant cervical carcinoma. The cells were cultivated in 75 cm^2 tissue culture vessels under meticulously regulated conditions, with 5% CO2 and a relative humidity of 37°C. The RPMI-1640 media (Thermo Fisher Company, USA) was utilized for cell cultivation, supplemented with 100 U/mL penicillin-streptomycin (100 µg/mL streptomycin) and 10% fetal bovine serum (FBS) **(42, 44)**.

The Human Foreskin Fibroblast (HFF) cell line, which is a non-cancerous, normal human cell line derived from neonatal foreskin tissue, was also obtained from the Iraqi Centre for Cancer and Medical Genetics Research.

2-3- Cytotoxicity study:

Cervical cancer cells and healthy human fibroblast cells cultured in a 96-well microtiter plate received treatments of metformin, metronidazole, cisplatin, and (metformin-metronidazole combination. The toxicity of the evaluated medications was examined throughout incubation periods of 24 and 72 hours **(45, 46)**.

Ten thousand cells make up each well. 10% fetal bovine serum is required as a seeding medium. To encourage cell attachment, the plates were treated for 24 hours at 37°C. An RPMI medium was used for serial dilutions; no extra serum was added. Metformin, metronidazole, cisplatin, and the metformin-metronidazole combination were diluted using the RPMI medium to provide a spectrum of values of about 0.1 to 1000 µg/ml for each treatment **(16, 47)**.

Following a 24-hour incubation period, each well containing a monolayer of cancer cells was treated with 200 µl of six replicates for each treatment and positive control (cisplatin), while the control well received 200 µl of maintenance media with a replicate number of around 20. The plates were bonded tightly using self-adhesive material and then put back into the incubator for 24 to 72 hours of incubation. MTT dye was applied to the cells.

At a wavelength of 550 nm for transmission, the optical density of every well was measured using an ELISA reader, more precisely, a microtiter plate reader **(48, 49)**.

The Growth ratio and the ability of drugs to suppress the growth are calculated using the following calculations **(49)**.

$$\textbf{Growth inhibition}\ \% = \frac{\textbf{optical density of control wells} - \textbf{optical density of treated wells}}{\textbf{optical density of control wells}} * \mathbf{100}\%$$

The IC50 values for metformin, metronidazole, cisplatin, and the (metformin-metronidazole) combination have been estimated for each incubation duration employed—GraphPad Prism, version 9.5.0, (2022).

2-4- Selective toxicity index:

A study inspected the selective toxicity of the metformin—metronidazole combination on cancer cells over two incubation periods: 24 hours and 72 hours. The selective cytotoxicity index was computed using the specified formula following the estimation of the combination's IC50 level through

cell proliferation curves for both HeLa and HFF cell lines (50).

$$\textbf{Selective toxicity Index } (SI) = \frac{IC\ 50\ of normal\ cell\ lines}{IC\ 50\ of cancer\ cell\ lines} \times 100$$

An SI greater than 1.0 suggests a drug is more effective at targeting tumor cells while exhibiting lower toxicity towards normal cells.

2-5- Drug combination study:

A study was conducted to analyze the combination of mixture constituents. It was done by creating concentration-effect curves, which involved graphing the percentage of cells that were affected (showing inhibited growth) against the medication amount following 24 and 72 hours of therapy. The interaction between agents was studied using Compusyn (Biosoft, USA), which employs a mathematical model to measure synergistic or antagonistic effects between compounds, providing numerical outputs such as the dose reduction index (DRI) and combination index (CI) (51).

2-6- Molecular docking:

Metformin and metronidazole structures were made using the ChemDraw Program (Cambridge Soft), modified, and energy minimized. Following this, the molecular structure of human Hsp70 and 60 was acquired from the Protein Data Bank codes (**PDB**: 1hjo and 4pj1). The application of AutoDock4 version (v4.2.6) optimized and modified protein structures, established the optimal conformation of the ligands, and generated a PDBQT file.

Following optimization, the ligand's metformin and metronidazole structures, and the human mitochondrial Hsp70 and Hsp60 complex were modeled using the AutoDock4 program version (v4.2.6). This program is also used to conduct the docking and calculate the scores. Finally, we used the Discovery Studio program (Dassault Systemes BIOVIA, USA)for visualization of the docking site (52, 53).

2-7- Research ethics:

This study did not involve any human participants.

2-8- Statistical assessment:

Six replicates were used for the MTT assay, and the results are presented as standard deviation (SD) calculations. One-way analysis of variance (ANOVA) was utilized. The study employed the Least Significant Difference (LSD) test and the paired two-sample t-test to examine the differences among the groups. The study employed SPSS version 20 for data analysis, with statistical significance determined at $p < 0.05$ (54).

The study utilized uppercase and lowercase letters in data tables to differentiate between statistical groups and significance levels. Means (averages) with the same letter indicate no significant difference, while means with different letters are significantly different. Uppercase letters are used to compare row means, and lowercase letters compare column means. This method provides a straightforward way to present complex statistical results clearly and concisely. Readers can easily determine which groups are similar or different based on the letters assigned.

3- Results:

3-1- cytotoxicity study:

3-1-1- Metformin:

The study demonstrated that metformin exerts a cytotoxic effect on cervical cancer cells. The results indicated that incubation time primarily affected the inhibition of cancer cell growth. There was a considerable variation in growth inhibition across the two incubation intervals for all metformin concentrations. The reduction in IC50 levels over the incubation periods indicates the influence of time on growth inhibition.

Furthermore, during each incubation period, significant changes between the concentrations were observed, see Figure 1 and Table 1.

Table (1): Metformin impact on HeLa cell line proliferation

Concentration (µg/ml)	Growth inhibition (mean ± SD)		*probability value*
	24 hr.	**72 hr.**	
0.1	**D** 0 ±0.00	**C** 4.00 ±.577	**0.002***
1	**D** 0 ±0.00	**BC** 17.00 ±1.155	**0.0001***
10	**C** 8 ± 1.732	**B** 27.00 ± 2.901	**0.005***
100	**B** 19.00 ±0.577	**B** 30.00 ± 2.887	**0.020***
1000	**A** 38.00 ±1.732	**A** 53.00 ± 1.692	**0.004***
LSD	**7.1**	**13.02**	-
IC 50	**1326 µg/ml**	**890.81 µg/ml**	-
A Probability value below 0.05 was regarded as statistically significant			

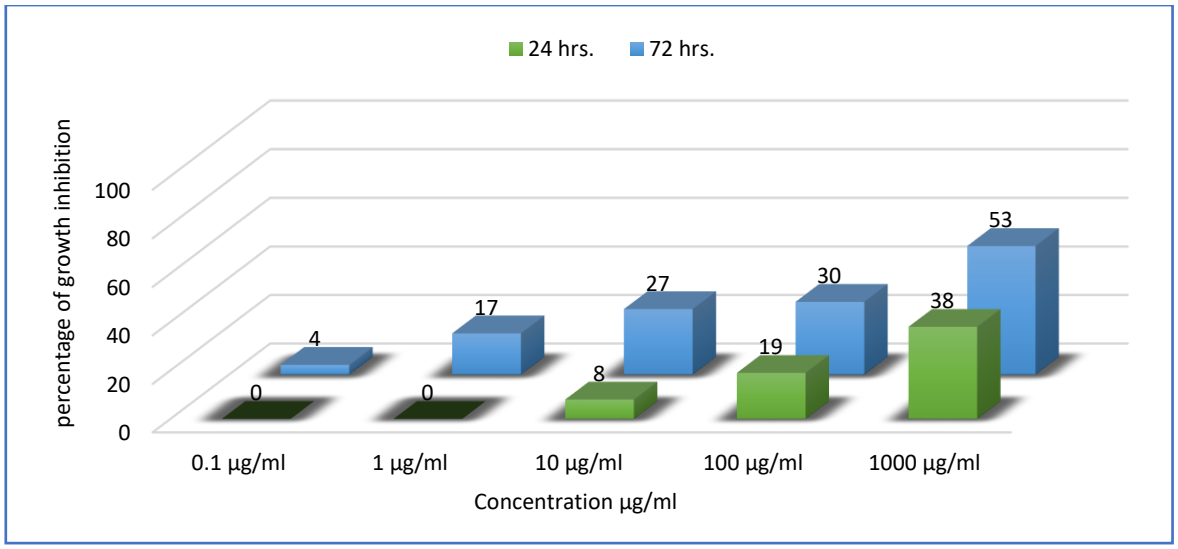

Figure (1) Metformin impact on Hela cell Line proliferation at 24 and 72 hours

3-1-2- Metronidazole cytotoxicity:

The study indicated that metronidazole's impact on the replicative capacity of cervical carcinoma cells (HeLa cell line) is contingent upon the concentration and duration of exposure. A marked difference in growth suppression was observed between the two incubation durations, particularly at concentrations of 10 and 1000 µg/ml. Furthermore, a notable difference exists in the concentration throughout each incubation period, especially between the lower doses (0.1 and 1) and the higher concentrations (10, 100, and 1000) µg/ml. Table (2), Figure (2).

3-1-3- Cisplatin cytotoxicity:

The cisplatin-induced cytotoxic on the HeLa cancer cell line demonstrates that increased drug concentrations and extended incubation periods are associated with higher rates of inhibition. "Growth inhibition varied significantly among the tested concentrations within each incubation interval. The findings displayed a significant difference in cytotoxicity across all tested concentrations and incubation durations, as evidenced by a decrease in the IC50 level at 72 hours. Comparison with 24-hour incubation. Table 3, Figure 3.

Table (2): Metronidazole impact on HeLa cell line proliferation

Concentration (µg/ml)	Growth inhibition (mean ± SD)		*probability value*
	24 hr.	72 hr.	
0.1	**B** 0.00 ±0.000	**B** 1.00 ±.577	**0.158**
1	**B** 1.00 ±0.577	**AB** 3.00±0.577	**0.070**
10	**A** 7.00±1.155	**A** 13.00±1.732	**0.045***
100	**A** 9.00 ±0.577	**A** 15.00 ±2.887	**0.111**
1000	**An** 11.00 ±1.155	**A** 20.00±1.160	**0.005***
LSD	**5.14**	**10.3**	-
IC 50	**6143 µg/ml**	**3222 µg/ml**	-
A probability value below 0.05 was regarded as statistically significant			

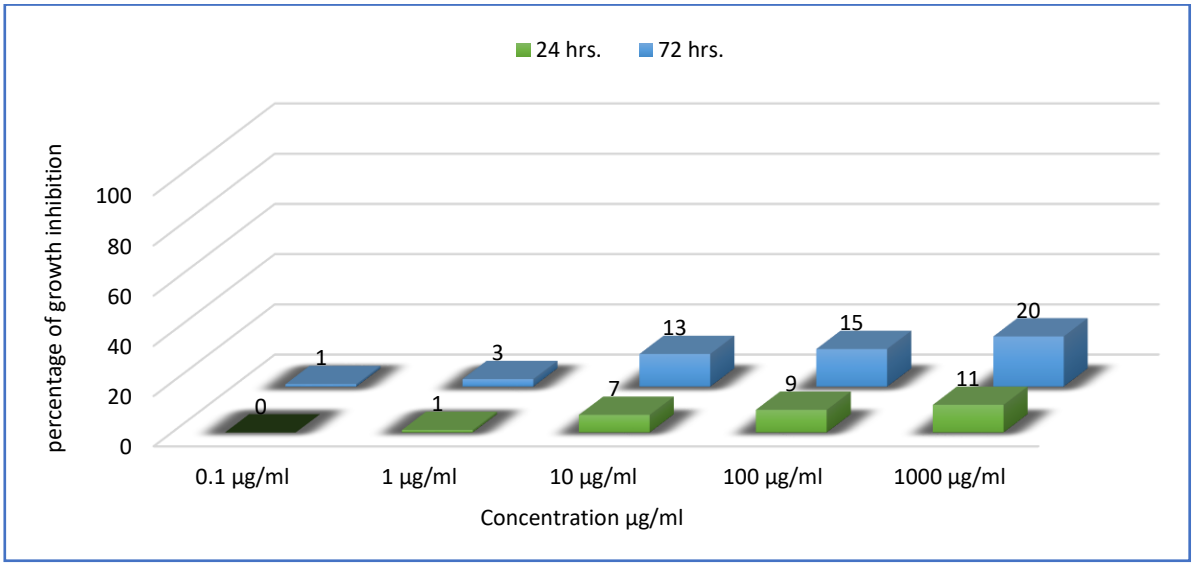

Figure (2): metronidazole impact on HeLa cancer cell proliferation at 24 and 72 hours

Table (2): Cisplatin impact on HeLa cell line proliferation

Concentration (µg/ml)	Growth inhibition % (mean ± SD)		*probability value*
	24 hrs.	72 hrs.	
0.1	**C** 1.00 ± 1.000	**D** 5.00 ± 2.000	**0.036***
1	**C** 2.00 ± 1.000	**D** 10.00 ± 5.000	**0.053**
10	**C** 6.00 ± 2.000	**C** 21.00 ± 3.000	**0.002***
100	**B** 27.00 ± 4.000	**B** 47.00 ± 3.000	**0.002***
1000	**A** 38.00 ± 1.000	**A** 68.00 ± 2.000	**0.0001***
LSD	**7.8**	**11.62**	
IC 50	**1325.4 µg/ml**	**605.8 µg/ml**	
A probability value below 0.05 was regarded as statistically significant.			

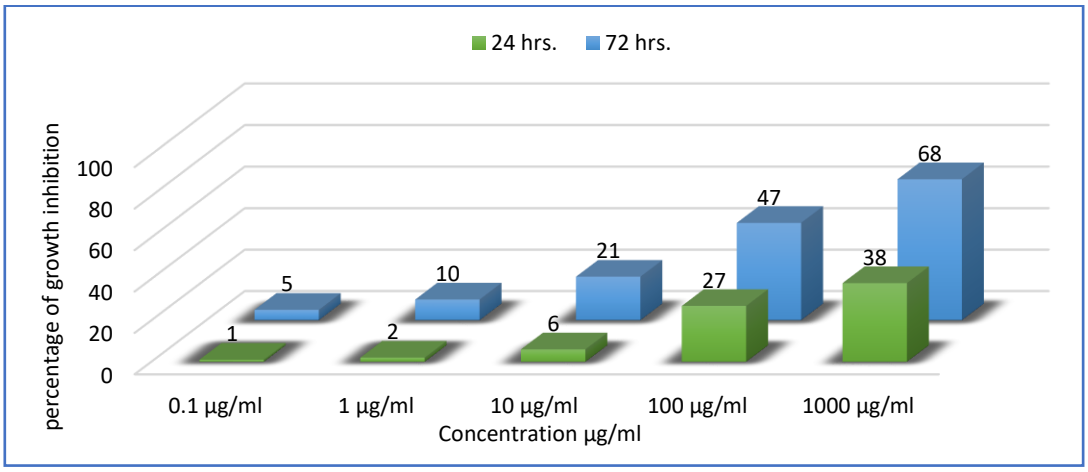

Figure (2): Cisplatin impact on HeLa cell line proliferation at 24 and 72 hours

3-1-4- Metformin-metronidazole combination cytotoxicity:

Metformin and metronidazole Mixture effectively inhibited the cell growth and proliferation of the cervical cancer cell line over time, evidenced by a significant reduction in cell proliferation at various concentrations during the incubation period; this effect was corroborated by a decrease in the IC50 at 72 hours compared to 24 hours. The degree of growth inhibition was contingent upon the concentration of the combination, especially at the 72-hour mark. Incubation periods. Table (4) figure (4)

Furthermore, the mixture's cytotoxicity was assessed on the human fibroblast cell line to evaluate potential toxicity on healthy cells stemming from pharmaceutical interactions among its components. Results demonstrated that the mixture exerted a significantly greater effect on the HeLa cell line than the HFF cell line at 24 and 72 hours of incubation (Tables 5, 6,7) (Figures 5, 6,7).

The cytotoxicity of the mixture was found to be significantly greater than that of its constituents across all incubation periods. The mixture demonstrated significantly greater cytotoxicity compared to cisplatin, especially at lower concentrations. At elevated concentrations, the results indicated no significant differences in cytotoxicity between the mixture and cisplatin. The IC50 levels in cells exposed to the mixture were lower than those in cells treated with metformin or metronidazole alone across all incubation periods and were less than those of cisplatin at 24 hours. While demonstrating equivalent levels of cisplatin at 72 hours. (Table 8,9) (Figures 8,9,17)

Table (4): The effect of the (metformin-metronidazole) combination on HeLa cell-line proliferation.

Concentration (µg/ml)	Growth inhibition (mean ± SD)		*probability value*
	24 hr.	72 hr.	
0.1	**B** 6.00±.577	**E** 12.00±1.155	**0.010***
1	**B** 12.00±1.155	**D** 23.00±1.732	**0.006***
10	**A** 23.00±1.732	**C** 34.00±2.309	**0.019***
100	**A** 33.00±2.887	**B** 47.00±1.155	**0.011***
1000	**A** 42.00±1.155	**A** 63.00±1.732	**0.001***
LSD	**10.68**	**10.54**	-
IC 50	**1256 µg/ml**	**604.7 µg/ml**	-
A Probability value below 0.05 was regarded as statistically significant.			

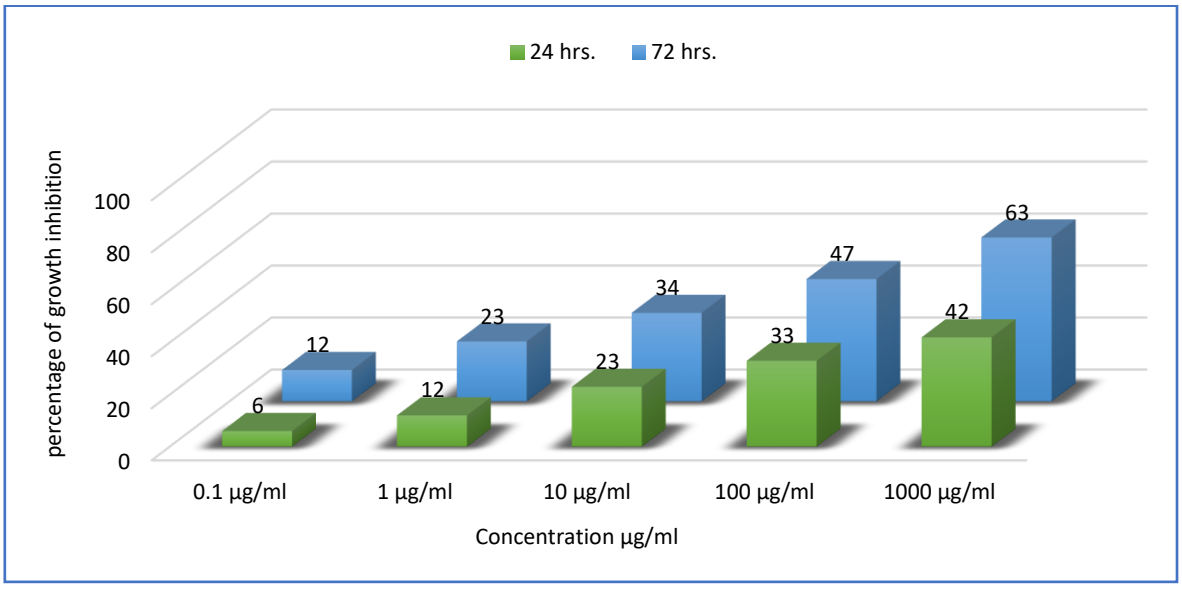

Figure (4): The potency of the (metformin-metronidazole) combination on HeLa cancer cell proliferation at 24 and 72 hours

Table (5): The effect of the (metformin-metronidazole) combination on HFF cell line proliferation

Concentration (µg/ml)	Growth inhibition (mean ± SD)		*probability value*
	24 hr.	**72 hr.**	
0.1	**B** 0.00 ± 0.000	**C** 0.00 ± 0.000	**N. S**
1	**B** 0.00 ± 0.000	**C** 0.00 ± 0.000	**N. S**
10	**B** 2.00 ± 1.000	**BC** 6.00 ± 2.000	**0.036***
100	**A** 9.00 ± 2.000	**AB** 14.00 ± 4.000	**0.125**
1000	**A** 11.00 ± 2.000	**A** 17.00 ± 3.000	**0.045***
LSD	**4.88**	**8.76**	-
IC 50	**5179 µg/ml**	**3401.5 µg/ml**	-
A probability value below 0.05 was regarded as statistically significant			

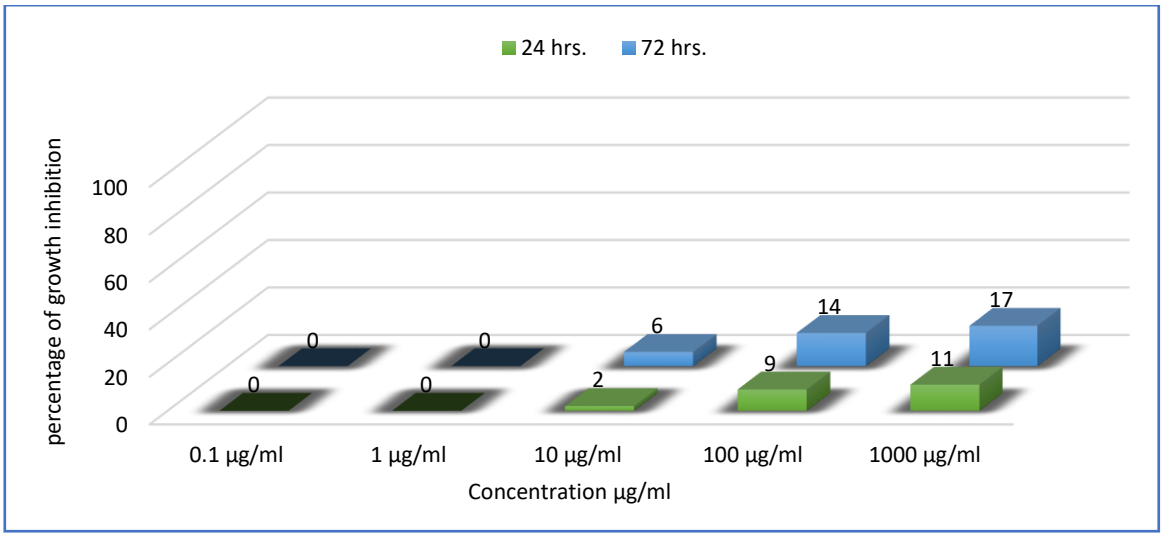

Figure (5): The effect of the (metformin-metronidazole) combination on HFF cell line proliferation at 24 and 72 hours

Table (6): A comparison of the growth inhibition effects of the metformin-metronidazole combination on HeLa and HFF cell lines after 24 hours of incubation.

Concentration (µg/ml)	Growth inhibition (mean ± SD)		*probability value*
	Hela	HFF	
0.1	**B** 6.00±.577	**B** 0.00 ± 0.000	**0.0001***
1	**B** 12.00±1.155	**B** 0.00 ± 0.000	**0.0001***
10	**A** 23.00±1.732	**B** 2.00 ± 1.000	**0.0001***
100	**A** 33.00±2.887	**A** 9.00 ± 2.000	**0.002***
1000	**A** 42.00±1.155	**A** 11.00 ± 2.000	**0.0001***
LSD	**10.68**	**4.88**	
IC 50	**1256 µg/ml**	**5179 µg/ml**	
A probability value below 0.05 was regarded as statistically significant			

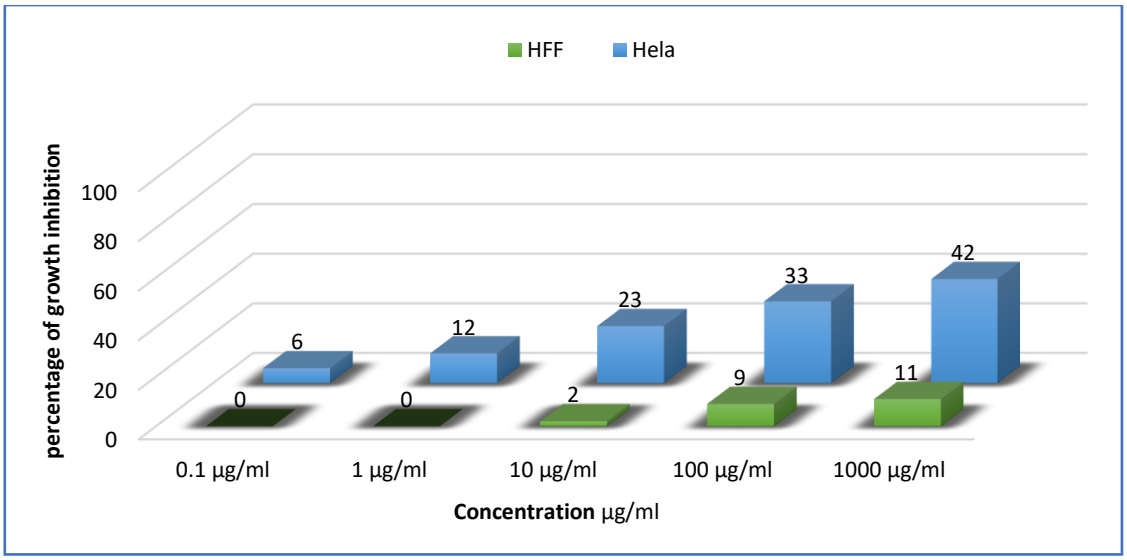

Table 6 compares the growth inhibition effects of the metformin-metronidazole combination on HeLa and HFF cell lines after 24 hours of incubation.

Table 7 compares the growth inhibition effects of the metformin-metronidazole combination on HeLa and HFF cell lines after 72 hours of incubation.

Concentration (µg/ml)	Growth inhibition (mean ± SD)		*probability value*
	HeLa	HFF	
0.1	**E** 12.00±1.161	**C** 0.00 ± 0.000	**0.0001***
1	**D** 23.00±1.732	**C** 0.00 ± 0.000	**0.0001***
10	**C** 34.00±2.309	**BC** 6.00 ± 2.000	**0.0001***
100	**B** 47.00±1.161	**AB** 14.00 ± 4.000	**0.0001***
1000	**A** 63.00±1.699	**A** 17.00 ± 3.000	**0.0001***
LSD	**10.54**	**8.76**	-
IC 50	**604.7 µg/ml**	**3401.5 µg/ml**	-
A probability value below 0.05 was regarded as statistically significant			

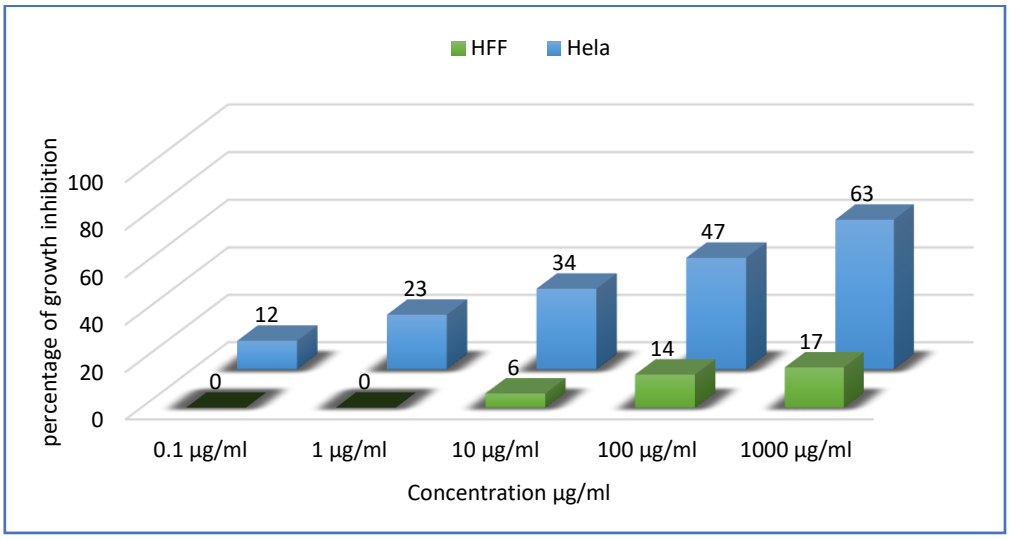

Table 7 compares the growth inhibition effects of the metformin-metronidazole combination on HeLa and HFF cell lines after 72 hours of incubation.

Table (8): Cytotoxic effect of Combination, metformin, metronidazole, and cisplatin, 24-hour.

Concentration (µg/ml)	Growth inhibition (mean ± SD)				LSD
	metformin	metronidazole	mix	cisplatin	
0.1	D 0 ±0.000 b	B 0.00 ±0.000 b	B 6.00±.577 a	C 1.00 ± 1.000 b	2.66
1	D 0 ±0.000 b	B 1.00 ±0.577 b	B 12.00±1.155 a	C 2.00 ± 1.000 b	4.62
10	C 8 ± 1.732 b	A 7.00±1.155 b	A 23.00±1.732 a	C 6.00 ± 2.000 b	9.6
100	B 19.00 ±0.577 bc	A 9.00 ±0.577 c	A 33.00±2.887 a	B 27.00 ± 4.000 ab	12.34
1000	A 38.00 ±1.732 a	A 11.00 ±1.155 b	A 42.00±1.155 a	A 38.00 ± 1.000 a	7.98
[b] LSD value	7.1	5.14	10.68	7.8	-
IC 50	1326 µg/ml	6143 µg/ml	1256 µg/ml	1325.4 µg/ml	-
The value is considered significant at (P<0.05)					

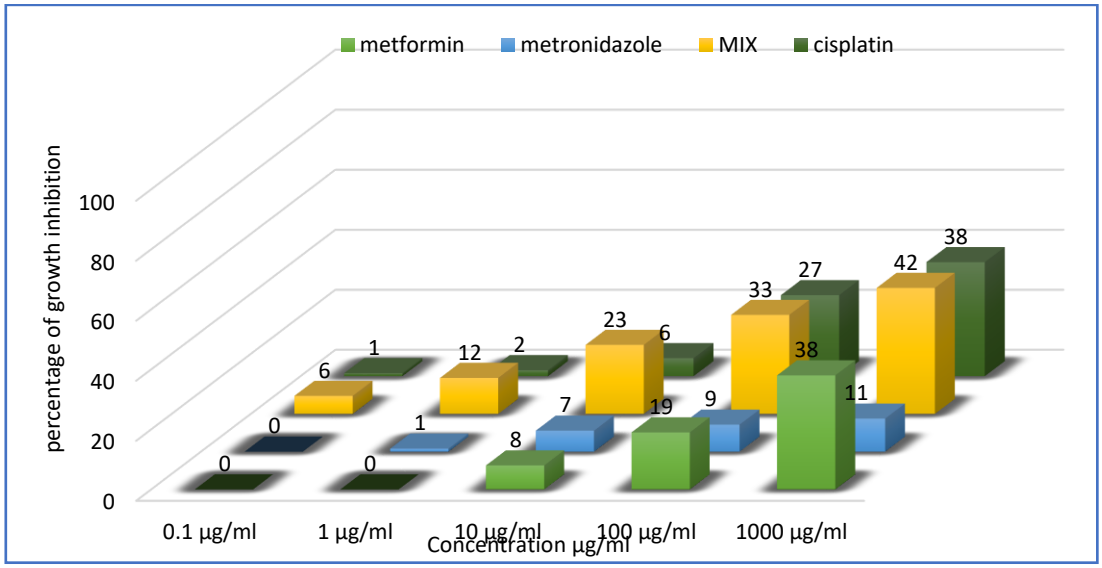

Figure (8): Comparison of 24-hour growth inhibition among the mixture of metformin, metronidazole, and cisplatin

Table (9): Comparison of 72-hour growth inhibition among the mixture of metformin, metronidazole, and cisplatin

Concentration (µg/ml)	Growth inhibition (mean ± SD)				LSD
	metformin	metronidazole	mix	cisplatin	
0.1	**C** 4.00 ±.577 **b**	**B** 1.00 ±.577 **b**	**E** 12.00±1.155 **a**	**D** 5.00 ± 2.000 **b**	**5.96**
1	**BC** 17.00 ±1.155 **ab**	**AB** 3.00±0.577 **c**	**D** 23.00±1.732 **a**	**D** 10.00 ± 5.000 **bc**	**11.76**
10	**B** 27.00 ± 2.887 **a**	**A** 13.00±1.732 **b**	**C** 34.00±2.309 **a**	**C** 21.00 ± 3.000 **a**	**14.46**
100	**B** 30.00 ± 2.887 **b**	**A** 15.00 ±2.887 **c**	**B** 47.00±1.155 **a**	**B** 47.00 ± 3.000 **a**	**14.94**
1000	**A** 53.00 ± 1.732 **b**	**A** 20.00±1.155 **c**	**A** 63.00±1.732 **a**	**A** 68.00± 2.000 **a**	**9.6**
[b] LSD value	**13.02**	**10.3**	**10.54**	**11.62**	
IC 50	**890.81 µg/ml**	**3222 µg/ml**	**604.7 µg/ml**	**605.8 µg/ml**	
The value is considered significant at (P<0.05)					

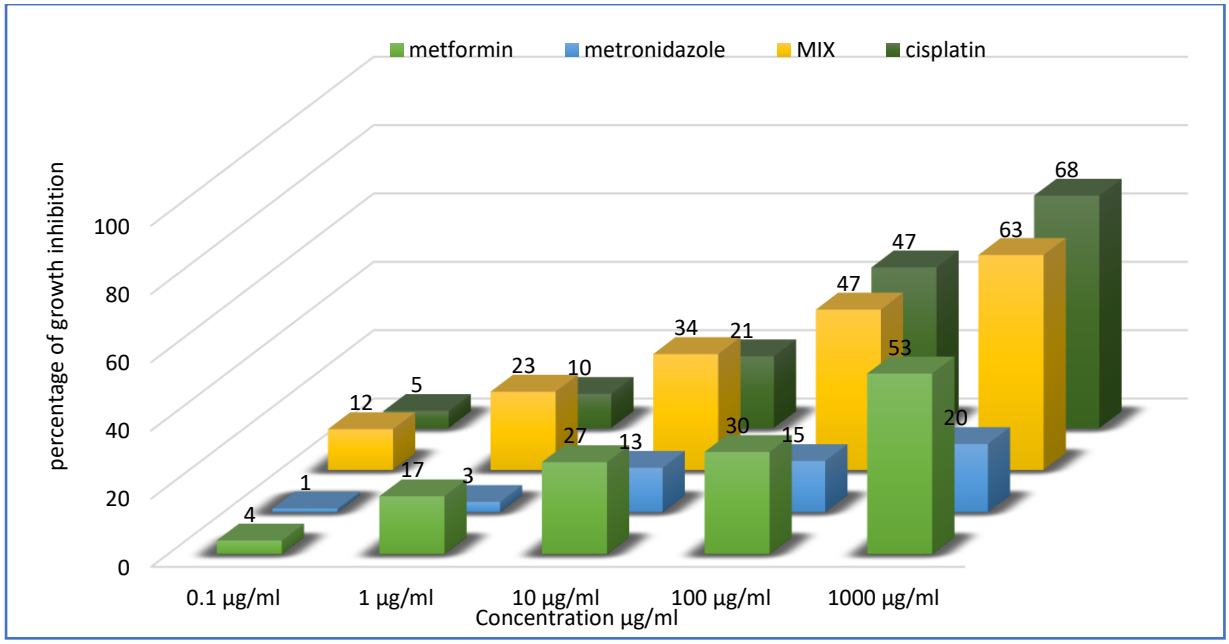

Figure (9): Comparison of 72-hour growth inhibition among the mixture of metformin, metronidazole, and cisplatin

3-2- Selective toxicity index:

The selective toxicity index score of the metformin–metronidazole combination was 4.12 and 5.62 for 24 and 72 hours, respectively. This suggests that the combination selectively targets cervical cancer cells over normal healthy cells, with an increase in the selectivity index corresponding to longer incubation times .Figure (10)

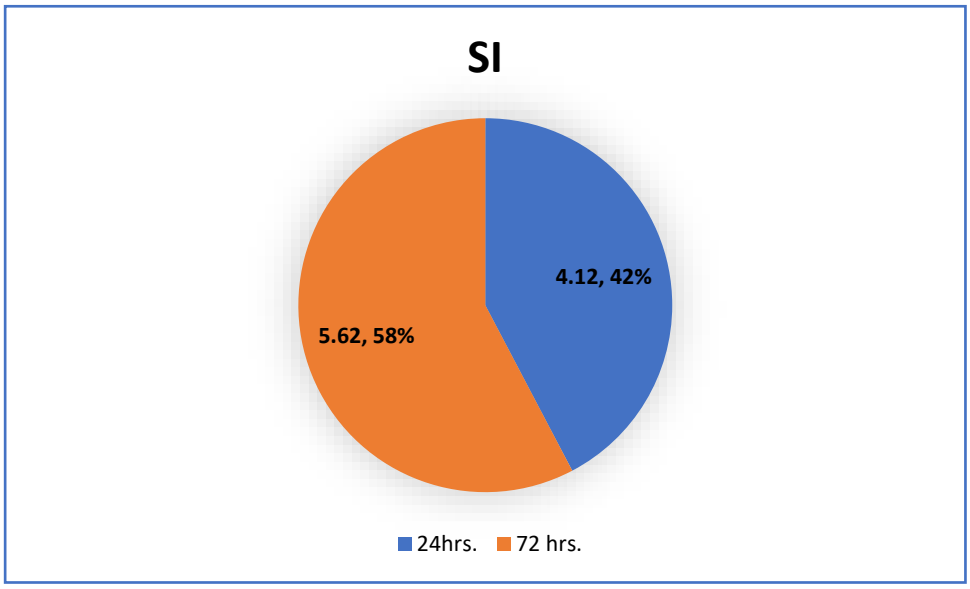

Figure (10): The selective toxicity index of metformin-metronidazole mixtures across two incubation periods. ***(An SI greater than 1.0 signifies a drug's enhanced efficacy against tumor cells compared to its toxicity towards normal cells.).***

3-3- Studying drug combinations:

The combined study of metformin and metronidazole yielded the following results. At each incubation period, a concentration of (0.1, 1, and 10) µg/ml for the combination demonstrated very strong synergism, while 100 and 1000 µg/ml exhibited strong synergism at 72 hrs. At 24 hrs. One hundred µg/ml showed synergism; however, strong antagonism was observed at 1000 µg/ml concentration at 24 hrs. Incubation times

The dose reduction index results indicated that the concentrations of the combination required to cause cytotoxicity declined compared with the effective cytotoxic concentration of each ingredient alone at each time interval, except for a concentration of 1000 µg/ml for metformin. Table (10, 11) Figure (11, 12)

Table (10): The metformin and metronidazole combination percentage and their cytotoxic effect on the Hela Cell line after 24 hours of incubation

Dose Reduction Index (DRI)	Interaction Type	Combination Index (CI)	Concentration Ratio	Concentration (µg/ml)
MET: 4343.04 / Met: 4745.72	Very Strong Synergy	0.0004	1:1	MET: 0.5 / Met: 0.5
MET: 1382.69 / Met: 816.473	Very Strong Synergy	0.00195	1:1	MET: 5 / Met: 5
MET: 457.326 / Met: 143.003	Very Strong Synergy	0.00918	1:1	MET: 50 / Met: 50
MET: 9.80787 / Met: 2.04453	Synergy	0.59107	1:1	MET: 500 / Met: 500
MET: 1.76572 / Met: 0.26930	Strong Antagonism	4.27970	1:1	MET: 5000 / Met: 5000

Notes:

The analysis of the interaction between metformin and metronidazole was conducted using CompuSyn software. The Combination Index (CI) values help categorize the drug interaction: - CI < 1 indicates a synergistic effect, - CI = 1 reflects an additive response, - CI > 1 denotes an antagonistic relationship.

Similarly, Dose Reduction Index (DRI) values highlight how much the dose can be lowered when drugs are used in combination compared to when used alone(54), **Met**: metformin, **MET**: metronidazole.

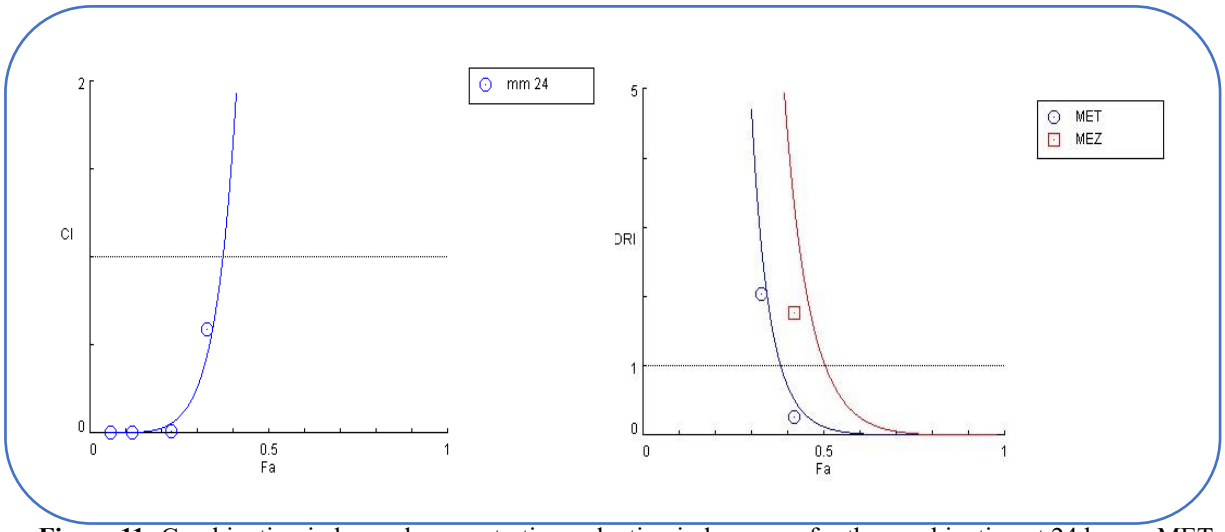

Figure 11: Combination index and concentration reduction index curve for the combination at 24 hours, MET**:** metformin, MEZ: metronidazole, **mm**: metformin – metronidazole mixture

Table (11): Interaction Profile of Metformin and Metronidazole on HeLa Cancer Cells After 72-Hour Incubation

Concentration (µg/ml)		Concentration Ratio	Combination Index (CI)	Interaction Type	Dose Reduction Index (DRI)	
Very Strong Synergy	MET: 2806.48 / Met: 12.5970	MET: 0.5 / Met: 0.5	1:1	0.07974	Very Strong Synergy	MET: 2806.48 / Met: 12.5970
Very Strong Synergy	MET: 5763.54 / Met: 36.8086	MET: 5 / Met: 5	1:1	0.02734	Very Strong Synergy	MET: 5763.54 / Met: 36.8086
Very Strong Synergy	MET: 4709.62 / Met: 38.4326	MET: 50 / Met: 50	1:1	0.02623	Very Strong Synergy	MET: 4709.62 / Met: 38.4326
Strong Synergy	MET: 382.083 / Met: 3.98070	MET: 500 / Met: 500	1:1	0.25383	Strong Synergy	MET: 382.083 / Met: 3.98070
Strong Synergy	MET: 472.212 / Met: 6.59719	MET: 5000 / Met: 5000	1:1	0.15370	Strong Synergy	MET: 472.212 / Met: 6.59719
Interaction Type	Dose Reduction Index (DRI)	Concentration (µg/ml)	Concentration Ratio	Combination Index (CI)	Interaction Type	Dose Reduction Index (DRI)

Notes:

The interaction assessment between metformin and metronidazole was performed using CompuSyn software. Combination Index (CI) values interpret drug interaction as follows:

- CI < 1 denotes synergy, - CI = 1 implies additive effect, - CI > 1 reflects antagonism.

Dose Reduction Index (DRI) values indicate the potential to lower drug doses when used in combination, thus possibly reducing toxicity if DRI > 1. (54) Met: metformin, MET: metronidazole

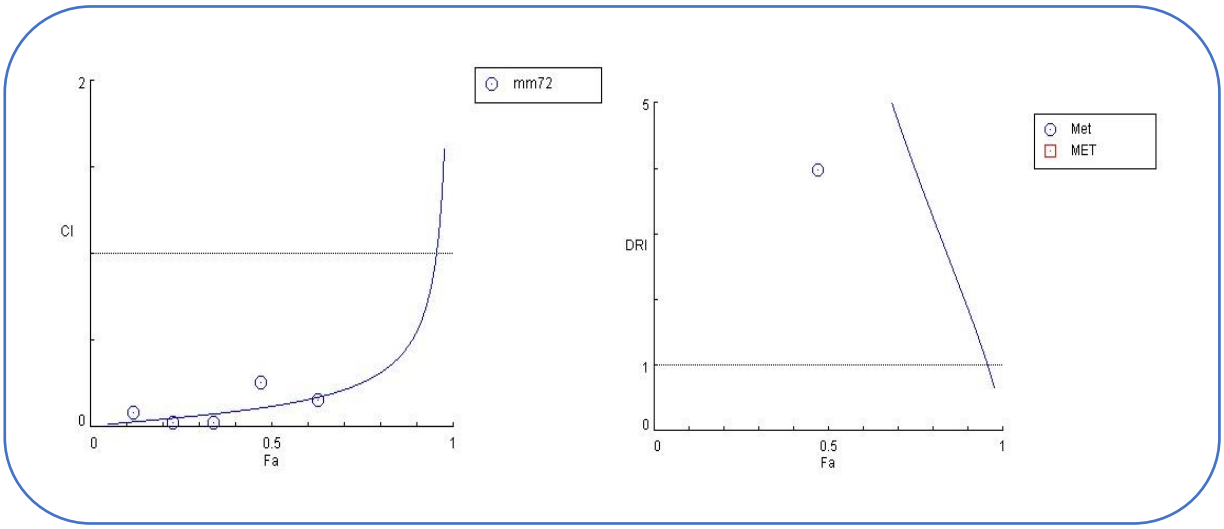

Figure (12): combination index and dose reduction index curve for the mixture at 72 hrs.., MET; metformin, MEZ: metronidazole, **mm**: metformin – metronidazole mixture

3-4- Molecular docking studies:

A molecular docking study examined the interactions of metformin and metronidazole with Hsp70 and Hsp60 (PDB codes: 1hjo and 4pj1) as a basis. The study employed AutoDock tools version 1.5.7 and BIOVIA Discovery Studio **(56)**

Molecular docking studies found that the best interaction of metformin occurs with Hsp70, with a molecular docking score equal to (-6.2) kcal/mol. Presented. Five conventional hydrogen bonds formed with one TRH A:13, one LYS A:71, one ASP A:10, and two ASP A:206 amino acid residues at 2.06 Å, 2.01 Å, 2.76 Å, 2.94 Å, 2.01 Å of distance. (Figure 13)

The results of the molecular docking study of 2-Phenylethynesulfonamide, a Hsp70 inhibitor, are provided for comparative analysis **(57-59)**. The total docking score was (-6.4) kcal/mol. Presented six Conventional hydrogen bonds with the one THR A:13, two THR A: 14, THR A:15, GLY A:202, and GLY A:203 amino acid residues at 2.67 Å, 2.03 Å, 2.21 Å, 2.07 Å, 1.83 Å, and 2.79 Å of distance, respectively. Finally, with one Pi-Pi stacked bond with TYR A:15 at 4.24 Å of distance. (Figure 15).

The molecular docking research results for metronidazole, which exhibited excellent interaction with Hsp60, revealed a total docking score of (-6.7) kcal/mol. Molecular docking analysis was conducted. Five conventional hydrogen bonds were established with one TRH A:89, one THR A:90, one THR A:21, and two THR A:91 amino acid residues at distances of 2.18 Å, 2.22 Å, 2.30 Å, 2.02 Å, and 1.90 Å. One pi-anion is linked to the amino acid residue ASP A:87 at a distance of 3.87 Å. (Figure 14). This study presents a molecular docking analysis of Epolactaene, a recognized Hsp60 inhibitor, for comparative evaluation **(60, 61).** indicated a total docking score of (-6.7) kcal/mol. Molecular docking analysis was presented. Four conventional hydrogen-bound with SER A:228 amino acid residue at 4.08 Å, SER A:229 amino acid residue at 1.89 Å, SER A:229 amino acid residue at 2.20 Å, and GLN A:231 amino acid residue at 2.39 Å. Two alkyls were also found with LYS A:225 amino acid residue at 5.39 Å and LEU A:310 amino acid residue at 4.74 Å. (Figure 16)

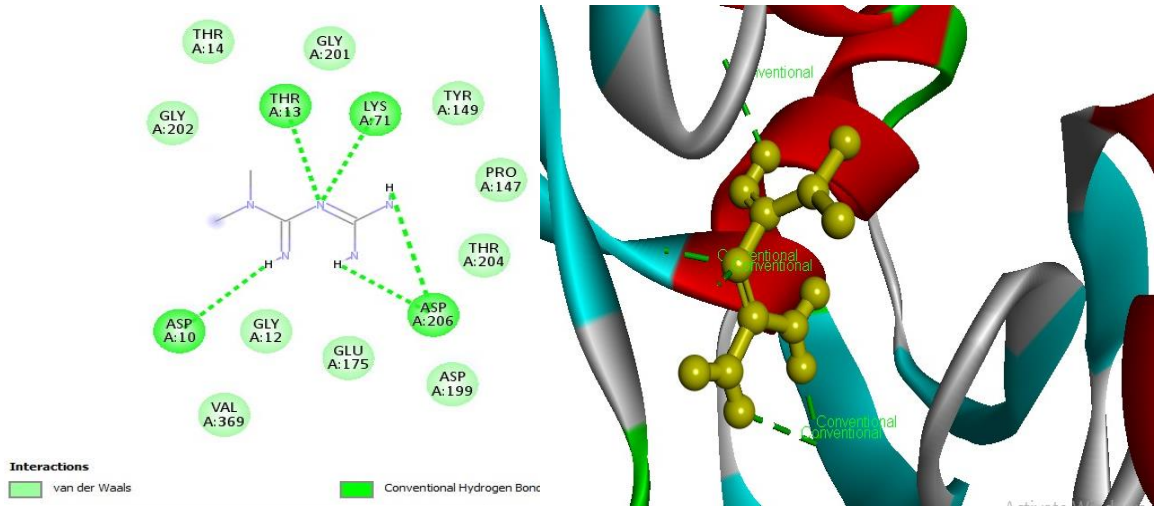

Figure (13): binding site of metformin with Hsp70

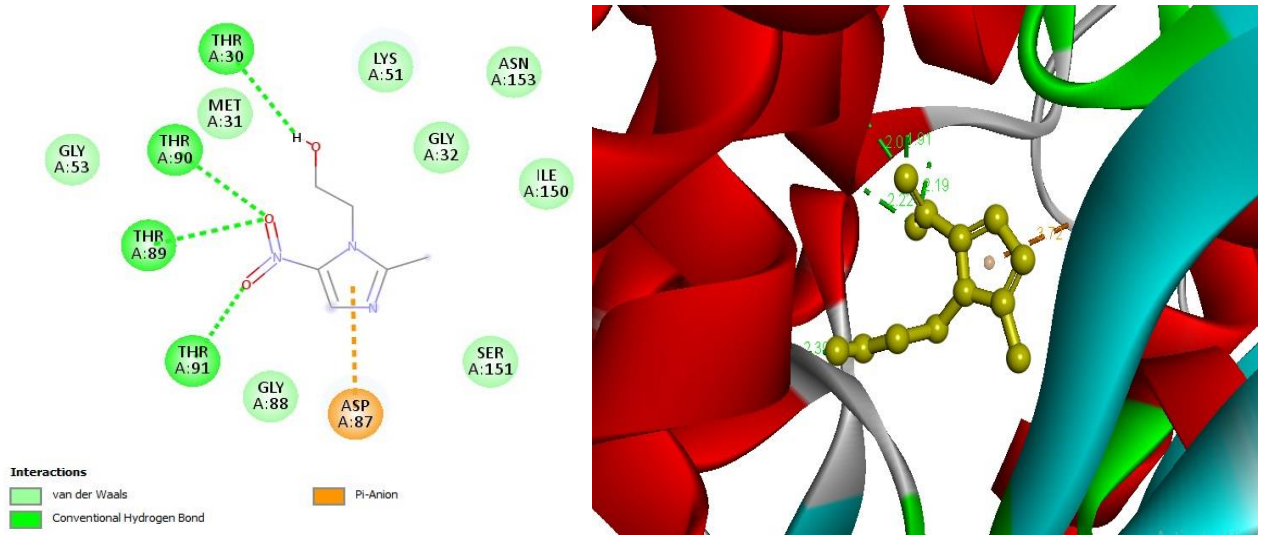

Figure (14): binding site of metronidazole with Hsp60

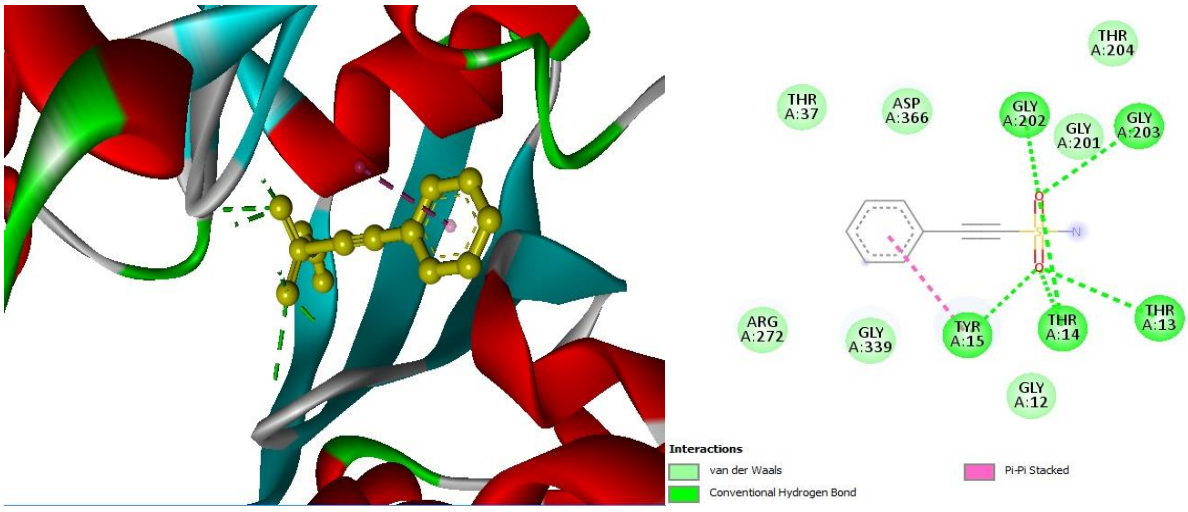

Figure (15): binding site of 2-Phenylethynesulfonamide with Hsp70

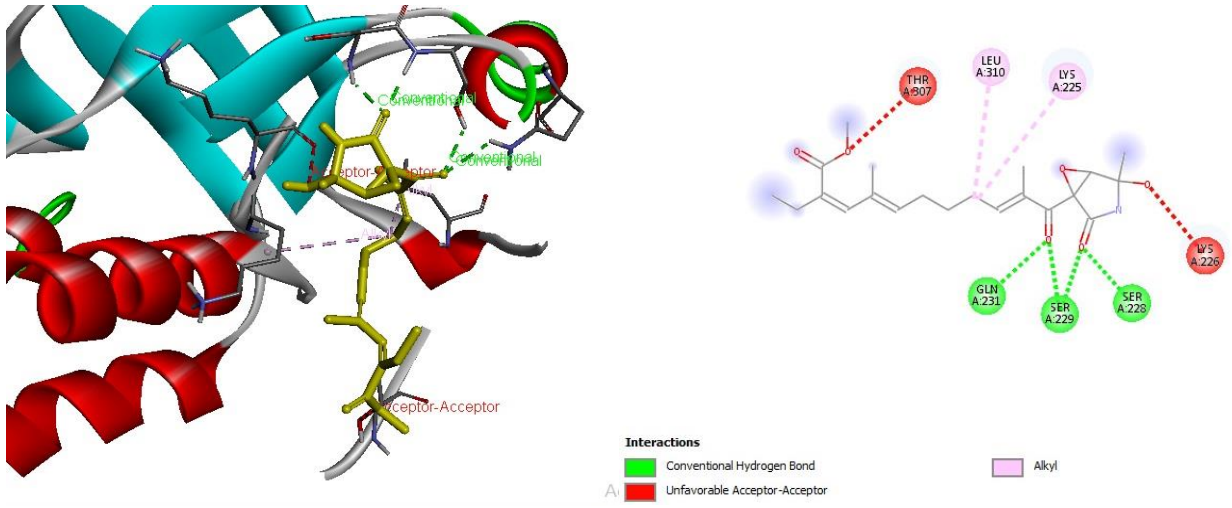

Figure (16): binding site of Epolactaene with Hsp60

Morphological Observations of HeLa Cancer Cells

Figure 17 illustrates the morphological changes observed in HeLa cancer cells subjected to different treatments over 72 hours.

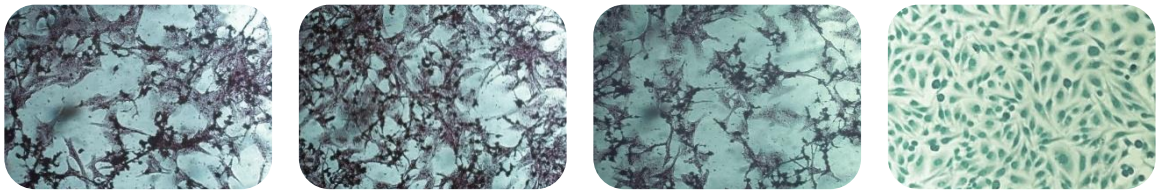

Figure (17): Morphological observation of HeLa cancer cells:

(A) Cells were exposed to metformin at a concentration of 1000 µg/ml for 72 hours.
(B) Cells were treated with 1000 µg/ml of metronidazole for the same time period.
(C) A combination treatment of metformin and metronidazole, each at 1000 µg/ml, was applied for 72 hours.
(D) Untreated cervical cancer cells were used as the control group.

4- Discussion:

This study evaluated the synergistic anticancer effects of metformin and metronidazole on HeLa cancer cell viability and investigated their targeting of the Hsp60 and Hsp70 chaperone proteins. The findings indicated that the antiproliferative effects of the metformin-metronidazole combination on cervical cancer cells were both cell cycle-specific and non-specific, and these effects were significantly greater than those of either component alone. This effect was consistently observed throughout each incubation period. For comparison, the mixture demonstrated superior cytotoxicity compared to cisplatin, particularly at lower concentrations, while exhibiting similar cytotoxicity at higher concentrations.

The combination index results indicated a synergistic interaction across all combinational concentrations at each incubation period, except the highest concentration at 24 hours. The dose

reduction index study indicated a significant decrease in the effective cytotoxic concentration of the mixture's components relative to that of each ingredient, implying improved safety and reduced adverse effects.

Moreover, the combination exhibited preferential toxicity towards cancer cells compared to healthy cells, supported by over one score of a selectivity index, which indicates a favourable selectivity index.

The chemical docking analysis revealed that each component of the combination binds to specific heat shock proteins. Metformin targets Hsp70, while metronidazole exhibits a higher affinity for Hsp60. This novel mechanism for each drug elucidates the anticancer mechanism of the mixture and the synergistic interactions among its components.

The mixture's cytotoxicity results in the viability of the HFF cell line, suggesting that the combination selectively targets cancer cells, as both malignant and healthy cells express Hsp70 and Hsp60. This behaviour elucidates the mixture's selective toxicity against cancer cells. The findings regarding the mixture's cytotoxicity on the HFF cell line suggest an absence of pharmaceutical interaction among the mixture's constituents.

Numerous prior studies indicate that each component of the mixture exhibits anticancer properties. A survey of metformin demonstrated its ability to inhibit cervical cancer cell proliferation via a time-dependent cytotoxic effect. Further research supports the conclusion that metformin, a widely used diabetes medication, effectively prevents the onset of various malignancies, including pancreatic cancer **(62, 63).** Another study has shown that metformin reduces the risk of and mortality from colon cancer **(64, 65)** Metformin demonstrated the capacity to decrease the development of adenomas and polyps in patients following polypectomy **(66)**. And reduces the risk of death in those with diabetes who have colon cancer **(67, 68)**. At the same time, another study demonstrates the capacity of metformin to reduce the occurrence and death rate associated with prostate cancer and liver cancer **(68-72)**.

Another in vitro study showed that metformin directly inhibits AMP deaminase, leading to increased AMP levels and activation of AMPK **(73).** Moreover, research suggests that agents that inhibit mitochondrial complex 1 activity in the respiratory system might elevate AMP levels and activate AMPK. This, therefore, results in the inhibition of mammalian target of rapamycin (mTOR) and the ensuing signaling pathways that enhance cell survival **(74, 75).** Also, Metformin showed the ability to eliminate active K-ras from the cellular membrane via a PKC-dependent mechanism. There is no evidence to indicate that metformin directly interacts with K-ras. Moreover, the research suggests that metformin establishes connections with and dismantles the PP2A complex in brain cells. Moreover, there is a possibility of inhibiting the activity of the PP2A-dependent phosphatase **(76).**

On the other hand, our findings demonstrated that the use of metronidazole significantly decreased the survival rate of cervical cancer cells, reducing it to 20%. This result is compatible with prior research investigating the cytotoxic impacts of metformin on pancreatic cancer **(77).** While other studies have demonstrated that metronidazole has the potential to diminish the growth of CHO (Chinese hamster ovary), HeLa (derived from cervical cancer), and human marrow cells, this impact seems to be dependent on the concentration of the drug and the degree of hypoxic conditions **(21, 78, 79).** Metronidazole displayed cytotoxic impacts on the MDA-MB-231 breast cell line. This cytotoxicity was observed at greater concentrations of up to 250 µg/ml after 72 hours of incubation **(20).**

Our study findings revealed that metronidazole's most significant antiproliferative impact was seen after 72 hours. When evaluating the effects of metronidazole on nucleic acid synthesis, it is seen that metronidazole is more effective in suppressing this process under anaerobic circumstances. This

inhibition is further enhanced after 72 hours due to the hypoxic environment. The cytotoxicity of metronidazole was predominantly seen during 72 hours, compared to the 24-hour periods **(80, 81).**

Our study introduces a novel anticancer mechanism for metformin and metronidazole, contrasting with previously proposed mechanisms. We investigated their capacity to target heat shock proteins, specifically Hsp70 and Hsp60. These types of heat shock proteins were selected based on the findings of molecular docking, which indicated a higher affinity of the mixture components for these types of heat shock proteins.

Heat shock protein 70 is vital for c-FLIP production, which inhibits apoptosis, contributing to cancer cell resistance against TNF-alpha, Fas-L, TRAIL, and chemotherapy **(82,83).** Furthermore, Malignant cells exhibit increased levels of Hsp70 relative to normal cells. Increased levels of Hsp70 correlate with a tumorigenic phenotype, often leading to resistance against chemotherapy and apoptosis **(84, 85).**

Hsp70 modulates multiple stages of apoptotic pathways, inhibiting the unwarranted onset of cellular death during stress conditions. Hsp70 regulates apoptosis, modulates the immune response, and aids in antigen delivery in conjunction with the MHC-I molecule. It engages innate and adaptive immune systems and is a potent immunomodulator **(86).** The expression of Hsp70, an evolutionarily conserved protein involved in apoptotic signalling, enhances cell viability under stress conditions. Cells with Hsp70 knockdown show heightened vulnerability to apoptosis **(87).** The overexpression of Hsp70 inhibits apoptosis either upstream or downstream of mitochondria **(88)**.

Hsp70 interacts with nerve growth factor and platelet-derived growth factor to promote cell survival via the PI3K pathway, activating Akt kinase, which inhibits apoptosis by targeting Bad and caspase-9 **(89-91)**. The Hsp70 protein enhances the stability of the Akt/PKB complex in K562 cells **(92)**.

Additionally, another study exhibited that HspA12B, a member of the Hsp70 family, is essential for blood vessel development in zebrafish. It facilitates endothelial cell migration and tube formation by maintaining Akt activation **(93).** The Hsp70 family plays a crucial role in regulating cell survival and differentiation. Hsp70 contributes to protein prephosphorylation and stability by promoting the activation of unphosphorylated protein kinases **(94).** Hsp70 acts as a suppressor of apoptosis signal-regulating kinase-1 in NIH3T3 cells, a kinase activated by stress. Down-regulation of Hsp70 leads to the generation of H2O2 and the activation of ASK-1, resulting in apoptosis **(95).**

Due to their importance in cancer pathogenesis, several studies focused on Hsp70 and Hsp60 as cancer therapeutic targets. suggested several agents as Hsp70 inhibitors, such as (2-phenyl ethyne sulfonamide), a Phenylethylsulfonamide-derived **(96, 97)**. (Apoptozole and Az-TPP-O3), an Imidazole-derived **(98-100)**. (YM-1 and JG-83) a Rhodocyanine-derived **(101, 102)**. (Epigallocatechin-3-gallate (EGCG), Quercetin, Kahweol, Cantharidin, and Veratridine), a Natural compound **(103-108).** And several agents, such as Hsp60 inhibitors, such as Epolactaene (60) , mytucommule **(109) .** and avrainvillamide **(110).**

Based on the earlier factors, agents able to target Hsp70 or Hsp60 show a promising anticancer property. Our study mixture exhibited an advantage over agents that inhibit the heat shock protein mentioned above, which is related to well-known pharmacokinetics and adverse effect profiles of mixture ingredients.

The study was limited by the lack of restrictions on drug concentration ranges. Various concentrations were used to identify the optimal concentrations for metronidazole and metronidazole.

5- Conclusion:

The results of this study demonstrate that the combination of metformin and metronidazole potentially suppresses the cancer growth of the HeLa

cell line. The inhibitory behavior exhibited both cell cycle-specific and cell cycle-nonspecific properties. The results indicate that individual medications had varying degrees of cytotoxicity. However, the combination showed synergistic suppression of the growth, which was evaluated using the combination index. Furthermore, this combination exhibited enhanced cytotoxicity compared to cisplatin, especially at lower doses, while displaying comparable cytotoxicity at higher levels. Computational molecular docking simulations suggested a novel anticancer mechanism for both metformin and metronidazole through their ability to target Hsp70 and Hsp60. This clarified the synergistic pattern among the combined ingredients, as each drug targets a specific type of heat shock protein, suggesting a complementary anticancer mechanism.

Furthermore, we suggested that the mixture demonstrated preferential toxicity towards cancer cells as opposed to healthy cells, as evidenced by the selectivity index score. The dosage reduction index results demonstrate that the concentration of drugs needed in the mixture to achieve substantial cytotoxicity is less than the cytotoxic concentration of each medication when used separately. These findings, along with well-known pharmacokinetics and adverse effect profiles of the mixture medications, indicate that the combination of metformin and metronidazole offers an effective, safe option for cervical cancer. We recommend conducting additional studies to evaluate the clinical testing of the mixture.

Author Contributions:

Design and development: Kawakeb N Abdulla, Nora Sabah Rasoul, Azal Hamoody Jumaa. Gathering and organizing data: Aqeela Hayder Majeed, Youssef Shakuri Yasin. Data analysis/interpretation: Nora Sabah Rasoul, Azal Hamoody Jumaa, Aqeela Hayder Majeed. Article composition: Kawakeb N Abdulla, Youssef Shakuri Yasin, Azal Hamoody Jumaa. Critique the essay for significant ideas: Youssef Shakuri Yasin, Azal Hamoody Jumaa. Statistical analysis expertise: Kawakeb N Abdulla, Nora Sabah Rasoul, Aqeela Hayder Majeed. Ultimate article endorsement and guarantee: Azal Hamoody Jumaa, Youssef Shakuri Yasin.

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Conflicts of interest:

No evidence indicating a conflict of interest was discovered.

Ethical statement

This article was conducted under the regulation of the ethical committee of the Iraqi Medical Research Center, 1021-2020

Abbreviations:

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide stain

LSD: Least Significant Difference

AMPK: 5' adenosine monophosphate-activated protein kinase

PKC: protein kinase C

PP2A: Protein phosphatase 2

DRI: dose reduction index

CI: combination index

SD: standard deviation

LSD: least significant difference

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