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Original Article

Apoptosis induction, cell cycle arrest, and tumor genes expression analysis of tamoxifen and ivermectin loaded chitosan nanoparticles against MCF-7 Cell line

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Abstract

One of the most dominant diseases in the world, particularly among women, is breast cancer. Breast cancer has tumor suppressor genes called *CHEK2* and *TP53*. When there is a mutation in *CHEK2* and *TP53* genes there are more chances of breast cancer. This study aimed to investigate the already prepared and characterized nanoparticles loaded with Chitosan for Cell death, Mitochondrial Membrane and cell cycle arrest estimated through Flow Cytometry and gene expression analysis of *CHEK2* and *TP53* genes by real-time PCR. The Livak method was used to evaluate the results. The mean (± S.D) comparison between the control and target genes were used to calculate gene expression. Results showed that Ivermectin and Tamoxifen NPs (B+C) represented 34.8% cell death that is better than other combinations with propidium iodide stain while with Acridine orange stain Tamoxifen+Ivermectin (A+B) combination showed the remarkable and maximum of the all cell cycle arrest with value of 69.7% cell arrest at G0/G1 phase, 7.11% of cell arrest at S Phase and 7.05% of G2/M Phase arrest. It was demonstrated that the expression levels of *CHEK2* and *TP53* genes were significantly increased ($P<0.001$) in Ivermectin+Tamoxifen NPs (B+C) compared with control groups. It is concluded that Tamoxifen nanoparticles with Ivermectin showed strong anti-proliferative activity against breast cancer cells. The expression levels of nanoparticles containing Tamoxifen were significantly increased compared to the other treatments and control groups ($P<0.001$). Gene expression change with change in dose concentrations.

Keywords: Breast cancer, Apoptosis, Cell cycle arrest, Pharmacogenomic, Gene expression

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Introduction

The primary cause of the events leading to cancer is disruption of the regular functioning of the cell cycle. Cancer is classified as the excessive growth and division of atypical cells. The uncontrolled metastasis leads to mortality and about two billion people affected by breast cancer, especially women, in 2018 (Wisesty et al., 2020). The lobules, ducts, and milk glands in the inner layers are most affected by breast cancer (Ataollahi et al., 2015). Understanding the gene expression profile of cancer cells is crucial for understanding the biological changes involved in disease development, finding new potential markers, predicting clinical outcomes, developing personalized pharmacological treatments for patients, and researching the molecular effects of drug exposure in an effort to increase treatment effectiveness (Rudloff et al., 2010). BRCA1/2, CHEK2 (cell cycle checkpoint kinase 2), and TP53 are a few of the breast cancer susceptibility genes that have been identified and are thought to be important in the response to DNA damage (Gutiérrez- Enríquez et al., 2014). A wide range of PCR arrays are currently accessible for the research of expression analysis in gene changes linked to hundreds of biological pathways. Since real-time PCR may provide information on tiny changes such as point mutations, expression analysis of genes, loss of genes, or gene strengthening, it is crucial for clinical testing. It is also helpful for looking at cancer markers (Mitas et al., 2001). Unfortunately, according to our experience, a lot of these RT-PCR arrays include technical issues that can make a gene expression analysis less successful. CHEK2 has become an important breast cancer susceptibility gene after the identification of BRCA1/2. Numerous investigations have revealed the critical roles that CHEK2 plays in the regulation of DNA repair, the cell cycle, and apoptosis (Gutiérrez- Enríquez et al., 2014). Cell cycle G1/S or G2/M phase arrest is thought to be caused by CHEK2, a crucial DNA double-strand break signaling protein.

The tetrameric transcription factor of the tumor suppressor gene is adaptable. Wild-type TP53 plays a crucial role in maintaining genomic stability, which is required for senescence, control of cell cycle progression, DNA repair, and apoptosis (Sharma et al., 2014).

The current investigation was a follow-up to the findings of a prior work that produced and analyzed chitosan carriers carrying both tamoxifen and ivermectin. Thus, in order to ascertain the apoptosis, we assessed the effects of these medications on breast cancer cells and cell cycle arrest by using MCF-7 cell line and tumor gene expression analysis by using CHEK2 and TP53 as compared to the normal tissue by using the real-time PCR technique.

Material and Methods

Cell death, mitochondrial membrane and cell cycle analysis through Flow Cytometry Propidium Iodide and Acridine Orange Staining Assay

Reconstitution of media

In 1 L of distill water, 12.5 g of GMEM (Glasgow Minimal Essential Media) (Caisson) were reconstituted. GMEM (Caisson's Glasgow Minimal Essential Media) 12.5 g/L, Caisson sodium bicarbonate (0.8 g/L), Capricorn fetal bovine serum (10%), Streptomycin sulphate (6 ml), Amphotericin B (10 ml), Benzylpenicillin sodium (1 ml), and tryptose phosphate broth (5 g/L) are the other ingredients. The media's pH was kept between 7 and 7.4. Before use, the media was filtered. Syringe filters, simple steel filtering assemblies with two filters, and capsule filters can all be used to accomplish this. A flask of the media was placed in an incubator at 37^{o}C overnight after filtering it to check for contamination. To check for bacterial growth, a tiny amount of media was placed over nutrition agar and blood agar.

Preparation of stock solutions

Already prepared nanoparticles of Tamoxifen and ivermectin and by using Propidium iodide and Acridine Orange dye on MCF-7 malignant cells, the interactions with the ratio of 1:1 between Tamoxifen and Ivermectin, Tamoxifen nanoparticles and Ivermectin, and Ivermectin Nanoparticles and Tamoxifen were examined. These combinations' inhibitory concentration (IC_{50}) values, obtained from the MTT Assay findings, were used to create the stock solution. The sample concentrations for flow cytometric analysis were those that inhibited MCF-7 cells to a maximal percentage in MTT experiment. Tamoxifen+Ivermectin (A+B) IC_{50} was 11.09 ug/ml as a result. Tamoxifen + Ivermectin Nanoparticles (A+D) had an IC_{50} of 9.36 ug/ml, whereas IC_{50} of Ivermectin + Tamoxifen Nanoparticles (B+C) was 8.0 ug/ml.

Preparation of dyes
Stock solution of Propidium Iodide and Acridine Orange dyes were prepared from 100 mg of solid dye. Stock solution was prepared by adding 100 mg powder in 100 ml of PBS (phosphate buffer solution)/water with continuous stirring in dark place results in final concentration of 1 mg/ml.

Flow cytometric Assay
After 24 hours of incubation, the medium was discarded and washed with 500 ul of PBS and then 1 ml trypsin was added and kept for 6 minutes in incubator. The cells were transferred to Eppendorf and centrifuged at 3500 rpm for 4 minutes. PBS (100 ul) was added to the pellet in a dropwise manner. Then, the cells containing samples (in combination) and control, were stained with 5 uL propidium iodide (PI) and 5 uL of Acridine Orange and kept in the dark at room temperature for 15 minutes. Then washed with 100 uL of PBS and centrifuged for 3000 rpm for 3 minutes. After removing the supernatant, 100 uL PBS was added and mixed with pellet of cells [20]. Then finally cells were analyzed using FACS AttuneXTM NxT acoustic focusing cytometer (Invitrogen®, Waltham, MA, USA). The absorbance peaks were checked at 520 nm.

Apoptosis detection by Annexin V-FITC
After treating MCF-7 cells (105 cells/well) with drug (IC50 value of Tamoxifen, Ivermectin and Nanoparticles respectively) for 72 hours in order to calculate the apoptosis/necrosis ratio, the cells were examined using the Annexin V/propidium iodide (PI) assay (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions. Control cells were MCF-7 ones that had not received any treatment.

Cell cycle analysis
Cell proliferation was assessed using propidium iodide (PI) and Acridine Orange staining, and because DNA content is used to determine the cell cycle stage, PI binding to DNA is proportional to DNA content. At first, in 6-well plates, cells were seeded at a density of 1×106 cells/well in complete medium and incubated overnight. After washing the cells with PBS, they were treated with drug patches for 72 h in a complete medium. Finally, the cells were fixed with ethanol 70% (18 h, 4 °C), and were stained using 500 µL of PI solution (containing RNase) in the dark for 20 min at room temperature and then investigated by flow cytometry [21]. All experiments were repeated three times. A flow cytometry device was used to perform the cellular analysis (Bio compare, USA).

Drugs treatments in genes expression analysis
The IC_{50} concentrations of tamoxifen (8.021 µg/ml), ivermectin (18.56 µg/ml), tamoxifen loaded chitosan nanoparticles TAM-CSNPs (5.86 µg/ml), ivermectin loaded chitosan nanoparticles IVM-CSNPs (9.14 µg/ml), tamoxifen + ivermectin (11.09 µg/ml), TAM-CSNPs + ivermectin (8 µg/ml) and IVM-CSNPs + tamoxifen (9.36 µg/ml) were applied to MCF-7 cells and left for 24 hours. Total RNA was extracted from the cells using the trizol reagent. Basically, trizol is a pink color solution of guanidinium isothiocynate and phenol. RNA was isolated from the Cell lines using the Trizol technique. The separation of RNA from proteins and DNA is governed by the Trizol's acidic pH. The trypan blue dye exclusion method was used to determine the viability of cellular populations at the conclusion of the treatment period (Amatori et al., 2016).

RNA extraction and cDNA synthesis
After treatment, RNA was isolated using the Trizol method. Cells were mixed with Trizol and chloroform in a sterile tube with an incubation period of 15 minutes at room temperature. The tube was centrifuged to get an aqueous layer containing RNA after the incubation period. The RNA was then precipitated by transferring this layer to a second, sterile tube and incubating it for 15 minutes at room temperature with 0.5 ml of isopropanol. The tube is treated with 75% ethanol before undergoing a second centrifugation to produce a pure RNA pellet. The pellet was then dried, resuspended in water that had undergone DEPC treatment to remove RNase, and stored between 4 and -20 °C for later use. The cDNA synthesis kit from Thermo-Fisher Scientific was used to produce cDNA. The proposed methodology was used to produce the PCR master mix. The optimum conditions for the amplification process were created by placing the PCR tube in a thermocycler. After amplification water (water that had undergone DEPC treatment) was used, the generated cDNA was stored for future use at 4 to -20° C (Jeromin et al., 2019).

Real-time PCR amplification
RT-PCR assay was used to analyze gene expression.

It was done by counting the number of transcript copies of the desired gene (CHEK2, TP53). Thermo Fisher Scientific's SYBER-GREEN master mix mixture, as described in table 2, was used to produce the desired results. The PCR master mix was created in accordance with the instructions supplied, and the reaction was conducted under optimal conditions. Thermal cycle is at 95 °C for 10 minutes, 40 times for 15 sec each, 64 °C for 30 sec, and 72 °C for 15 seconds was used. An examination of the melting curve was conducted with a ramp from 60 °C to 90 °C and an increase of 0.5 °C every 2 second. The 2-ΔΔCT approach was used to Identify changes in gene expression in terms of fold induction from the control population of untreated cells (Amatori et al., 2017).

Table-1: Recipe for cDNA master mix

Sr.no	Reagents	Quantity
1	RNA template	≤500 ng
2	DNTPs	1.5 µl
3	Reverse primer	2 µl
4	Buffer (5x)	3 µl
5	RevertAid	1 µl
6	Nuclease free water	To make 20µl

Statistical analysis

Using SPSS version 22, the significant difference between the dose exposure samples and the control group was examined using the t-test, mean, standard deviation, and one-way ANOVA. The level of relevance had been considered as $p < 0.05$ (5% probability).

Results

Flow cytometry-based detection and quantification of cell death in MCF cell line using Propidium Iodide

Histogram showing cell death by Tamoxifen+Ivermectin (A+B), Tamoxifen+Ivermectin NPs(A+C) and Ivermectin+Tamoxifen NPs(B+C) drugs combination on MCF cells. Overlay histogram showing cell death by drugs combination on MCF cells. Data is representative from three independent experiments with each condition done in triplicates. FSC= Forward scatter, SSC= Side scatter, PI= Propidium Iodide. Scale bar= 100 µm

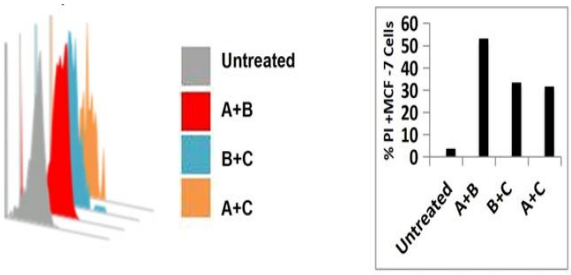
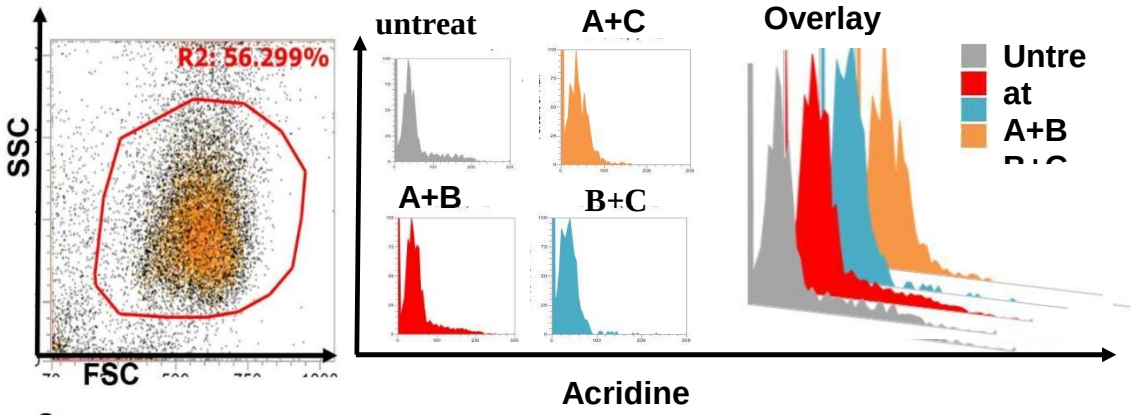


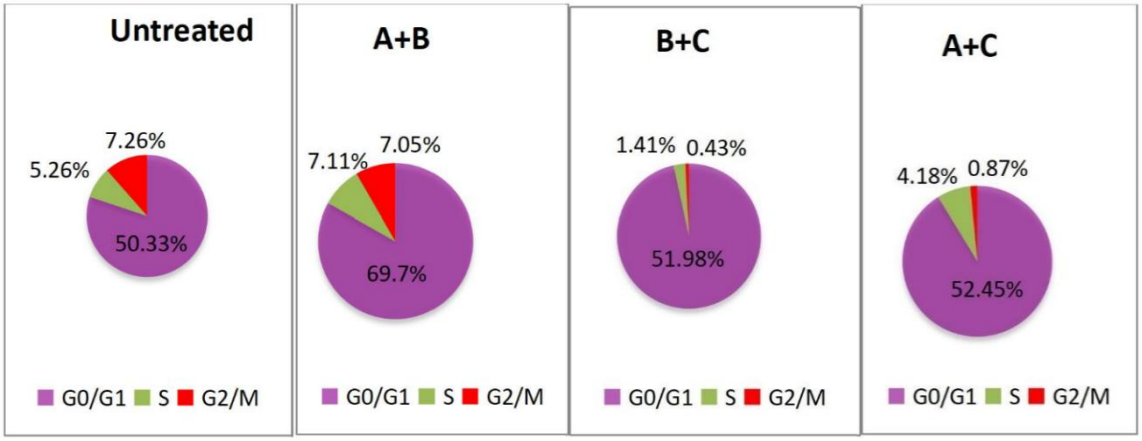
Figure-1. Flow cytometry-based detection and quantification of cell death in MCF cell line using Propidium Iodide (PI) after incubation with different combination of Tamoxifen (A), Ivermectin (B) and NPs (C).

After treating the MCF-7 cells with Propidium Iodide dye with different combinations of Tamoxifen+Ivermectin (A+B), Tamoxifen+Ivermectin NPs(A+C) and Ivermectin+Tamoxifen NPs(B+C), it was showed that Tamoxifen and Ivermectin has represented maximum level of cell death as the propidium iodide dye binds with DNA of dead cells and which was 56.01%. After this, Ivermectin and Tamoxifen NPs (B+C) represented 34.8% of cell death which was still little bit better than 32.7% of cell death shown by the combination Tamoxifen and Ivermectin NPs (A+C). Untreated cells showed minimum 4.36% of cell death which represented maximum number of live cells.

Flow cytometry-based detection and quantification of cell cycle stages in MCF cell lineusing Acridine Orange dye (AO).

A. Scatter plot showing gating of MCF cells using Attune™ NxT Acoustic Focusing Cytometer B. Histogram showing untreated cells stained with AO dye, Histogram showing cell cycle changes by Tamoxifen+Ivermectin (A+B), Tamoxifen+Ivermectin NPs(A+C) and Ivermectin+Tamoxifen NPs(B+C), drugs combination on MCF cells. Overlay histogram showing cell cycle alterations by different drug combination on MCF cells, while Pie charts showing data from control untreated MCF cells and different drug combinations. Bar graph showing data from control untreated MCF cells and different drug combinations. Data is representative from three independent experiments with each condition done in triplicates. FSC= Forward scatter, SSC= Side scatter, Orange=AO, Scale bar= 100 µm.





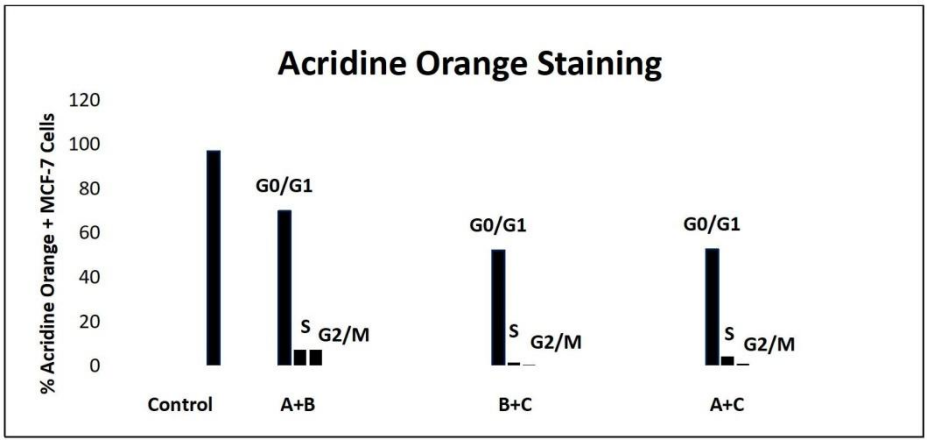


Figure-2. Flow cytometry-based detection and quantification of Cell cycle stages in MCF cell line using Acridine Orange dye (AO).

Table-2. Flow cytometry-based detection and quantification of cell cycle stages in MCF cell line using Acridine Orange dye (AO)

Treatment	Cell cycle Phases	% Cell Arrest
Untreated	**G0/G1**	50.33%
	S	5.26%
	G2/M	7.26%
Tamoxifen+Ivermectin (A+B)	**G0/G1**	69.7%
	S	7.11%
	G2/M	7.05%
Ivermectin+Tamoxifen NPs (B+C)	**G0/G1**	51.98%
	S	1.41%
	G2/M	0.43%
Tamoxifen+Ivermectin NPs (A+C)	**G0/G1**	52.45%
	S	4.18%
	G2/M	0.87%

After treating the MCF-7 cells with Acridine Orange dye with different combinations of Tamoxifen+Ivermectin (A+B), Tamoxifen+Ivermectin NPs(A+C) and Ivermectin+Tamoxifen NPs(B+C), the results achieved described that Tamoxifen+Ivermectin (A+B) combination showed the remarkable and maximum of the all cell cycle arrest with value of 69.7% cell arrest at G0/G1 phase, 7.11% of cell arrest at S Phase and 7.05% of G2/M Phase arrest. Ivermectin+Tamoxifen NPs (B+C) represented evidential cell cycle arrestat G0/G1 phase with value of 51.98% alongwith negligible value of 1.41% cell arrest at S phase and 0.43% of arrest at G2/M phase. Tamoxifen+Ivermectin NPs (A+C) displayed significant cell arrest at G0/G1 phase with 52.45% value along with 4.18% of S phase cell arrest and 0.87% of G2/M phase cell arrest. Untreated cells showed 50.33% of cell arrest at G0/G1 Phase which is less than the cell cycle arrest percentage exhibited by all the combinations, 5.26% of cell arrest at S phase which is more than 4.18% and 1.41% as exhibited by Tamoxifen+Ivermectin NPs (A+C) and Ivermectin+Tamoxifen NPs (B+C) respectively at S phase, and 7.26% of cell cycle arrest at G2/M phase which is also slightly more than 7.05% of cell

Expression analysis by RT-PCR

Using real time-PCR, an investigation of gene expression was performed out. The newly produced cDNA was used for the gene expression investigation. Thermo-Fisher Scientific's SYBER GREEN system was used to create both reaction mixtures for the two genes, and the reaction was carried out under optimal conditions. Reaction results were displayed on the computer screen in real time as peaks and graphs.

C_T value of expression analysis

Real-time PCR gene amplification was followed by gene expression analysis to determine the fold change in gene expression. The fold change from CT values was calculated for this purpose using the Livak method. Tamoxifen+Ivermectin (A+B), Tamoxifen+Ivermectin NPs(A+C) and Ivermectin+Tamoxifen NPs(B+C) have been shown in studies to influence the expression of various genes in breast cancer cells. To see how Chitosan nanoparticles formulation affects the treatment of breast cancer cells and the synergistic effect of Tamoxifen+Ivermectin (A+B), Tamoxifen+Ivermectin NPs(A+C) and Ivermectin+Tamoxifen NPs(B+C), expression of two different genes (CHEK2 and TP53) was measured within the MCF-7 breast cancer cell line. It was demonstrated that the expression levels of CHEK2 and TP53 genes were significantly increased ($P<0.001$) in Ivermectin+Tamoxifen NPs(B+C) compared with Tamoxifen+Ivermectin (A+B), and control groups. The expression levels of nanoparticles containing Tamoxifen were significantly increased compared to the other treatments and control groups ($P<0.001$). Gene expression change with change in dose concentrations.

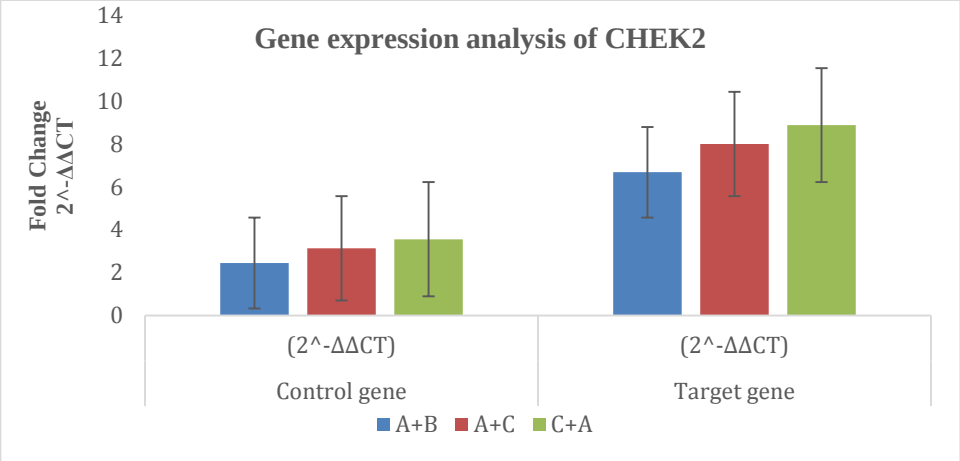


Figure-3. The expression of CHEK2 gene in MCF-7 cells after treatment with samples by real-time PCR method.

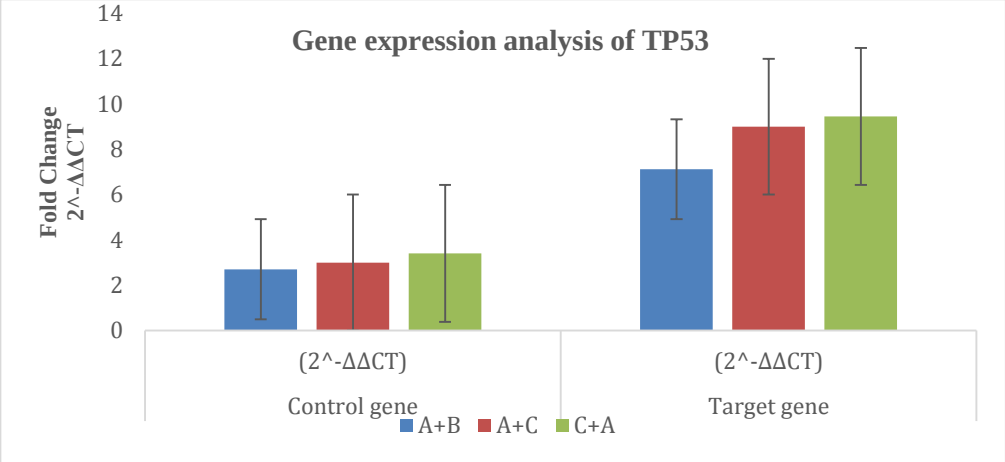


Figure-4. The expression of TP53 gene in MCF-7 cells after treatment with samples by real-time PCR method.

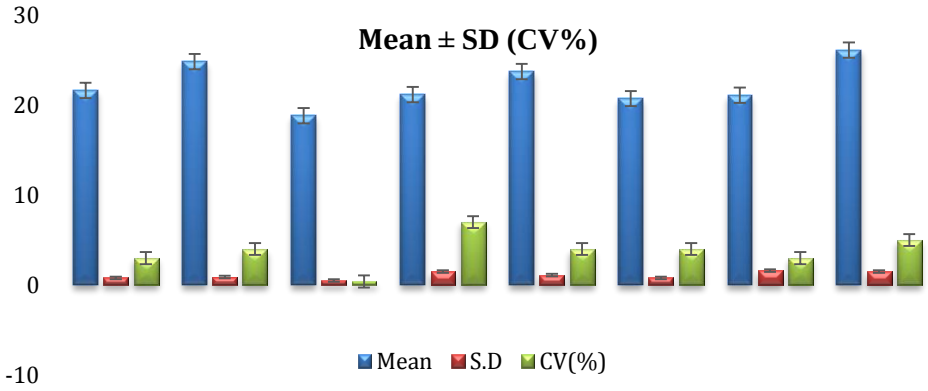


Figure-5. Mean of C_T value of Genes

Comparison of mean and standard deviation

Gene expression was also calculated by mean (+ S.D) comparison between control and target genes. For *CHEK2* and *TP53* the mean and S.D was 21.80 ±1.3, 21.81 ±1.3, 18.80 ±0.2, 19.22 ±0.9, 24.43 ±1.4, 26.41±0.8 and 24.22 ±0.6 for control gene and 27.06 ±0.7, 25.77 ±2.0, 22.02 ±1.1, 22.72 ±0.6, 27.06 ±0.9, 30.74 ±4.1 and 28.62 ±0.7 for target gene at different doses respectively. The mean values represent that the expression was upregulated in treated or target genes as compared to control.

Discussion

Quantifiable cytology in the sort of flow cytometry has largely modernized the clinical interpretation of tumor cell heterogeneousness using probes that distinguish cancerous from normal cells and evaluate differentiative and proliferative cancerous cell attributes. Survival of cancerous cells depends on its ability to enhance anti-apoptotic markers (El-Magd et al., 2019). Tamoxifen can increase apoptosis and has prominent anti-cancer effects against MCF7 cell line both in vivo and in vitro (Badawy et al., 2021). In current studies, we have examined Flow cytometric gating and standardization of MCF cell line using Propidium Iodide (PI) and Acridine Orange filters on unstained cells with all showing cell population of 28.712%. Flow cytometric evaluation Live-Dead MCF cells using Propidium Iodide (PI) filter showed 0.321% with minimum auto fluorescence and minimum cell death. Flow cytometric evaluation Cell Cycle of MCF cell line using Acridine Orange (AO) showed 0% G0/G1, S, G2/M activity exhibiting 0 cell arrest.

It has been observed through various experiments that tamoxifen has several side effects which are directly associated with its dose and concentration used in breast cancer patients. Therefore, giving low dose is pragmatic approach to reduce its harmful impacts. Nanotechnology is one of the possible solutions to improve efficiency of the drug as well as to reduce its harmful impacts (Mahmood et al., 2024).

Another research revealed that when MCF-7 cells was treated with Tamoxifen. It significantly increased the Go and G1 phase percentage from 52.7% to 62.6% (Guney Eskiler et al., 2018). Another study revealed that Ivermectin cause cell autophagy in human breast cancer cells (Deng et al., 2018). In another research, ivermectin was given and after 24hrs, its effect on cell viability was evaluated by using luminous ATP viability assay on breast cancer cells. Before conducting experiment, the cell viability was 95% which was significantly reduced after treatment with ivermectin (Güler and Günaydin akyildiz, 2023). Another study revealed that 5um ivermectin has antitumor response (Juarez et al., 2020). Another research study concluded that the multiplication of several breast cancer cell line MCF-7, MDA-MB-231 and MCF-10 significantly lowered when they were treated with ivermectin. Ivermectin inversely act on Akt/mTOR pathway to activate autophagy and target p-21 activated kinase 1 (Dou et al., 2016). Previous studies showed that Tamoxifen alone through flow cytometric analysis of cell cycle exhibited global cell cycle arrest in MCF-7 cells by increasing expression of p21 protein, that causes cell cycle arrest (Liu et al., 2011). In another study, the Propidium Iodide staining using FCM analysis were used to evaluate cell apoptosis effect on Cisplatin and tamoxifen. The results showed that these two drugs in combination elicited a noteworthy increase in apoptotic cells from 11.2% Cisplatin, 20.0% Tamoxifen to 29.7% of their combination, as the expression of P53, Bax, Caspase 3,7,9, which are pro-apoptotic molecules, got upregulated and enhanced in combination treatment and in cell cycle arrest, the combined treatment of drugs, remarkably enhanced the inhibition of cell cycle in the G2/M phase arrest from 16.1% Tamoxifen and 17.39% Cisplatin to 35.13 of combined treatment, as compared with single drugs (Li et al., 2021). In case of Ivermectin on MCF-7 cells was studied to show enhanced expression of p53, p21 and caspase-3 as compared to untreated cells and cause cell arrest through DNA fragmentation (Seresht et al., 2019). Through flow cytometry analysis, Ivermectin showed cell cycle arrest at G0/G1 phase along with apoptosis (Jaafari et al., 2012).

Real-time PCR gene amplification was followed by gene expression analysis to determine the fold change in gene expression. The fold change from CT values was calculated for this purpose using the Livak method. Another study discovered that when suppressor genes were chosen (ATM, CHEK2, BRCA1, BRCA2, and TP53) were analyzed for expression using the real-time PCR method, there was no visible difference between the expression of the ATM gene in ovarian cancer and that of the control group (74.46% ± 4.55 vs. 99% ± 4.25 with $p > 0.05$) and no discernible changes in the transcriptional levels of TP53, BRCA1, BRCA2, or CHEK2 were found. The mRNA level of ATM1 was lowered by 25% in ovarian cancer patients, and there is no expression, respectively (Olbromski et al., 2022). A study concluded that in response to DNA stranded double breaks, the ataxia telangiectasia protein activates the CHEK2 protein. CHEK2 phosphorylates cell cycle proteins like p53, BRCA1 and BRCA2, which causes cell cycle arrest or apoptosis, which results in low expression while treating suppressor proteins to become expresser (Näslund-Koch et al., 2016). CHEK2 mutations raise the risk of breast cancer in women with a stronger

family history than in those without such a history, just like PALB2 mutations do. These people have a 28–44% lifetime cumulative chance of getting breast cancer compared to women without a family history of the disease (Cybulski et al., 2012). Either its capacity to express itself or its capacity to function as a signaling molecule is lost when CHEK2 is damaged. Protein-protein interactions that are wrong as well as defective interactions of the CHEK2 protein with its substrates, including as BRCA1, p53, and Cdc25A, are caused by mutations in the SQ/TQ regulatory domain that prevent the upstream kinase ATM from properly activating CHEK2. The catalytic activity of CHEK2 is inhibited by flaws in the kinase domain (Kumpula et al., 2023).

After amplification, the CT values and gene expression in this study range from 21 to 27, coupled with GAPDH control. The research states that TP53 controls the G1 checkpoint and can cause to stop cell cycle growth, restoration, or apoptosis if DNA damages are severe with a CT value of 24 (Wiesmüller, 2001). The wild type p53 protein has the ability to transcriptionally trans activate the p21Cip1 protein, a potent inhibitor of the majority of cyclin-dependent kinases associated with cell cycle arrest (Ocker and Schneider-Stock, 2007). TP53 is mutated in about 30% of breast cancers (Olivier et al., 2006). Several investigations have looked into the potential associations between p53 mutations and the pathological or clinical characteristics of breast cancers. According to the gene-expression profiles of the illness, there are at least four main molecular subgroups luminal-like, basal-like, normal-like, and HER-2 positive subtypes of breast cancer. 15% of all breast cancers are basal-like tumors, also referred to as triple-negative breast cancers (TNBCs). TNBCs are actual malignancies because they exhibit no HER2, progesterone, or estrogen receptors (Gluz et al., 2009), including some weakly differentiated luminal breast tumors as well as basal-like breast cancers. They also have a dismal prognosis and are related to younger ages. TP53 mutations also occur more frequently in TNBCs (Chae et al., 2009).

Conclusion

According to the results of the current study showed that combinations give better results than each agent alone. Combination relatively safe or one can say less cytotoxic drug to normal cells may influence the anticancer drug effects by reducing its dose can help us to determine optimal combination therapy. CHEK2 and TP53 gene expression is higher in treated samples compared to untreated samples, and considering the decline in their usual performance can contribute to the advancement of breast cancer. These genes' aberrant expression demonstrates that certain hereditary and epigenetic variables are the underlying cause of atypical expression of genes.

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Contribution of Authors

Naeem UB & Rasheed MA: Conceived idea, designed research methodology, data collection and analysis and prepared and edited manuscript

Ashraf M: Critically analyzed and interpreted the results and approved for submission.

Zahoor MY: Determined the expression profile with CT value and did literature review.

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