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ORIGINAL ARTICLE

Advances in Basic Medical Sciences

In vitro synergistic cytotoxic activity of oral hypoglycemic agent (Metformin) with Methotrexate against cancerous cells

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ABSTRACT

OBJECTIVE: Evaluation of synergistic cytotoxic effects of add-on therapy of Metformin with Methotrexate in different cancerous cells.

Methodology: This interventional study was undertaken BMSI, JPMC, in conjunction with PCMD. The trial lasted three months. To appraise the Cytotoxic activity of metformin only and in combination with methotrexate, we employed cells from breast cancer (MCF-7 and MDA-MB-231), vaginal cancer (Hela), and colorectal adenocarcinoma (HT-29 cell line). We employed the most appropriate MTT tests to assess cytotoxic effects.

Results: When the percentage viabilities of the examined cell lines were compared, it was discovered that combination therapies of Metformin and Methotrexate dramatically lowered the percentage viabilities and had synergistic cytotoxic effects. There was a significant difference in the percent viability of cells representing breast cancer (MCF-7, MDA-MB-231), colorectal carcinoma (HT-29), and vaginal carcinoma representative cells (Hela cell lines) as assessed by MTT assay between Metformin alone and Metformin with Methotrexate. CDI values of each cell line for combination therapy were 0.702 ± 0.034, 0.67 ± 0.019, 0.69 ± 0.019, and 0.73 ± 0.040 for MDA-231, Hela, MCF-7, and HT-29 cells respectively.

Conclusion: This research showed that Metformin also has synergistic effects with Methotrexate, in addition to have antiproliferative effects on the studied cancer cell lines.

Key words: MDA-231,CDI index,MCF-7, Hela,HT-29, MTT assay

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INTRODUCTION

Cancer is a condition in which the proliferation of cells is irregular and uncontainable and may also embrace adjoining tissue.[1] Greater than 200 diverse cancer forms have already been established, individually each of which develops in a specific way. Nonetheless what they all share is the fact that they are all caused in the same way: a shift in a cell's normal internal structure.[2]

Communal characteristics among different types of this condition are: 1) unwarranted evolution signals; 2) inadequate response to anti-growth signals; 3) unplanned cell demise; 4) inexhaustible capacity for proliferation; 5) formation of newer blood vessels under the influence of various angiogenetic factors; and 6) invasion of tissue and metastasize so that cells may spread to other areas of the body by bloodstream or lymphatic[3,4].

According to the WHO, cancer is the second leading worldwide cause of death, with 8.8 million deaths in 2015.[5] About one in six people worldwide are estimated to die from cancer, involving death from different kinds of cancer such as liver cancer associated deaths were 788,000, colorectal cancer related 774,000 deaths, stomach cancer leads to 754,000 deaths and breast cancer complications leads to 571,000 deaths.[6]

Diabetes Mellitus is a communal disease worldwide and associated with various systemic complications. Besides of that systemic complications the diabetic's patients are more likely to develop a variety of diseases, including colon cancer, rectal cancer, pancreas and hepatic cancer, compared to non-diabetic patients. Systemic insulin resistance and mitogenic

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hyperglycemic effects increase the prevalence of several malignancies in persons with type 2 diabetes mellitus.[7]

Because of its specific effects on the treatment of type II diabetes, Metformin is a commonly used drug in modern societies. Metformin can significantly diminish the blood glucose levels stand-in marker for glycemic regulation HbA1c (1–1.5%) and insulin resistance and thus reducing the insulin levels.[8]

Metformin's insulin-reduction effects are important as insulin partakes a mitogenic and survival promoting effects and in addition to that, cancerous cells often have an unusual quantity of the insulin receptor, indicating a possible susceptibility to growth evolving effects of the insulin.[9]

Hence metformin has contributed to a decrease in the expression of insulin receptor for cancers.[10] Furthermore, the most substantial reductions in blood insulin, tumour insulin receptor expression and p-Akt associated with greater decrease in cancer cell development were examined independently in an imperative investigation. Beside of these, Metformin generally inhibits mTOR activity by activating LKB1 and AMPK and thereby prevents the synthesis and cell growth of proteins.[11]

Metformin alone or together with radiation therapy has also been shown to reduce tumor growth in a range of animal models in various carcinoma cancers, including ovarian, melanoma, prostate and breast cancers.[12]

Therefor in this trial we analyzed the in vitro cytotoxic activity of Metformin against cancer cells lines of breast cancer, colorectal adenocarcinoma and vaginal carcinoma.

METHODOLOGY

In the BMSI Department of Pharmacology, JPMC, collaboration with PCMD, such interventional trial was carried out. This study protocol is endorsed by JPMC's ethical committee.

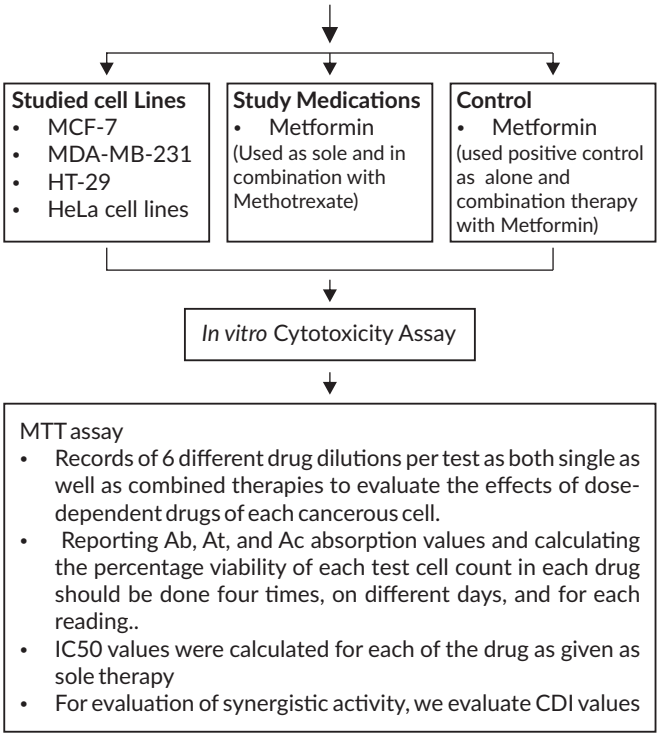
For evaluation of in vitro cytotoxic properties of Metformin as a sole therapy and add-on therapy with Methotrexate against cancer cells, we used 4 cell lines MCF-7 (representing Breast cancer), MDA-MB-231 (representing triple negative breast cancer cell), Hela (representing vaginal cancer) and HT-29 (representing colorectal carcinoma).

We used MTT assay for analysis of independent or as Methotrexate combination therapy cytotoxic effects of Metformin.

The combination drug file (CDI) was utilized to close the synergistically inhibitory impact of medication blend. CDI was surveyed by Calcusyn framework and as indicated by various scopes of mix drug lists drug blends can be delegated Synergistic in the event that they had CDI <1, as added substance on the off chance that they had CDI equivalent to 1 and demonstrated hostility on the off chance that they had CDI >1, as portrayed in succeeding table:[14]

(Bijnsdorp IV et al. 2011)[14]

Plan for evaluation of synergistic cytotoxic activity of Metformin with Methotrexate



Range of Combination Index	Treatment Effect
<0.1	Very strong synergism
0.1-0.3	Strong synergism
0.3-0.7	Synergism
0.7-0.85	Moderate synergism
0.85-0.9	Moderate synergism
0.9-1.10	Slight synergism
1.10-1.20	Nearly additive
1.20-1.45	Slight antagonism
1.45-3.3	Moderate antagonism
3.3-10	Antagonism
>100	Very Strong antagonism

RESULTS

Comparison of % decrease in At values of MCF-7 cells among various dose of alone treatment showed non-significant differences, as for metformin treated cells % decrease of At was -61.651 ± 1.699 at dose 6, whereas for Methotrexate treated cells it was -64.378 ± 3.062 (p=0.149). As depicted in Table 1.

Cells treated with MDA-MB-231 also exhibit non-significant changes between the two groups. As for Dose 6 % decrease of

Percentage decrease	Treatment Groups		P-value
	Metformin Mean ± SD	Methotrexate Mean± SD	
Dose 0 – 1st Dose	-11.024 ± 1.169	-14.248 ± 2.363	0.021
	(-12.08 – -9.572)	(-16.77 - -12.09)	
Dose 0 – 2nd Dose	-21.913 ± 1.583	-25.346 ± 2.539	0.083
	(-23.91 – -20.112)	(-28.062 - -21.936)	
Dose 0 – 3rd Dose	-31.638 ± 2.509	-35.048± 3.577	0.148
	(-34.367– -28.752)	(-38.757 - -30.384)	
Dose 0 – 4th Dose	-42.467 ± 2.178	-45.307± 3.226	0.149
	(-44.436– -39.665)	(-47.935- -40.899)	
Dose 0 – 5th Dose	-51.299 ± 1.982	-54.428 ± 3.311	0.149
	(-53.614 – -48.864)	(-57.88- -49.463)	
Dose 0 – 6th Dose	-61.651 ± 1.699	-64.378 ± 3.062	0.149
	(-62.983 – -59.218)	(-66.402- -59.826)	
P-value	< 0.001**	< 0.001**	

Table 1: The percentage decrease in At values of MCF-7 treated cells among the various dose of alone treatments

Percentage decrease	Treatment Groups		P-value
	Metformin Mean ± SD	Methotrexate Mean± SD	
Dose 0 – 1st Dose	-8.197 ± 1.133	-10.251 ± 1.788	0.149
	(-9.594 – -6.837)	(-12.098 - -7.803)	
Dose 0 – 2nd Dose	-16.414 ± 2.087	-18.599 ± 2.868	0.248
	(-18.75 – -13.675)	(-21.807 - -14.845)	
Dose 0 – 3rd Dose	-25.929 ± 3.109	-28.404± 3.489	0.149
	(-28.594 – -21.51)	(-31.761 - -23.521)	
Dose 0 – 4th Dose	-33.504 ± 3.763	-36.111± 3.436	0.386
	(-36.875 – -28.119)	(-39.663 - -31.408)	
Dose 0 – 5th Dose	-40.923 ± 2.703	-43.046 ± 3.073	0.149
	(-42.844 – -37.094)	(-45.238- -38.647)	
Dose 0 – 6th Dose	-48.453 ± 3.403	-50.628 ± 3.462	0.382
	(-52.955 – -45.726)	(-66.402- -59.826)	
P-value	< 0.001	<0.001	

Table 2: Comparison of the % decrease in At values for MDA-MB-231 treated cells at different doses between the treated groups of alone therapy

At was -48.453 ± 3.403 and -50.628± 3.462 for Metformin and Methotrexate treated cells (p=0.382). As shown in table 2.

Table 3 compares the dose-dependent effects of metformin and methotrexate alone therapy on the percentage decrease in At values for cells treated with HT-29. This shows non-significant differences among both treated groups, as for dose 6 % decrease was -58.151 ± 5.659 and –60.874± 5.239 for Metformin and Methotrexate treated cells (p=0.248).

Comparison of effects on % viabilities of MCF-7 of alone therapy of Methotrexate and add on therapy of Metformin with Methotrexate shows statistically significant differences among both groups. For dose 6 there was statistically significant difference (p=0.001), with % viabilities were 36.7 ± 3.1 and 13.5 ± 0.51 for Methotrexate alone and Combination Therapy respectively. As revealed in Table 4.

Percentage decrease	Treatment Groups		P-value
	Metformin Mean ± SD	Methotrexate Mean± SD	
Dose 0 – 1st Dose	-9.364 ± 1.479	-11.851 ± 1.588	0.021
	(-11.178 – -7.568)	(-13.362 - -9.913)	
Dose 0 – 2nd Dose	-19.286 ± 1.500	-21.527 ± 1.615	0.043
	(-20.677 – -17.168)	(-22.84 - -19.207)	
Dose 0 – 3rd Dose	-28.648 ± 2.781	-31.112± 3.152	0.149
	(-30.811 – -24.937)	(-33.469 - -26.766)	
Dose 0 – 4th Dose	-38.259 ± 3.414	-40.574± 3.628	0.248
	(-41.409 – -34.135)	(-44.172 - -36.059)	
Dose 0 – 5th Dose	-47.059 ± 4.358	-49.433 ± 4.655	0.149
	(-50.859 – -42.155)	(-53.618- -43.99)	
Dose 0 – 6th Dose	-58.151 ± 5.659	-60.874± 5.239	0.248
	(-63.151 – -5.659)	(-65.662- -55.266)	
P-value	< 0.001	< 0.001	

Table 3: Comparison of the % decrease in At values for HT-29 human colorectal adenocarcinoma treated cells at various doses between the treated groups

Doses (µM)	N= 28	% Viability of Methotrexate Alone Therapy	% Viability of Metformin + Methotrexate Combination Therapy	P-value
0	4	99.85 ± 0.1	99.725 ± 0.215	0.332
		(99.75 - 100)	(99.5 - 100.00)	
1st Dose	4	86.67 ± 3.0	82.29 ± 0.422	0.027
		(84 - 90.43)	(82.05 - 82.92)	
2nd Dose	4	75.71 ± 3.3	68.94 ± 1.61	0.010
		(72.5 - 80.2)	(66.99 - 70.45)	
3rd Dose	4	64.49 ± 4.2	52.55 ± 0.463	0.001
		(60.75 - 70.5)	(51.87 - 52.87)	
4th Dose	4	54.67 ± 3.6	37.07 ± 3.144	0.003
		(51.48 - 59.28)	(33.27 - 40.65)	
5th Dose	4	44.98 ± 3.1	26.56 ± 2.04	0.001
		(41.38 - 48.73)	(24.07 - 28.7)	
6th Dose	4	36.7 ± 3.1	13.5 ± 0.51	0.001
		(34.15 - 41.13)	(12.75 - 13.85)	

Table 4: Effects of metformin plus methotrexate therapy on MCF-7 cell line viability in comparison to methotrexate alone, with respect to dose.

Association of effects on % viabilities of MDA-MB-231 treated cells displays noteworthy differences amid both Methotrexate alone and Methotrexate combined with Metformin. As for dose 6 the % viability was 49.69 ± 4.2 and 31.45 ± 3.12 for alone therapy and combined therapy of Methotrexate respectively. As shown in table 5.

When the dose-dependent effects of methotrexate alone and in combination therapy are compared, the percentage viability of the HT-29 cell line reveals statistically significant variations between the two groups. As for dose 6 % viabilities were 41.19 ± 2.5 and 20.78±2.34 for Methotrexate alone and combination therapy respectively. As shown in table 6.

Table 7 shows the comparison of methotrexate as alone therapy

Doses (µM)	N= 28	% Viability of Methotrexate Alone Therapy	% Viability of Metformin + Methotrexate Combination Therapy	P-value
0	4	99.88 ± 0.19	99.93 ± 0.085	0.648
		(99.75 - 100)	(99.82-100)	
1st Dose	4	90.38 ± 1.6	87.42 ± 0.184	0.010
		(88.7 - 92.5)	(87.27-87.67)	
2nd Dose	4	82.44 ± 2.7	75.81± 1.76	0.006
		(79.53 - 86.05)	(73.6-77.77)	
3rd Dose	4	72.61 ± 3.2	64.55 ± 1.92	0.005
		(69.75 - 77.25)	(62.9-67.3)	
4th Dose	4	64.47 ± 3.5	53.69 ± 1.67	0.001
		(61.9 - 69.4)	(52.0-55.85)	
5th Dose	4	57.12 ± 3.7	43.13 ± 2.46	0.007
		(52.68 - 61.6)	(39.97-45.9)	
6th Dose	4	49.69 ± 4.2	31.45 ± 3.12	0.004
		(43.8 - 53.2)	(27.47-35.1)	

Table 5: The effect of Metformin in combination with methotrexate against methotrexate alone on the viability of MDA-MB-231 cell lines was compared in a dose-dependent manner.

Doses (µM)	N= 28	% Viability of Methotrexate Alone Therapy	% Viability of Metformin + Methotrexate Combination Therapy	P-value
		Mean ± SD	Mean ± SD	
0	4	99.6 ± 0.1	99.79±0.14	0.069
		(99.75 - 100)	(99.72-100)	
1st Dose	4	89.23 ± 1.1	89.82±1.95	0.617
		(88.18 - 90.65)	(87.725-92.45)	
2nd Dose	4	79.63 ± 2	76.06±1.07	0.019
		(76.93 - 81.58)	(75.25-77.62)	
3rd Dose	4	69.86 ± 2.9	63.3±1.932	0.009
		(67.15 - 73.9)	(61.0-65.68)	
4th Dose	4	60.4 ± 2.9	49.99±3.25	0.003
		(58.23 - 64.7)	(45.725-53.625)	
5th Dose	4	51.34 ± 3.3	36.82±3.38	0.008
		(48.95 - 56.18)	(32.7-40.97)	
6th Dose	4	41.19 ± 2.5	20.78±2.34	0.001
		(39 - 44.68)	(18.17-23.4)	

Table 6: The viability of the HT-29 cell line was compared in a Concentration-dependent manner between Metformin + Methotrexate and Methotrexate alone.

and as combination therapy on % viability of HeLa cell lines. This shows that significant differences between both groups, as for % viability of for dose 6 HeLa cell line were 44.04 ± 1.5 and 18.756±1.09 for Methotrexate as alone therapy and combination therapy respectively.

This was further supported by CDI values. The CDI values of MCF- 7 and Hela cell lines shows synergism as there values are fall in ranges between 0.3-0.7. For MDA-MB-231 and HT-29 cell lines combination therapy of Metformin and Methotrexate shows moderate synergism as they fall in range between 0.7-0.85. As depicted in Table 8.

Doses (µM)	N= 28	% Viability of Methotrexate Alone Therapy	% Viability of Metformin + Methotrexate Combination Therapy	P-value
0	4	99.76 ± 0.2	99.81±0.092	0.665
		(99.58 - 99.93)	(99.7-99.92)	
1st Dose	4	88.96 ± 1.1	88.05±1.24	0.314
		(87.6 - 89.88)	(86.25-88.87)	
2nd Dose	4	80.51 ± 1.6	75.73±2.09	0.011
		(78.6 - 82.25)	(73.87-78.37)	
3rd Dose	4	71.49 ± 1.5	62.04±0.55	0.001
		(69.53 - 72.6)	(61.22-62.45)	
4th Dose	4	63.08 ± 1.6	48.29±1.26	0.001
		(61.05 - 64.93)	(46.85-49.75)	
5th Dose	4	54.55 ± 1.6	34.49±0.79	0.001
		(52.33 - 55.8)	(33.35-35.1)	
6th Dose	4	44.04 ± 1.5	18.756±1.09	0.001
		(42.05 - 45.83)	(17.775-19.725)	

Table 7: The effect of Metformin + Methotrexate therapy on HeLa cell line viability was compared to Methotrexate alone therapy.

Cell lines	Combination drug Index
	Mean±SD (Min-Max)
MCF-7	0.69±0.019
	(0.67-0.72)
MDA-MB-231	0.702±0.034
	(0.66-0.74)
HT-29	0.73±0.040
	(0.69-0.77)
HeLa	0.67±0.019
	(0.65-0.70)

Table 8: Metformin CDI values were compared amid treated cells

DISCUSSION

Cancer is one of the most serious diseases in which abnormal cell growth invades and spreads to other parts of the body. Cancer has become a major scourge in Pakistan in recent years. Cancer incidence in Pakistan has been steadily increasing, according to the World Health Organization. Based on the current study, the five categories of most prevalent malignancies were carcinoma of the breast (24.1%), the oral cavity (9.6%), colorectum (4.9%), esophageal (4.2%), and carcinoma of the liver (3.9%).[16]

Cancer as well as diabetes are both common diseases throughout the world. Diabetes is associated with a rise in the incidence of cancer. Diabetes patients are at a significantly greater risk of developing common malignancies such as pancreatic, liver, breast, colorectal, urinary system, gastric, and female genital tumors, according to epidemiologic studies. Cancer mortality is slightly higher in diabetic patients than in non-diabetics.[17]

Diabetes mellitus is a disorder that is etiologically complex,

guided by a multitude of cellular pathways. Given that glucose is an important cellular metabolic substrate and that insulin signaling has mitogenic effects, the proliferation and spread of breast cancer is intimately related to cellular glucose metabolism. In the treatment of breast cancer, rising attention has been focused on anti-diabetic agents.[18]

Epidemiological studies show that in type 2 diabetic patients, metformin reduces the incidence of cancer and mortality. Preclinical in vitro and in vivo research provides an intriguing insight into the cellular mechanisms behind metformin's anticancer effects.[19]

In this study we evaluated that addition of Metformin can increase the cytotoxic activity of the Methotrexate in different cancer cell lines. For this we used MCF- 7 and MDA-MB231 cell lines which are representative of Breast cancer, Hela cell line which represented Cervical cancer and HT-29 colorectal adenocarcinoma cell line. For evaluation of cytotoxic activity of drugs, we used MTT assay.

In our study, the percentage viability of all cancer cell lines decreased substantially in the combined use of Methotrexate + Metformin as compared to monotherapy of Methotrexate. As for MCF-7 % viability was significantly reduced in combination therapy (MTX and Metformin) to 13.50 ± 0.51 while in alone therapy of MTX % viability was about 36.5±3.10. The CDI for combination therapy of MTX and Metformin was 0.69±0.019 which indicates synergism.

While for MDA-MB-231 cell line % viability was reduced to 49.9±.4.20 in alone therapy of MTX while % viability was reduced up to 31.45±3.12 in combination therapy of MTX and Metformin. The CDI index was 0.702±0.03, which indicate slight synergism. Similarly for HT-29 % viability was significantly reduced in combination therapy of MTX and Metformin and CDI index was 0.72±0.04 which shows slight synergism. Moreover, for Hela cell line % viability was also significantly reduced in combination therapy of MTX and Metformin and CDI index shows synergism (0.67±0.02).

These results in agreement with the study conducted by Yang et al. (2019).[20] As they evaluated that either Metformin can complement the chemotherapeutic effects of current standard chemotherapeutic drugs (Methotrexate or DDP) on ovarian cancerous cell lines. The DDP and methotrexate (MTX) half-inhibitory concentration (IC50) values (that were assessed by MTT assay) were 14.35 and 4.21 μg/ml for SKOV3 cells and 70.26 and 15.27 μg/ml for SKOV3/DDP cells, respectively. Moreover, the SKOV3/DDP resistance index was 4.89 and 3.62 for DDP and MTX, respectively. Furthermore, the SKOV3/DDP susceptibility index showed 4.89 for DDP as well as 3.62 against MTX. The concentrations of the IC50 to SKOV3 cells following the combo treatment comprising metformin, DDP, and MTX included 11.20 & 2.80 μg/ml, and 6.21 and 2.74 μg/ml, respectively. Following mixing metformin plus DDP and MTX, the IC50 results for SKOV3 lines included 11.20 and 2.80 μg/ml, and 6.21 and 2.74 μg/ml for SKOV3/DDP cells.

Metformin diminished the IC50 of DDP and MTX by 11.31-and 6.18-fold in drug-resistant SKOV3/DDP tumor cells. This suggested that while treated with the combination of metformin and chemotherapeutic drugs, cell proliferation was diminished compared with chemotherapeutic agents as sole therapy.

Such results were also endorsed by Iliopoulos et al. (2011). They considered that Metformin's combinatorial effect could reduce the dosage of different chemotherapeutic drugs (Cisplatin, doxorubicin also paclitaxel) and thereby reduce the chances of side effects of existing antitumor drugs.[21]

Metformin's anti-proliferative effectiveness alone or in conjunction with chemotherapeutic drugs and mTOR system antagonists has been investigated to successfully minimize the proliferative capabilities of numerous cell lines of breast cancer. They established that Metformin can reduce the viability of breast cancer cell lines, particularly MDA-MB-2311 cells, either in combination or as a only treatment.[22]

The reason behind the synergistic effects is that by stopping cell growth by affecting the mTOR pathway or directly inhibiting cell growth by affecting Cyclin D, Metformin can increase the antitumor activity of Methotrexate and thus inhibit its dependent kinases and further progression of cell growth. [23]The molecular targets of Metformin in malignancy cells (e.g., mTOR, HER2) are like those currently used for organized tumor care. Be that as it may, Metformin is non-toxic and can be very effective in enhancing the activity current available chemotherapeutic agents.[24]

The main draw backs of Current anticancer therapies are that, they are usually costly and in addition to that mostly they are available as parenteral therapies and used as combination therapies. All of these can increase the economic burden on nations for treating cancers.[25] So, researchers are currently paying attention in exploration of the newer economical anticancer options, which can have antiproliferative actions so this can compliment the effects of current anticancerous drugs for particular tumor. So, this study aimed to emphasize the favorable complementing effects of Metformin with existing chemotherapeutic agents.

CONCLUSION

This research showed that Metformin also has synergistic effects with Methotrexate, in addition to have antiproliferative effects on the studied cancer cell lines.

Conflict of interest

No conflict of interest to declare

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CONFLICT OF INTEREST

Author declare no conflict of interest.

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AUTHORS CONTRIBUTIONS

FR: Conception, Design of the work, Data collection, and Drafting, Reviewed, Final approval, Agreement to be accountable.

DATA SHARING POLICY

The data that support the findings of this study are available from the corresponding author upon reasonable request

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