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The cytotoxicity and anti-proliferative effect of Metformin on solid tumors in vitro cell lines.

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Abstract:

Background: Cancer is a pathogenesis that happens when modification in collections of normally occurring cells inside the people body occurs leading to non-controlled growth causing a lump called the tumor; this applies to all types of cancers except leukemia (cancer of the blood). Doxorubicin (DOX) is one of the highly effective anti-neoplastic drugs of the anthracyclines family used to treat many pediatric and adult cancers, e.g. solid tumors, lymphomas, leukemia and breast cancer. DOX is known to produce severe cytotoxicity. Metformin (Met) is a biguanide used for type 2 diabetes mellitus. Met have cytoprotective effect in addition to reducing basal and postprandial levels of glucose by decreasing the production of ROS, maintaining energy homeostasis and apoptosis regulation by its activation of adenosine monophosphate-activated protein kinase (AMPK). Met has also the ability to increase apoptotic factors and suppression of proliferation thus MET consider as cytotoxic and anti-proliferative drug.

Objectives: This study was designed to investigate the cytotoxic and antiproliferative effect of Met comparing to DOX as a control in solid tumor cell lines by using annexin V detection and p21 detection assay.

Materials and Methods: Cell lines that used in our research (Epithelial cells as a normal cell line and MCF-7 cell line was taken from human breast cancer) cultured in suitable media potentiated with different concentrations of heat-inactivated human serum. MTT assay using for detection of cytotoxicity in Epithelial and MCF-7 cell lines for duration 72hrs. Annexin v detection kit applied for MCF-7 cell lines for detection of apoptosis for duration 72hrs.Finally, P21 detection kit applied for MCF-7 cell lines for detection of proliferation arrest for duration 72hrs.

Results: The results showed that treatment with DOX,MET and combination DOX with MET there is significant ($p \leq 0.5$) cytotoxic , apoptotic and atiproliferative effect and there is potentiation effect between MET and DOX on tumor cells when treated by combination DOX with MET .

Conclusion: From the results, it can be concluded that Metformin have a good cytotoxic and antiproliferative effect on tumor cell lines. Met showed a valuable cytotoxic effect through the detection of IC_{50} , annexin V and p21 expression .

Keywords: Doxorubicin, Metformin, IC_{50}, Combination, cell line,MCF-7.

INTRODUCTION:

Cancer is a pathogenesis that happens when modification in collections of normally occurring cells inside the people body occurs leading to non-controlled growth causing a lump called the tumor; this applies to all types of cancers except leukemia (cancer of the blood). If remain with no treatment, the tumors could be grow up and spreads into the surrounding normally another tissues, or to other regions of the body by the blood stream and lymph system, and can also effect on the digestive tract, circulatory and nervous systems. People often used the word 'cancer' as synonymous with 'tumor', but in fact they are not the same [(1)] . A tumor in a simple form a space-occupying lesion (something that should not be there, that is; a "lump") occurs by abnormal cell replication (medically, the word "tumor" literally means "swelling). While cancer is a disease in which cells division is *totally out of control* and there is destruction to the genes (DNA) which normally *stop* cell division when it needs to be stopped[(2)] .

Cancer considers the second causable factor of death in the world, and was leaded to for 8.8 million death case in 2015. Around 70% of death cases occurs due to cancer in middle- and low- income countries. Cancer treatment may involve surgery, radiation and/or chemotherapy. Most people may receive combination of chemotherapeutic agents [(3)].

Chemotherapy is a type of cancer therapy that uses drugs to kill cancer cells these cancer therapy that uses drugs to damage cancer cells. It is also named "chemo." there are many different types of chemotherapy such as antibiotics group (doxorubicin). DOX is effective anticancer drugs that used to treat many pediatric and adult cancers, e.g. solid tumors, lymphomas, leukemia . DOX have severe toxicities, e.g. hematopoietic suppression, alopecia, extravasation, nausea and vomiting, yet the cardio toxicity being the most important. The beginning of cardio toxicity may be delayed up to 10 –15 years after stopping of therapy [(4)] .

DOX reduced to doxorubicinol which also have biological activity .DOX also undergo reduction to a semiquinone radical by several intracellular oxido reductases. Reoxidation of semiquinone radical yield reactive oxygen species (ROS). The ROS production considered as one mechanism of its anticancer and antibiotic capabilities. DOX excretion is done by the liver and kidney through a biphasic half-life of 5 minand30–40 hrs [(5)].

The drug of first-line regime in treating of type 2 diabetes is currently MET. Besides its glucose-declining effect, there is important effect of the drug of potential relevance to cardiovascular and cancer diseases. However, the mechanisms of action persist elusive. Metformin's mechanism of action in diabetes and may also be of importance in cardio vascular and cancer diseases [(6)].

MET by mechanisms thought to involve the activation of AMPK (AMP-activated protein kinase) [(7)] and/or suppressing of adenylate cyclase [(8)] in response to energetic stress, and/or the direct inhibition of mitochondrial glycerol phosphate dehydrogenase [(9)] lead to decreases blood glucose.

Certain types of cancers, like chorionic carcinoma and Burkitt's lymphoma, can be cured by therapy with a single agent, although these are extremely exceptional cases. It is impossible to uniformly kill a group of cells with high heterogeneity and thus difficult to obtain a good outcome through administration of a single anticancer agent because the group of cells is likely to comprise cells that are responsive to the treatment as well as those that are resistant. As a result, therapy combining multiple agents that have different mechanisms of action has evolved, i.e., combination chemotherapy. The goal of combination chemotherapy is to eradicate tumor cells through potent therapy before the appearance of resistant cells or an elevation in the number of the resistant cells[10] .

Identification and development of safe and potent combination therapy composed of multiple drug resistance reversing agents such as biguinide (MET) with a potent chemotherapeutic agent such as anthracyclin (DOX) is still a big deal among researchers, most of studies showed there is a good targeting correlation can be represented through potentiation or synergism effect to one of them of combining therapy lead to obtaining the desired effect [11].

Apoptosis and Cell proliferation

Apoptosis is over twenty times faster than mitosis. Apoptotic cells are undergoing engulfment and destruction by neighboring cells without a trace [12]. Apoptosis is represented by specific morphologic features, including lossing of plasma membrane structure and binding , plasma membrane blebbing , the cytoplasm and nucleus condensation, and inter nucleosomal cleavage of DNA. Lossing of plasma membrane structure consider one of the earliest features of apoptosis. In apoptosis, the cellular membrane phospholipid phosphatidylserine (PS) translocation from the inner to the outer leaflet of the cellular plasma membrane, there by exposing PS to the outer cellular environment[13] .

Annexin V is a recombinant phosphatidyl serine binding protein that has been highly and specifically interacts with phosphatidyl serine residues and can be used for the assessing of apoptosis [14] .

Apoptosis and mitosis shared in common of morphological features such as chromatin condensation, cell shrinkage and membrane blabbing. Thus, the equilibrium between apoptosis and cell proliferation must be strictly maintained to support tissue homeostasis [15].

Cell proliferation, cell differentiation and cell death are important processes in multicellular organisms, and many lines of event link apoptosis to proliferation, uncontrolled proliferation can be associated with a high level of apoptosis [16].

Apoptosis can happen at any stage of the cell cycle, as the metabolic machine responsible for induction of caspase-3. When caspase-3 activated by TGFB1 lead to cleaves p21, p27andPARP that lead to induction of cell cycle arrest that may be initiate the conversion of cell-cycle arrest to apoptosis .p21, also known as cyclin-dependent kinase 1, participate in an important role in a many of cellular processes including cell cycle regulation, apoptosis, and autophagy Treatment of cancer depends on the type and stage of growth. Some patients with cancer will have only one treatment [15] .

METHOD

Epithelial cell line and MCF-7 Human Breast cancer cell line in falcon was obtained from the Biotechnology center/ Al-Nahrain University. The cell culture medium was DMEM (Dulbecco's modified Eagle's medium) .The cells were incubatedat 37°C at a humidified atmosphere 5% CO2 a Cells were passaged in to new T25 or T50 cell culture flasks every 4 days by washing with Phosphate Buffer Saline (PBS) followed by harvesting via Trypsin EDTA. All cell cultures were incubated in a humidified incubator supplemented with 5% CO2 and 95% air at 37°C. Annexin v detection kit applied for MCF-7 cell line for detection of apoptosis for duration 72hrs and P21 detection kit applied for MCF-7 cell lines for detection of proliferation arrest for duration 72hrs.

Study design

Two cell lines has been used:

1. Epithelial cell as normal cell treated by MET,DOX and combination DOX with MET for duration 72hrs.
2. MCF-7 cell line of breast cancer treated by MET,DOX and combination DOX with MET for duration72hrs.
4. MTT assay using for detection of cytotoxicity in Epithelial and MCF-7 cell lines 72hrs.
4. Annexin v detection kit applied for MCF-7 cell line for detection of apoptosis for duration 72hrs..
5. P21 detection kit applied for MCF-7 cell line for detection of proliferation arrest for duration 72hrs.

Cytotoxic activity assay (MTT) :

The tow cell lines (Epithelial and MCF7) accounted by cell counter technique where (1 x 10^2/µl) in 96 well microplate with 100µl in each well and incubated for 24 hours in starved media at 37°C.

First, discard whole media from tissue flask then washed it with 2ml PBS to remove the waste product of cells, after that add 1ml of trypsin EDTA for 1 min to detach the cells from surface.

Cytotoxicity of Doxorubicin.

Six serial dilutions of doxorubicin was prepared (40µM, 20µM, 10µM, 5µM, 2.5µM, 1.25µM) and added to normal epithelial cell line and cancer MCF-7 cell lines to determine the cytotoxic effect of DOX after 72hrs incubation periods.

Cytotoxicity of Metformin.

Six serial dilutions of Metformin was prepared (40mM, 20mM, 10mM, 5mM, 2.5mM, 1.25mM) and added to normal epithelial cell line and MCF-7 cancer cell lines to determine the cytotoxic effect of MET after 72hrs incubation periods .

Cytotoxicity of combination
(Doxorubicin with Metformin) .

Six serial dilutions (1.25µM, 2.5µM, 5µM, 10µM, 20µM, 40µM) of DOX were prepared in combination with (10

mM) of MET then added to the tow cell lines to determine the cytotoxic effect.
CompoSyn software system used to determine the type of interaction (synergism, agonism or antagonism effect) occurs between combination (DOX with MET) after 72hrs incubation periods [17] .
The result that showed after 72hrs consider the dependable result for apoptosis and p21 detection assay due to the best reults occure during this time.

Apoptosis detection assay (Annexin V test) :
Apoptosis or programed cell death , is a normal physiological process for removal of unwanted cells, in this process phosphatidylserine (PS), marker of apoptosis, located in the cytoplasmic face of cell membrane is exposed to cell surface allow to exhibit pro-inflammatory and pro-coagulant activities. Annexin v will bind with PS and inhibit those activities cellular death. Total endogenous annexin V can be detected via ELISA which will suggest further insights in the pathophysiological role of Annexin V[18].
The human Annexin V ELISA kit (purchased from Thermo Fisher Scientific Company invitrogen) is an enzyme-linked immunosorbent

Detection of P21.`
Cell division relies on the activation of cyclins, which bind to CDKs to induce cell-cycle progression towards S phase and later to begin mitosis. The CDKs function is tightly regulated by cell-cycle inhibitors such as p21 Waf1/Cip1 and p27Kip1 proteins. Following anti-mitogenic signals or DNA damage, p21 Waf1/Cip1 and p27Kip1 bind to cyclin-CDK complexes to reduce their catalytic activity and induction of cell cycle arrest.
Total endogenous P21 can be detected via ELISA. The P21 ELISA kit (purchased from Thermo Fisher Scientific Company invitrogen) is an enzyme-linked immune sorbent assay for the quantitative detection of P21.

Statistical analysis
Throughout the thesis, the experiments were performed three times independently and results were expressed as the mean ± the standard deviation and compared using analysis of variance of one way ANOVA followed by post hoc LSD software . p value < 0.05 was considered as statistically significant.

RESULTS

Cytotoxicity of Doxorubicin, Metformin and combination of Doxorubicin with Metformin on epithelial cell line.
The results showed significant dose dependent inhibition of growth in normal epithelial cell line induced by DOX (1.25µM,2.5 µM,5 µM,10 µM,20 µM and 40 µM), MET (1.25mM,2.5 mM,5 mM,10 mM,20 mM and 40 mM) and the combination of DOX (1.25µM,2.5 µM,5 µM,10 µM,20 µM and 40 µM) with MET (10Mm) , as shown in Table (1,2 and 3) and Fig(1) during 72hrs.

Cytotoxicity of Doxorubicine, Metformin and Combination of Doxorubicin with Metformin on MCF-7 cell line.
The results showed significant dose dependent inhibition of growth in MCF-7 cell line induced by DOX (1.25µM,2.5 µM,5 µM,10 µM,20 µM and 40 µM), MET (1.25mM,2.5 mM,5 mM,10 mM,20 mM and 40 mM) and the combination of DOX (1.25µM,2.5 µM,5 µM,10 µM,20 µM and 40 µM) with MET (10Mm) , as shown in Table (4,5 and 6) and Fig(2) during 72hrs.

Table (1) Effect of DOX1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM on epithelial cell line (72hrs).

DOX conc. (µM)	% Growth inhibition ± SD
1.25	38±0.07
2.5	45±0.03
5	53±0.08
10	65±0.04
2O	74±0.01
40	80±0.1

Table (2) Effect of MET1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM on epithelial cell line (72hrs).

MET conc. (mM)	% Growth inhibition ± SD
1.25	26±0.01
2.5	38±0.05
5	49±0.02
10	57±0.08
20	68±0.07
40	72±0.2

Table (3) Effect of combination (DOX1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM with MET10 µM) on epithelial cell line (72hrs).

DOX conc.(µM)	% Growth inhibition ± SD
1.25	43±0.03*
2.5	52±0.04*
5	62±0.1*
10	72±0.07*
2O	79±0.1*
40	86±0.04*

Each value expressed as mean ±SD. The statistical analysis done by using one way ANOVA followed by Post Hoc, LSD .
* significant difference ($p < 0.05$) when compared between DOX with MET and DOX with combination (DOX+MET) .

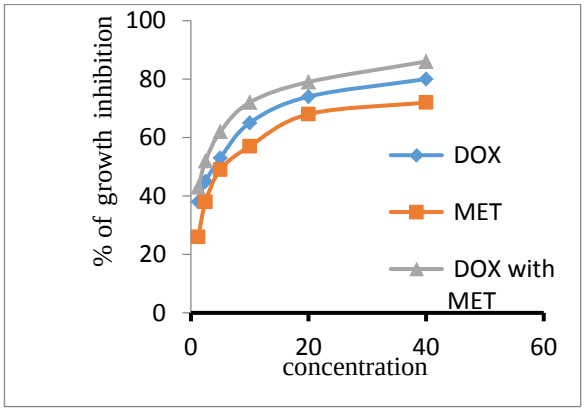

Figure (1) effect of DOX1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM, MET1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM and DOX1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM with MET 10 µM on epithelial cell line after 72hr.

Table (4) Effect of DOX 1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM on MCF-7 cell line (72hrs).

DOX conc. (µM)	% Growth inhibition± SD
1.25	21±0.07
2.5	37±0.01
5	49±0.04
10	60±0.001
2O	71±0.05
40	83±0.2

Table (5) Effect of MET1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM on MCF-7 cell line (72hrs).

MET conc. (mM)	% Growth inhibition ± SD
1.25	18±0.002
2.5	31±0.02
5	40±0.4
10	52±0.06
20	61±0.001
40	76±0.1

Table (6) Effect of combination (DOX1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM with MET10 µM) on MCF-7 cell line (72hrs).

DOX conc.(µM)	% Growth inhibition ± SD
1.25	30±0.07*
2.5	39±0.03*
5	51±0.2*
10	67±0.07*
2O	75±0.02*
40	87±0.05*

Each value expressed as mean ±SD. The statistical analysis done by using one way ANOVA followed by Post Hoc, LSD .
* Significant difference ($p < 0.05$) when compared between DOX with MET and DOX with combination (DOX+MET) .

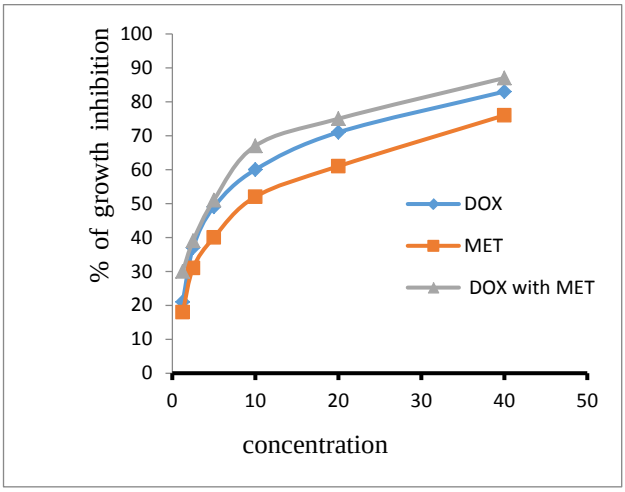

Figure (2) Effect of DOX1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM, MET1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM and DOX1.25µM, 2.5 µM,5 µM,10 µM,20 µM and 40 µM with MET 10 µM on MCF-7 cell line after 72hrs.

DISCUSSION

Based on the IC_{50} values, the cells showed a wide variation in sensitivity of cancer cell line (MCF-7) to the treatment (DOX,MET and DOX with MET) more than sensitivity of normal cell (epithelial) cell line .

MTT assay revealed significant dose dependent effects after 72hrs of incubation periods for DOX, MET and combination (DOX with MET) .
However, at lower than 1.25 µM no more effect produced even after incubation for a longer time.
The IC_{50} values after application of DOX (1.25, 2.5,5,10,20 and 40 µM) on epithelial and MCF-7 cell lines in different time intervals can be illustrated in (Table 4):

Table (7) IC_{50} of DOX for Epithelial,MCF-7 during 72hrs.

IC_{50} of DOX (µM)	72hrs
Epithelial	3
MCF-7	2

That showed the low values of IC_{50} at 72hrs this is due to DOX is trafficked straight to the nuclear area without any accumulation in the cytoplasm up to 24hrs. Some trace amounts do appear in the cytoplasm at 48hrs and increase accumulation in 72hrs, cytoplasmic DOX mechanism of action associated with oxidative stress as a result of reactive oxygen species production. To determine the cytotoxicity of MET, MTT assay has been used and the effects after 72hrs of incubation in vitro was studied.
The IC_{50} values after application of MET (1.25, 2.5,5,10,20 and 40 µM) on epithelial and MCF-7 cell lines in different time intervals can be illustrated in (Table 8):

Table (8) IC_{50} of MET for Epithelial and MCF-7 during 72hrs.

IC_{50} of MET (µM)	72hrs
Epithelial	6
MCF-7	4

That showed the low values of IC_{50} during 72hrs refers to sensitivity of cells to MET that means the MET induced DNA synthesis alteration , the highly cytotoxic effect of MET and strong reduction in DNA synthesis in all experimental cultures after 72 hours of incubation[108].
This study was showed the combination of (DOX and MET) has cytotoxic effect on epithelial and MCF-7 cell lines *in vitro* and MET has synergism effect to DOX on all cell lines, all that can be shown through percent of inhibition values 72hrs that is can be illustrated by table (9) :

Table (9) IC_{50} of DOX with MET for Epithelial and MCF-7 during 72hrs.

IC_{50} of DOX with MET (µM)	72hrs
Epithelial	2
MCF-7	0.7

That showed the low values of IC_{50} refers to high sensitivity during 72hrs that is refer to potentiation effect of DOX by MET.
In the present study, we observed that combining MET, with DOX leads to potentiation effect to DOX and decreasing the IC_{50} of DOX in all lines of cells *in vitro* that is occurs through Multi drug resistance shows to be one of the most challenging problems in chemotherapy agents. Such as, doxorubicin induces drug resistance by over

expressing numerous drug transporters (e.g., *ABCB1*) to reduce intracellular drug accumulation. *ABCB1* encoded P gp is up regulated in various drug resistant tumors and accounts for thee efflux of different drugs. The reduction of Pgp expression or function is feasible to reverse multi drug resistance. The combination of DOX with MET shows synergistic effects in all cell lines. Since P gp consumes two ATPs to export one substrate, MET could weaken Pgp function by reducing ATP production. This leads to elevation intracellular and nuclear DOX accumulation, which facilitates DOX cytotoxicity. Furthermore, MET suppress *ABCB1* (Pgp) expression on the mRNA and protein levels, whereas DOX lead to enhancement the drug-resistant phenotype. This result suggested that MET may be reverse acquired multi drug resistance during prolonged DOX therapy. Additionally, it was found that MET could selectively eliminate cancer stem cells, which are responsible for intrinsic drug resistance and prolong remission in multiple cancer cell types [19] .

Apoptosis induction assay.

The cell cycle represents a successive steps which lead to proliferation of the cell under a strict control of many factors e.g. CDKs,p21,p27 and p53 which is the tumor suppressor factors[20] .

Many of anticancer drugs display antiproliferative activity by targeted apoptosis in the cancerous cells ,which might be through interference with DNA replication and the integrity of DNA [21] .

In the present study the highly expression of Annexin V assay was selected to study the effect of DOX,MET and combination (DOX with MET) on the induction of apoptosis in the cancerous cell lines. This test depends on the hypothesis of normal viable cells.

After apoptosis induction , fast alterations in the organization of phospholipids in most cell types happens leadingto exposure of PS on the cell surface. Recognition of PS by phagocytes *in vivo* results in the eliminates of cells programmed to die thus apoptosisis not always associated with the local inflammatory response which associated with necrosis[22].

Externalized PS *In vitro* detection can be achieved through interaction with the annexin V. In case of calcium presence, fast high affinity binding of annexin V to PS happens. PS translocation to the cell surface preceding nuclear degradation. DNA fragmentation, and the expression of most apoptosis-associated molecules making annexin V binding a marker of early-stage apoptosis[23].

There was a dose dependent increase in the expression of PS with DOX and MET in both cell lines .Additionally, there was a potentiation in expression of PS after the application of combination (DOX with MET) . Previous researches showed that DOX may enhances apoptosis by enhancing production of H2O2 which will induces nitric oxide synthase (eNOS) ,Antisense eNOS depressed DOX-induced oxidative stress and apoptosis in addition to p53 dependent pathways[24] .

The same protocol showed that MET, also , have the ability to produces dose dependent increase in the translocation of PS to the extracellular membrane due to decline the activation of IRb, Akt and ERK1/2, increased pAMPK, FOXO3a, p27, Bax and cleavedcaspase-3, and decreased phosphorylation of p70S6K and Bcl-2 protein appearance also there's elevation in H2O2 that lead to elevation in oxidative stress which was associated with declining in cell numbers [25] , and combination of DOX with MET showed enhanced effect in a dose dependent that is due to MET and DOX co-treatment markedly decreased tumor volume, increased survival rate, and improved other parameters compared to single therapy effect was mediated by down regulation of cyclin D1 while elevating the level of the tumor suppressorgeneP53. The activity of AMPK and NF-κB was modulated so that they triggered apoptotic pathway rather than proliferative one [26].

Detection of P21.

The cell division relies on the activation of cyclins, which bind to CDKs to induce cell-cycle progression towards S phase and later to begin mitosis. Their function is tightly regulated by cell-cycle inhibitors such as p21 and p27 proteins. P21 is involved ingrowth arrest induced by cell-cycle checkpoints, senescence, or terminal differentiation. Following anti-mitogenic signals or DNA damage, p21and p27 bind to cyclin-CDK complexes to inhibit their catalytic activity and induce cell cycle arrest[27].

There was a dose dependent increase in the expression of P21 with DOX and MET in both cell lines .Additionally, there was a potentiation in expression of p21 after the application of combination (DOX with MET). In the present study P21 consider as indicator of cell death by inhibition of proliferation and cell cycle arrest after application of DOX (1.25, 2.5, 5, 10, 20 and 40µM) through highly expression of p21 from the inner to the outer membrane of both cancerous cells. That is refers to the ability of DOX to inhibit proliferation of cells due to again strictly dependent on FHL2 expression the protein responsible for an up- regulation of p21in cancer cells in response to DOX treatment [28] .

Due to the expression of p21 impedes DOX-induced cell death by inactivation of cyclin-dependent kinase (CDK) activity, which in turn blocks the cell cycle at the G1 and G2 phases . The present findings here reinforced this idea by showing p21's ability of abrogating DOX-induced cell death correlated with its inhibition of cell cycle progression after reducing p65 in p53 cells[29].

The previous study showed that MET, also , have the ability to produces dose dependent increase in the expression of P21 to the extracellular membrane in both cancerous cells that is refer to the MET caused cell cycle arrest accompanied by decreased cyclin D1 and increased p21 protein expression also MET can indirectly shows antiproliferative effects as well as directly suppress cell proliferation through cell cycle modulation, up regulation of tumor suppressor genes and by inducing cell death mediated by increased oxidative stress[30] .

The combination of DOX with MET treatment shows enhanced effect in a dose dependent that refers to ability of combination to cause dysfunction of cell cycle checkpoint proteins can alter the progression of the cell cycle also dysfunction the G1/S phase proteins cyclin D1, CDK4 and

p21. Cyclin D1 and CDK4, which are responsible for the transition from G0/G1 phase to S phase, were down regulated following combined treatment[31].
Additionally, p21, a cyclin-dependent kinase (CDK)inhibitor, was obviously induced following combined treatment thus; these results demonstrated that the combined treatment group arrested cancer cells in the G0/G1 phase[32].

CONCLUSION

From the results above, it can be concluded that metformin have a good cytotoxic and antiproliferative effect on tumor cell lines. Met showed a valuable cytotoxic effect through the detection of IC_{50} , annexin V and p21 expression .

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Conflict of interest

The authors report no conflicts of interest in this work.

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