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# POTENTIATION OF ANTI-PROLIFERATIVE EFFECT OF DOXORUBICIN USING METFORMIN AND HESPERIDINON HUMAN BREAST CANCER CELL LINE MDA-MB-468

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**Keywords:**

Breast cancer, Doxorubicin, Hesperidin, Metformin, TNBC

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**ABSTRACT:** Among various subtypes of breast cancer, triple-negative breast cancer (TNBC) is aggressive breast cancer. Doxorubicin is the standard therapy used for treating TNBC, but poses a risk of severe adverse effects affecting patient compliance. Hence, there is a need for therapy that reduces the adverse effect and increase compliance for better overall pathological complete response (pCR). Metformin, widely used as an anti-diabetic, has gained momentum for its preventive potential and delaying various cancers, including breast cancer, owing to its high safety and better tolerability. Hesperidin, commonly found in citrus fruits, is an integral part of daily diet and consumed worldwide. The objective of the current study was to investigate the potentiation of anti-proliferative effect of doxorubicin using metformin and hesperidinon MDA-MB-468 human breast cancer cell line. The IC50 concentration of doxorubicin (1 µM) was combined with non-toxic doses of metformin (0.5, 1, and 2 mM) and hesperidin (5, 10, and 20 µM). Metformin (1 mM) and hesperidin (5 µM) in combination with doxorubicin (1 µM) inhibited the migration of MDA-MB-468 cells in a scratch assay. In Annexin V-FITC assay, a combination of doxorubicin, metformin, and hesperidin exhibited an increase in cells in late and early apoptosis phase.Further, when cell cycle analysis was performed, cells shifted from G0/G1 phase to S phase, ultimately leading to the arrest of cells in S phase. Hence, the results are conclusive of potentiation of the anti-proliferative effect of doxorubicin using metformin and hesperidin on human breast cancer cell line MDA-MB-468.

**INTRODUCTION:** Breast cancer (BC) is the leading cause of cancer, accounting for 2.3 million cases worldwide [1]. Triple-negative breast cancer (TNBC) is the most aggressive cancer among various breast cancer subtypes. TNBC accounts for 10-20 % of cases of BC [2].

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Chemotherapy is the main treatment for treating TNBC, and doxorubicin, combined with other chemotherapeutic agents, is widely used as first line therapy for treating breast cancer [3]. Even though doxorubicin has better overall pathological complete response (pCR), there is a risk of severe adverse cardiotoxicity effects that limit its use [4].

Hence, there is a need for therapy that can reduce the risk of severe adverse effect of doxorubicin, thereby potentiating the anti-proliferative effect of doxorubicin. Metformin, an oral antidiabetic drug, showed a reduced risk of breast cancer along with reduced cancer-related mortality in diabetic and

non-diabetic patients [5]. Metformin displayed better pCR irrespective of breast cancer patients' diabetic or non-diabetic status [6]. AMPK activation by metformin leads to inhibition of mTOR and ROS generation, ultimately inhibiting protein synthesis thereby controlling cancer cell proliferation [7]. An *in-vitro* study on the MCF-7 cell line demonstrated the anti-proliferative effect of metformin through apoptosis and cell cycle arrest via oxidative stress and AMPK stimulation, ultimately leading to FOXO3a activation, responsible for tumor suppression [8]. Metformin by binding to complex 1 of the mitochondrial respiratory chain, inhibited ROS (reactive oxygen species) generation, which was increased in various cancers [9]. Metformin causes inhibition of the initial triggering inflammatory response through NF-κB inhibition [10].

Hesperidin, a bioflavonoid obtained from citrus fruits, is known for its antioxidant activity, anti-inflammatory [11], analgesic [12], vasorelaxant [13], antioxidant and anticarcinogenic activity [14]. Hesperidin also possesses hypoglycemic, hypolipidemic [15], antiviral, antibacterial, antifungal, antiparasitic and antiallergic activity, hepatoprotective and neuroprotective activity [16, 17]. Hesperidin revealed a cytotoxic effect in an *in-vitro* study on MCF-7 cell lines through DNA damage and increased apoptotic protein expression (p53 and caspase-3) ultimately leading to apoptosis [18]. A study involving MCF-7 and T47D cells showed a cytotoxic and synergistic effect of hesperidin, piperine, and bee venom in combination with tamoxifen. There was a significant increase and decrease in the mRNA levels of Bax and Bcl2, respectively, along with cell cycle arrest at G0/G1 phase observed [19]. Moreover, hesperidin showed a cytotoxic effect on doxorubicin-resistant MCF-7 breast cancer cell line (MCF-7/Dox) along with a synergistic effect when given in combination with doxorubicin [20]. Hesperidin ameliorated the doxorubicin-induced cardiotoxicity in rats [21].

In the light of these reports, metformin, and hesperidin when combined with doxorubicin as combinatorial therapy, may potentiate the anti-proliferative effect of doxorubicin. This combinatorial therapy may further reduce the severe adverse effects of doxorubicinin the clinical setting. To the best of our knowledge, no previous study has been carried out to evaluate the effect of combinatorial therapy of doxorubicin, metformin and hesperidin on human breast cancer cell line MDA-MB-468. The objective of the current study is to evaluate the potentiation of anti-proliferative effect of doxorubicin using metformin and hesperidin on human breast cancer cell line MDA-MB-468.

## MATERIAL AND METHODS:

**Cell lines and Chemicals:** Breast cancer cell line MDA-MB-468 was procured from National Centre for Cell Sciences (NCCS), Pune. All the consumables and chemicals were procured from HiMedia®. Doxorubicin (Dox) was received as a generous gift sample from Sun Pharmaceuticals Ltd., Vadodara, and metformin (Met) was received as a gift sample from Abhilasha Pharma Pvt. Ltd., Ankleshwar. Hesperidin (Hes) (~95%) was purchased from Spectrochem Pvt. Ltd. Doxorubicin and metformin were prepared by dissolving in sterile distilled water and then in a complete growth medium. Hesperidin was dissolved in 1 % DMSO and then suspended in a complete growth medium.

**Cell Culture:** MDA-MB-468 cell lines were cultured in complete growth media consisting of Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% antibiotic-antimycotic solution in 37 ± 0.5°C and 5 % $CO_2$.

**Cell Viability Assay:** For cytotoxicity assay, 2.5 x $10^4$ viable cells of MDA-MB-468 per well in 200µl were seeded in 96 well plates. Plates were incubated at 37°C for 24 hours in a 5% $CO_2$ incubator. After 24 hrs, cells were treated with 200 µl of different concentrations of the Dox, Met, and Hesintriplicates. Plates were incubated in a 5% $CO_2$ incubators at 37°C for 24 and 48 hours. After 24 and 48 hrs, the cDMEM was discarded, and fresh cDMEM was added. To each well 10µlof MTT (5 mg/mL) was added to a final concentration of 0.5 mg/ml. Plates were then again incubated for 4 hours at 37°Cin a 5% $CO_2$incubator until the form azancrystals are formed that could be seen under the microscope. cDMEM was decanted gently without disturbing form azancrystals, and 100µl of DMSO was added per well to solubilize the crystals and incubated for 10 minutes in the dark, followed by measuring absorbance at 570nm in a multi-

mode plate reader [22]. The percentage (%) cell viability was calculated using the formula:

Percentage cell viability = (Absorbance of treated cells/absorbance of control cells) ×100 %

Where, the absorbance of treated cells is the absorbance obtained after subtracting the average absorbance of test concentration of drug from the absorbance of blank wells. Using the absorbance values of different drugs, $IC_{50}$valueswere calculated for Dox, Met, and Hes using Graphpad Prism.

**Scratch Assay:** For scratch assay (wound healing assay), 2000 µL containing 2 × $10^6$ cells were seeded in each well of 6-well plates and cultured overnight. Plates were incubated for 24 hrs at 37°C in a 5% $CO_2$ incubator. After 48 hrs, the scratch was made gently and slowly on the monolayer with a new sterile pipette tip (10 µL) across the center of the well. The cDMEM containing 2 % FBS was aspirated, and wells were washed twice with PBS to remove any detached cells. After washing, images were captured at 0 hr with the help of an inverted phase-contrast microscope (ECLIPSE Ts2, Nikon). The scratched cells were treated with different concentrations of Dox, Met and Hes for 24 and 48 hrs. An inverted microscope captured images at 0, 24, and 48 hrs [23]. The width of the scratch was measured, and relative migration (%) was analyzed by determination of the migration rate of cells using the following formula:

Relative migration (%) = (Distance at 0 hr-Distance at 24/48 hrs) / Distance at 0 hr x 100 %

**Annexin-V-FITC/PI Assay:** MDA-MB-468 cells were seeded in 6-well plates at a density of 5x$10^5$ cells per well and incubated for 24 hrs at 37°C in a 5% $CO_2$ incubator. After incubation, cells were treated with different concentrations of Dox, Met and Hes were added to the wells, and plates were incubated again for 24 and 48 hrs at 37°C in a 5% $CO_2$ incubator. After incubation, cells were trypsinized, centrifuged at 1000 rpm for 5 min, and washed with PBS. Each sample cell pellet was resuspended in a 200 µL 1X binding buffer containing 5 µL annexin-V-FITC and incubated at room temperature for 10 min in the dark. Then each sample was washed with 1X binding buffer by centrifugation. Again the cell pellet was resuspended into 200 µL 1X binding buffer containing 10 µl PI and incubated at room temperature in the dark [24]. After incubation, cells were analyzed by flow cytometer (BD FACSAria™ Fusion, BD biosciences). The distribution of live cells and cells undergoing early apoptosis, late apoptosis, and necrosis were determined by flow cytometry analysis.

**Cell Cycle Analysis:** MDA-MB-468 cells were seeded in 6-well plates at a density of 5x$10^5$ cells per well and incubated for 24 hrs at 37°C in a 5% $CO_2$ incubator. After incubation, cells were treated with different concentrations of Dox, Met and Hes and plates were incubated again for 24 and 48 hrs at 37°C in a 5% $CO_2$ incubator. After incubation, cells were trypsinized, centrifuged at 1000 rpm for 5 min, and washed with cold PBS. The cell pellet was fixed by adding chilled 90% methanol dropwise to the sample while vortexing. Fixed cells were kept at 4°C overnight and then washed with PBS at 850 x g in a centrifuge and discard the supernatant carefully. Again the cell pellet was resuspended in 500 µL PBS with 5 µL RNase and 50 µg/ml propidium iodide and incubated at room temperature in dark for 30 min. Further, the samples were analysed through a flow cytometer to measure the forward scatter (FS) and side scatters (SS) to identify single cells. Forward and side scatter analysis was used to identify the cells [25]. The gates were combined applied to the PI histogram plot. Using the markers set within the analysis program, the percentage of cells in each cell cycle phase was quantified.

**Statistical Analysis:** All the values were expressed as mean± standard error mean (SEM). Statistical analysis was performed using GraphPad Prism (Version 6.0 for Windows, Graph Pad Software Inc., San Diego, California, USA). One-way analysis of variance (ANOVA) was performed for comparisons between groups, followed by Dunnett's multiple comparison tests as a post hoc analysis. $P<0.05$ was considered to be statistically significant.

## RESULTS:

**Cell Viability Assay of Doxorubicin and Hesperidin on Human Breast Cancer Cell Lines MDA-MB-468:** The cell viability assay was performed on MDA-MB-468 cells to determine the $IC_{50}$ values of doxorubicin, metformin and

hesperidin. On MDA-MB-468 cells, the $IC_{50}$ value of doxorubicin was found to be 0.95 µM, metformin 20.48 µM and for hesperidin was greater than 320 µM that could not be determined at 24 hrs.

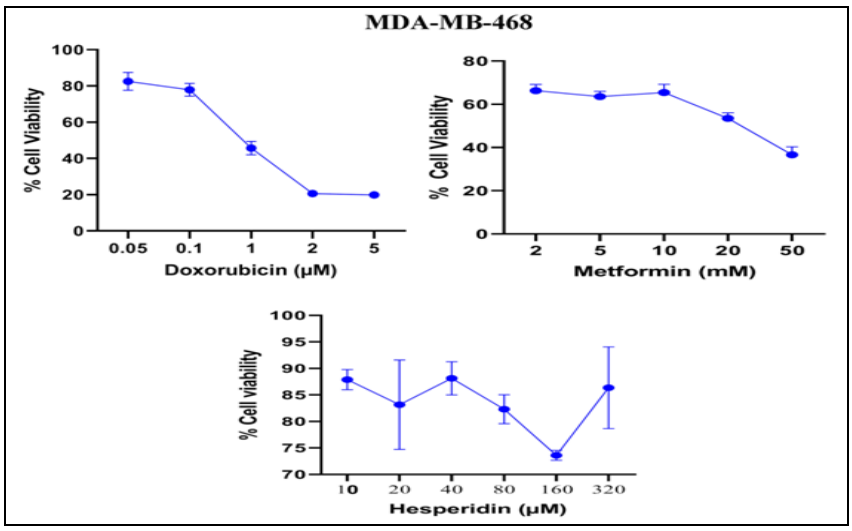


**FIG. 1: EFFECT OF DOXORUBICIN, METFORMIN AND HESPERIDIN ON % CELL VIABILITY OF MDA-MB-468**

Based on the cell viability assay results, $IC_{50}$ concentration of doxorubicin 1 µM and half the dose of $IC_{50}$ *i.e.*, 0.5 µM was used for further assays on MDA-MB-468. In the case of metformin, non-toxic and lower doses 0.5, 1 and 2 mM were taken for further assays. Hesperidin at the doses of 5, 10 and 20 µM was evaluated for further assay. The doses selected for metformin and hesperidin were based on the hypothesis of combining non-toxic and low doses of metformin and hesperidin with doxorubicin for evaluating potential anti-proliferative and synergistic effect.

**Anti-proliferative Effect of Doxorubicin, Metformin, and Hesperidin on MDA-MB-468:**

**Scratch Assay:** Scratch assay was performed to evaluate migratory potential on MDA-MB-468 of doxorubicin, metformin, and hesperidin at 24 and 48 hrs. The analysis of scratch assay involved Mosaic 2.1 software for calculating scratch width for images. **Fig. 2** represents the effect of doxorubicin, metformin, and hesperidin on inhibition of % relative migration of MDA-MB-468 using scratch assay at 24 and 48 hrs.

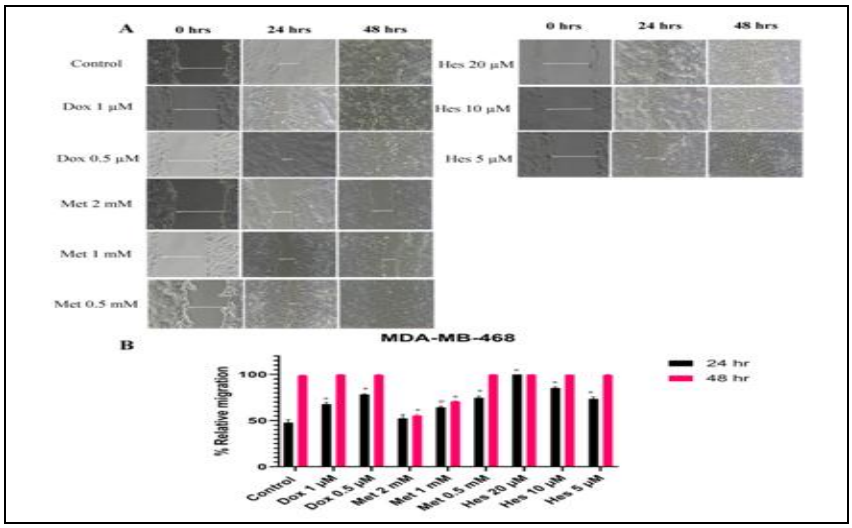


**FIG. 2: EFFECT OF DOXORUBICIN, METFORMIN AND HESPERIDIN ON INHIBITION OF % RELATIVE MIGRATION OF MDA-MB-468 USING SCRATCH ASSAY AT 24 AND 48 HRS.** (A) Representatives images of scratch captured using inverted microscope. (B) Inhibition of percentage (%) relative migration. The results are obtained from 3 independent experiments and values are expressed as Mean±SEM. @p<0.05, $p<0.01, #p<0.001 and *p<0.0001 are considered statistically significant when compared with control.

In the case of MDA-MB-468, doxorubicin at a concentration of 1 and 0.5 µM inhibited percentage relative migration at 24 hrs compared to control cells. Moreover, metformin inhibited percentage relative migration in a dose-dependent manner. Hesperidin was not able to inhibit percentage relative migration. Moreover, metformin showed a protective effect on migration and inhibited % relative migration at 48 hrs in a dose-dependent manner. However, doxorubicin at 0.5 µM and hesperidin (20, 10 and 5 µM) were not able to inhibit % relative migration of MDA-MB-468 at 48 hrs. Hence doxorubicin, metformin (except at 2 mM) and hesperidin were not having any inhibitory effect on cell migration of MDA-MB-468 as compared to control.

**Annexin-V/FITC Assay:** Annexin-V/FITC assay was performed to evaluate the effect of drugs to induce apoptosis in MDA-MB-468 using flow cytometry. A percentage (%) apoptotic cells was calculated as the total of late and early apoptotic cells. For statistical analysis, all the cells treated with drugs were compared with the control cells. In the case of MDA-MB-468, doxorubicin 1 and 0.5 µM treated cells showed a significant ($p<0.0001$) increase in the % apoptotic cells as compared to the control cells at 24 and 48 hrs. Metformin at a dose of 2 and 1 mM showed a significant increase in the % apoptotic cells in a dose-dependent manner at both 24 and 48 hrs. Moreover, hesperidin 20 and 10 µM and 5 µM treated cells did not show apoptotic cells at 24 and 48 hrs.

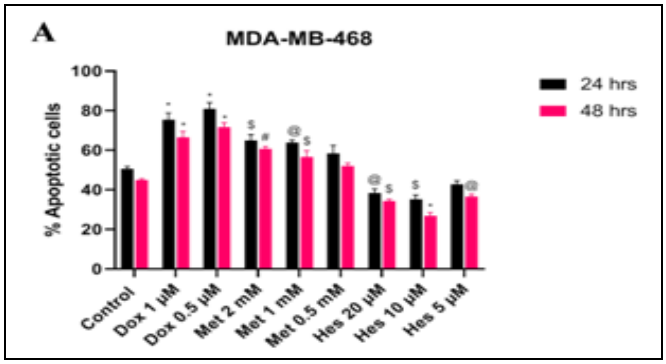


**FIG. 3: EFFECT OF DOXORUBICIN, METFORMIN AND HESPERIDIN ON % APOPTOTIC CELLS OF (A) MDA-MB-468 AND USING ANNEXIN-V/FITC ASSAY AT 24 AND 48 HRS**. % Apoptotic cells were calculated as a total percentage of cells in late apoptosis and early apoptosis. The results are obtained from 3 independent experiments and values are expressed as Mean±SEM. @$p<0.05$, \$$p<0.01$, #$p<0.001$ and *$p<0.0001$ are considered statistically significant when compared with control.

**Cell Cycle Assay:** Cell cycle analysis was performed to evaluate the effect of doxorubicin, metformin, and hesperidin. The control untreated cells were classically into maximal G0/G1 or resting phase, minimally into S phase, and residual G2/M phase or mitotic phase for both MDA-MB-468. In the case of MDA-MB-468 at 24 hrs, doxorubicin treated cells at a dose of 1 and 0.5 µM showed a significant ($p<0.0001$) decrease in the cells in G0/G1 phase along with the significant shift of cells arresting in S phase ($p<0.0001$) and G2/M phase ($p<0.01$, $p<0.05$) was observed in doxorubicin 1 and 0.5 µM compared to control cells.

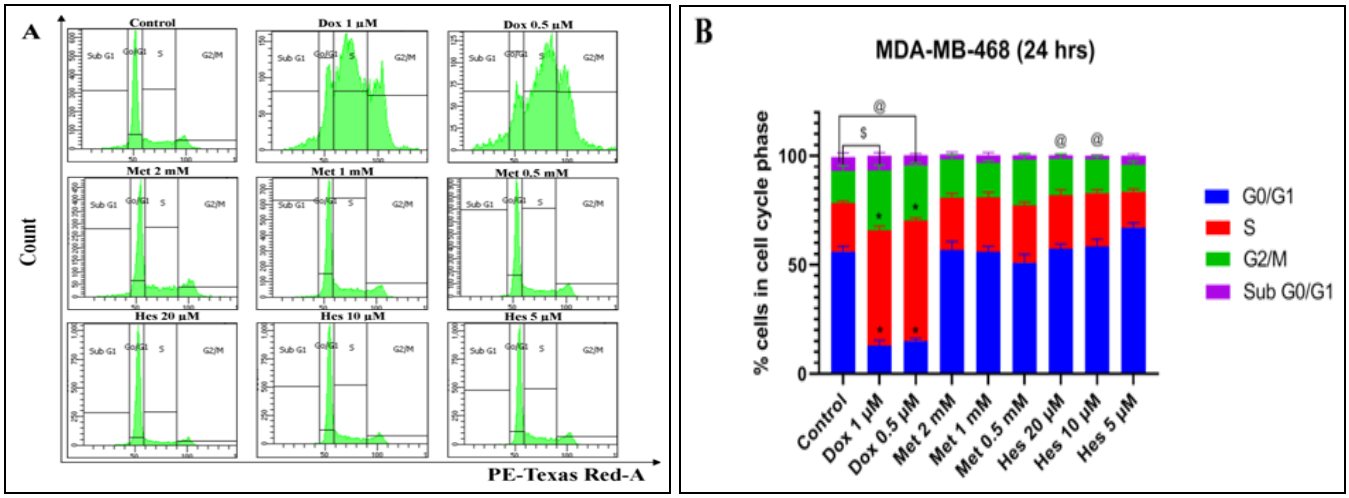


**FIG. 4: EFFECT OF DOXORUBICIN, METFORMIN AND HESPERIDIN ON % CELLS IN CELL CYCLE PHASE OF MDA-MB-468 AT 24 HRS.** (A) Representatives images of cell cycle analysis. (B) Graphical representation of % cells in different cell cycle phases. The results are obtained from 3 independent experiments and values are expressed as Mean±SEM. @$p<0.05$, \$$p<0.01$, #$p<0.001$ and *$p<0.0001$ are considered statistically significant when compared with control.

Metformin-treated cells at a concentration of 20,10, and 5 mM was not able to affect the cells in any cell cycle phase. However, hesperidin at a concentration of 20 and 10 µM showed a significant ($p<0.05$) decrease in the cells from sub-G0/G1 phase of cell cycle at 24 hrs.

In the case of 48 hrs treatment on MDA-MB-468, there was a significant ($p<0.0001$) decrease in the cells in G0/G1 phase of the cell cycle in doxorubicin (1 and 0.5 µM) as compared to the control cells. Moreover, a significant shift of cells into S phase and G2/M phase was observed for doxorubicin 1 and 0.5 µM treated cells. Metformin (2, 1 and 0.5 mM) treated cells did not show a significant change in cells in cell cycle arrest compared to the control cells.

Hesperidin 20 µM treated cells at 48 hrs showed a significant ($p<0.05$) decrease in the cells in G0/G1 phase of cell cycle compared to control untreated cells at 48 hrs. Hesperidin 10 and 5 µM treated cells also showed no changes in cell cycle arrest at 48 hrs.

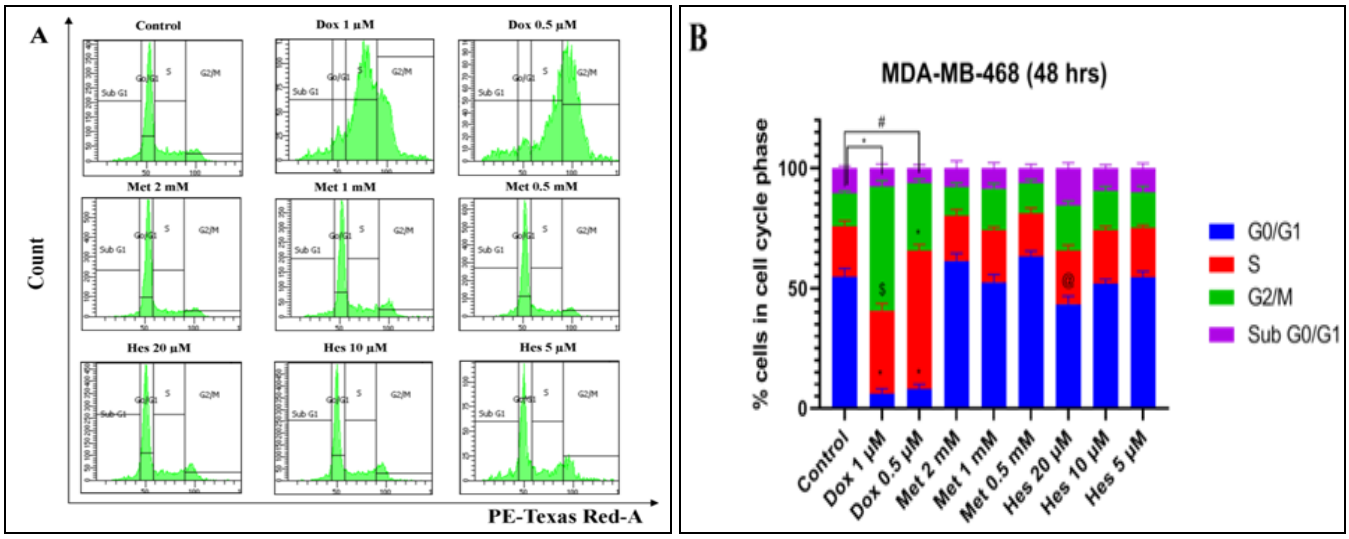


**FIG. 5: EFFECT OF DOXORUBICIN, METFORMIN AND HESPERIDIN ON % CELLS IN CELL CYCLE PHASE OF MDA-MB-468 AT 48 HRS.** (A) Representatives images of cell cycle analysis. (B) Graphical representation of % cells in different cell cycle phases. The results are obtained from 3 independent experiments and values are expressed as Mean±SEM. @p<0.05, $p<0.01, #p<0.001 and *p<0.0001 are considered statistically significant when compared with control.

**Synergistic Anti-proliferative Effect of Hesperidin in Combination with Doxorubicin on MDA-MB-468:** Based on the results obtained from scratch, apoptosis and cell cycle analysis assay, doxorubicin at an $IC_{50}$ dose was selected for combination with metformin and hesperidin to evaluate the synergistic anti-proliferative effect on MDA-MB-468.

The MDA-MB-468 cells were treated with the combination of doxorubicin 1 µM, metformin 1 mM and hesperidin 5 µM. The concentration of doxorubicin 0.6 µM, metformin 0.5 mM and hesperidin 5 µM was selected based on the rationale to combine $IC_{50}$ value of doxorubicin with non-toxic low dose of metformin and hesperidin for potential synergistic anti-proliferative effect.

**Scratch Assay:** The scratch shown in **Fig. 5** represents combination of doxorubicin, metformin and hesperidin on inhibition of % relative migration of MDA-MB-468 cells.

The combinatorial effect of drugs on MDA-MB-468 cells was a significant decrease in the % relative migration at 24 hrs with a combination of doxorubicin 1 µM with metformin 1 mM, doxorubicin 1 µM with hesperidin 5 µM, metformin 1 mM and hesperidin 5 µM and doxorubicin 1 µM, metformin 1 mM and hesperidin 5 µM as compared to the control cells.

A significant ($p<0.0001$) decrease in % relative migration was observed for doxorubicin 1 µM with metformin 1 mM, doxorubicin 1 µM with hesperidin 5 µM, metformin 1 mM and hesperidin 5 µM and doxorubicin 1 µM, metformin 1 mM and hesperidin 5 µM at 48 hrs as compared to the control cells at 48 hrs.

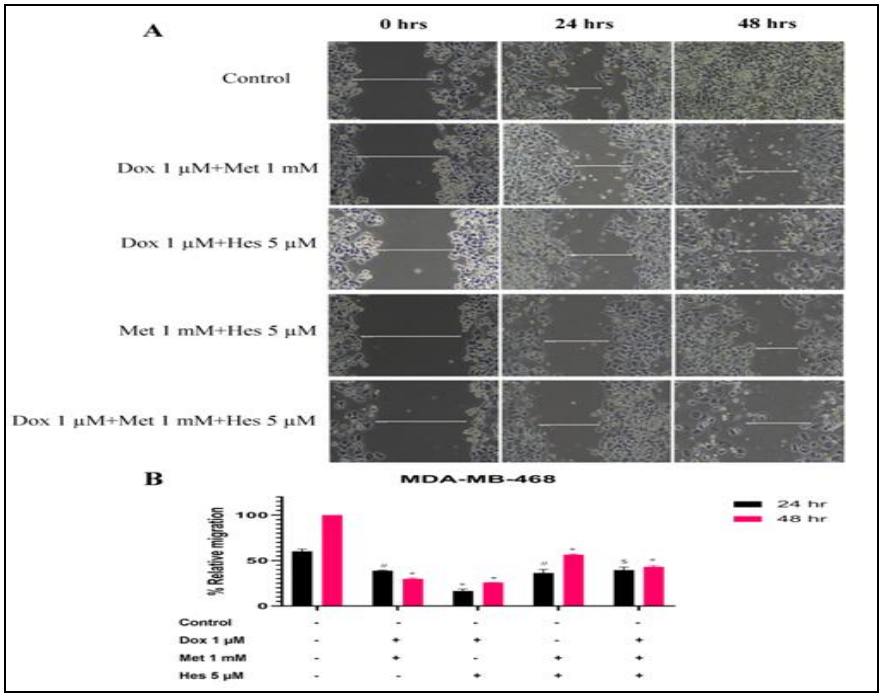


**FIG. 6: EFFECT OF COMBINATION OF DOXORUBICIN, METFORMIN AND HESPERIDIN ON INHIBITION OF % RELATIVE MIGRATION OF MDA-MB-468 USING SCRATCH ASSAY AT 24 AND 48 HRS**. (A) Representatives images of scratch captured using inverted microscope. (B) Inhibition of percentage (%) relative migration. The results are obtained from 3 independent experiments and values are expressed as Mean±SEM. @p<0.05, $p<0.01, #p<0.001 and *p<0.0001 are considered statistically significant when compared with control.

**Annexin-V FITC Assay: Fig. 6** represents the effect of different doxorubicin, metformin and hesperidin on % apoptotic cells of MDA-MB-468 cells. The MDA-MB-468 cells were treated with doxorubicin 1 µM, metformin 1 mM and hesperidin 5 µM showed significant increase (p<0.0001) in the % apoptotic cells as compared to the control cells at 24 and 48 hrs.

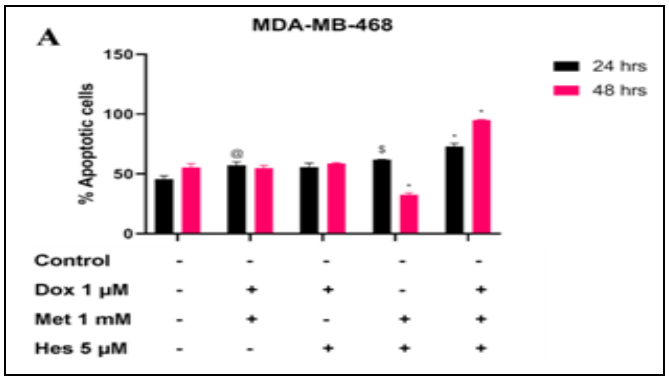


**FIG. 7: EFFECT OF COMBINATION OF DOXORUBICIN, METFORMIN AND HESPERIDIN ON % APOPTOTIC CELLS OF MDA-MB-468 USING ANNEXIN-V/FITC ASSAY AT 24 AND 48 HRS.** % Apoptotic cells were calculated as total percentage of cells in late apoptosis and early apoptosis. The results are obtained from 3 independent experiments and values are expressed as Mean±SEM.@p<0.05,$p<0.01,#p<0.001 and *p<0.0001 are considered statistically significant when compared with control.

**Cell Cycle Assay:** The cell cycle shown in figure 4.13 and 4.14 represents the effect of the combination of doxorubicin, metformin, and hesperidin on % cells in the cell cycle phase of MDA-MB-468 at 24 hrs and 48 hrs, respectively. MDA-MB-468 cells treated with doxorubicin 1 µM, metformin 1 mM and hesperidin 5 µM showed a significant decrease in the cells in G0/G1 phase for combination groups involving as compared to the control. A significant (p<0.01, p<0.001, p<0.001) increase in the % cells in the S phase, indicating arrest of cells in the S phase, was observed for doxorubicin 1 µM and metformin 1 mM, doxorubicin 1 µM and hesperidin 5 µM and doxorubicin 1 µM, metformin 1 mM and hesperidin 5 µM as compared to the control at 24 hrs. Moreover, there was a significant (p<0.0001) increase in the cells in sub G0/G1 phase of cell cycle was observed during the treatment of doxorubicin 1 µM, metformin 1 mM and hesperidin 5 µM at 24 hrs as compared to the control. Doxorubicin 1 µM, metformin 1 mM, and hesperidin 5 µM treated cells showed a significant decrease (p<0.001) in cells in G0.G1 phase and a significant (p<0.05, p<0.001) increase in cells arresting in G2/M phase and sub G0/G1 phase of cell cycle as compared to the control at 48 hrs.

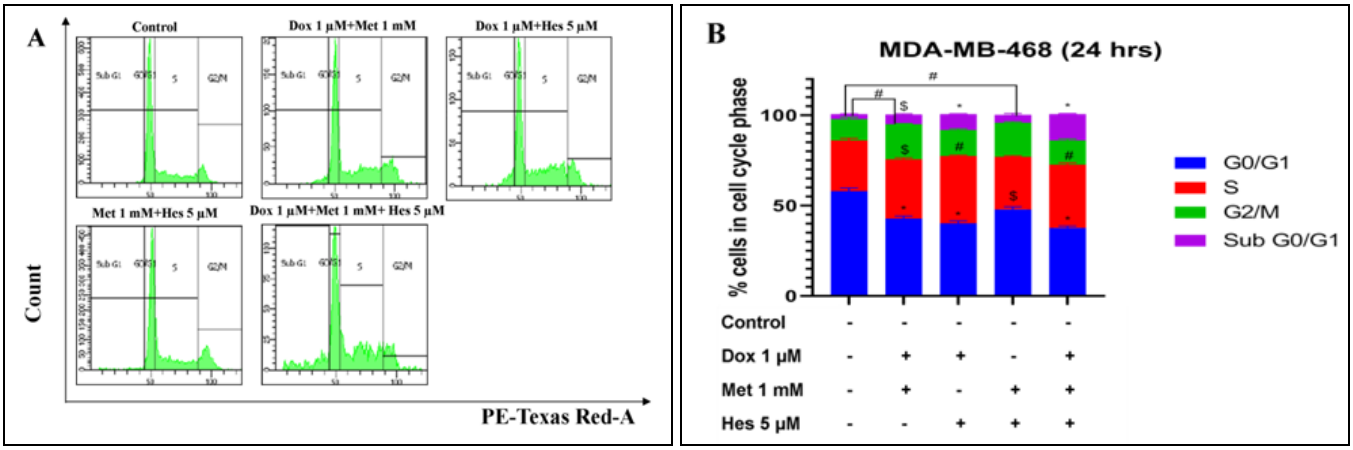


**FIG. 8: EFFECT OF COMBINATION OF DOXORUBICIN, METFORMIN AND HESPERIDIN ON % CELLS IN CELL CYCLE PHASE OF MDA-MB-468 AT 24 HRS.** (A) Representatives images of cell cycle analysis. (B) Graphical representation of % cells in different cell cycle phases. The results are obtained from 3 independent experiments and values are expressed as Mean±SEM. @p<0.05, $p<0.01, #p<0.001 and *p<0.0001 are considered statistically significant when compared with control.

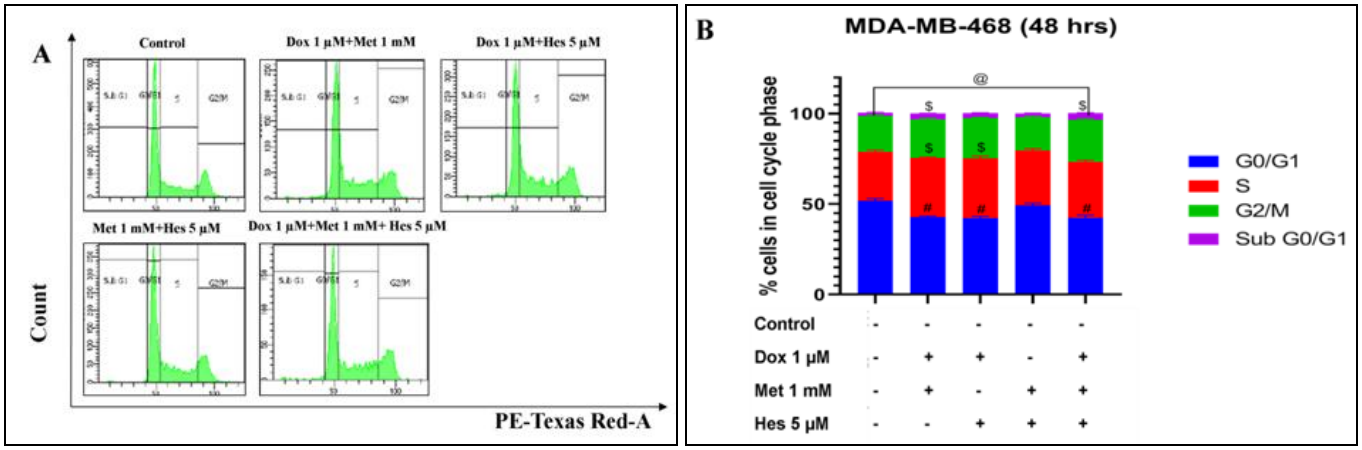


**FIG. 9: EFFECT OF COMBINATION OF DOXORUBICIN, METFORMIN AND HESPERIDIN ON % CELLS IN CELL CYCLE PHASE OF MDA-MB-468 AT 48 HRS.** (A) Representatives images of cell cycle analysis. (B) Graphical representation of % cells in different cell cycle phases. The results are obtained from 3 independent experiments and values are expressed as Mean±SEM. @p<0.05, $p<0.01, #p<0.001 and *p<0.0001 are considered statistically significant when compared with control.

**DISCUSSION:** Looking at the data of GLOBOCAN for breast cancer incidence and mortality, there is a need to search for therapy that effectively controls cancer proliferation and reduces mortality. According to WHO, cancer incidence and mortality can be prevented by modifying or avoiding risk factors, early diagnosis, and management of BC patients [26]. Despite advancements, current chemotherapy used in the treatment of BC has several serious adverse effects, leading to poor compliance and discontinuation of the treatment [27]. TNBC is the most aggressive cancer among various breast cancer subtypes. Chemotherapy is the standard treatment for TNBC, but there is a poor overall survival rate and complete pathological response (pCR) [28]. However, in TNBC patients, anthracycline-based therapies showed higher pCR and overall survival. Doxorubicin is the most commonly used anthracycline for breast cancer treatment with higher response rates, but cardiotoxicity limits its use [29]. Metformin, an oral antidiabetic drug, has a high safety profile and is known to possess anti-proliferative action. Hesperidin, on the other end, is commonly found in citrus fruits consumed daily worldwide and possesses potent antioxidant activity. The present study aims to evaluate the effect of metformin and hesperidin in combination with doxorubicin on human breast cancer cell lines. Cytotoxic potential of doxorubicin, metformin, and hesperidin was performed using MTT assay to find out $IC_{50}$ concentration of doxorubicin, metformin, and hesperidin. $IC_{50}$ concentration for doxorubicin, metformin, and hesperidin was found to be 0.95

µM (≈ 1 µM), 20.48 µM, and greater than 320 µM that could not be determined at 24 hrs. $IC_{50}$ concentration of doxorubicin (1 µM) and its half dose (0.5 µM) was used for further assays to be combined with the non-toxic doses of metformin (0.5, 1 and 2 mM) and hesperidin (5, 10 and 20 µM). Wound healing assays are employed to estimate cell proliferation and migration rates of cells [30]. Doxorubicin, metformin, and hesperidin were evaluated individually using scratch assay; migration rates were higher, and complete scratch closure was observed. However, when the MDA-MB-468 cells were treated with a combination of doxorubicin, metformin, and hesperidin, migration rates of cells were decreased compared to the control.

Annexin V/FITC assay estimates the number of cells that undergo apoptosis. This method discriminate to discrimination between viable, apoptosis, and necrotic cells [31]. When individual drugs were evaluated for % of apoptotic cells, it was observed that at 24 and 48 hrs the number of apoptotic cells was more compared to the control cells. However, when a combination of all the three drugs was evaluated for % apoptotic cells, more cells were in the apoptotic phase than that control cells. This indicates that the cells treated with combination of doxorubicin, metformin and hesperidin showed higher apoptosis compared to control cells. Cell-cycle analysis by flow cytometry utilizes the change in DNA content through the stages to identify cells in different stages of division at a single cell level [25]. Doxorubicin treated cells showed shift of cells from G0/G1 phase to S phase and leading to the arrest of cells in S phase of cell cycle. Whereas metformin and hesperidin treated cells showed no change in cells in the cell cycle phase. When given in combination the doxorubicin, metformin and hesperidin showed a significant decrease in cells in G0/G1 phase and an increase in cells in sub G0/G1 phase, indicating the cell cycle arrest in sub G0/G1 phase of the cell cycle.

**CONCLUSION:** Our results suggest the potentiation of the anti-proliferative effect of doxorubicin using metformin and hesperidin on the MDA-MB-468 cell line. When non-toxic doses of metformin and hesperidin were combined with doxorubicin, it showed antiapoptotic effect, decreased relative migration, and shift of MDA-MB-468 cells from G0/G1 to S phase of cell cycle. The exact molecular mechanism is yet to be evaluated for a better understanding of the potentiation of the anti-proliferative effect. Thus, metformin and hesperidin can be used with doxorubicin as adjuvant therapy, and further clinical studies are warranted to confirm the same.

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**CONFLICTS OF INTEREST:** The authors declare that they have no competing interests.

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