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Paclitaxel induced oxidative stress suppresses proliferation of breast tumor MCF-7 cells

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ABSTRACT:
Background: Breast cancer is the most common malignancy in women, affecting one in eight to ten women during their lifetime. Paclitaxel (PTX) is a taxane-based chemotherapeutic agent used as a treatment in both early-stage and metastatic breast cancer. Cancer cells (relative to normal cells) demonstrate alterations in oxidative metabolism characterized by increased steady-state levels of reactive oxygen species.

Objectives: The aim of this study was to evaluate the antioxidant potential and the *in vitro* anticancer efficacy on MCF-7 breast cancer cell line.

Material and methods: Total ROS of the cells were assessed. The biochemical estimations on MCF-7 include determining the activity of the activities of enzymatic antioxidants (superoxide dismutase (SOD) and the non-enzymatic antioxidants reduced glutathione (GSH). The reactive nitrogen species were estimated by assessing the NO levels.

Results: Treatment with paclitaxel effectively suppressed breast cancer. Dose-dependent decrease in viability (MTT assay) in MCF-7 cells were observed. The calculated IC_{50} value of PTX was 5.56 µg/mL for 48 hrs. Total ROS levels determined were<250 in case of PTX treatment. Increased LDH release indicated loss of membrane integrity as LDH release is considered as a general marker of cell injury. This further revealed by the concomitant increase in the activities of enzymatic antioxidants and glutathione transferase (GST)) and non-enzymatic antioxidants (reduced glutathione (GSH). Further, increased levels of NO and decreased SOD levels post- treatment of PTX indicated the involvement of reactive nitrogen species too.

Conclusion: The study concludes that PTX suppressed proliferation of the breast cancer cells *via* oxidative stress.

KEYWORDS: Paclitaxel, MCF-7 cell line; breast cancer, reactive nitrogen species, oxidative stress.

I. INTRODUCTION:

Excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS) production induces oxidative damage in cells that is primarily caused by generation of singlet oxygen, hydroxyl radicals, as well as superoxide anions [1]. Plethora of literature suggests that oxidative damage maybe correlated with the pathophysiological alterations occurring in many diseases including cancer, diabetes, atherosclerosis, neurological diseases etc. [2, 3]. However, it is established that oxidative damage causes an imbalance between the pro-oxidant and anti-oxidant defense system, DNA destruction, lipid and protein functions [4]. Breast cancer is the most common malignant tumor that poses a serious threat to health of women worldwide. Accumulative evidences have implicated the correlation between inflammation and oxidative damage in the etiology of breast diseases [5]. Furthermore, researches have suggested that antioxidants prevented or delayed ROS-triggered apoptosis that might be a rational way for treating breast cancer [6, 7]. Among all kinds of antioxidants, natural substances usually obtained by chromatographic separation technology from natural plants showed merits than synthetic chemicals because synthetic chemicals have acute by-effects though strong radical scavenging abilities [8]. Besides, natural antioxidants can prevent the body injury through removing excessive ROS, enhancing activities of antioxidant enzymes [9]. Presently, substantial researches had been concentrated on hunting for natural active ingredients with breast protective potential, which could scavenge excess free radicals and prevent cell oxidative damage.

Currently, numerous medical research manuscripts suggest the most effective drugs used as the first-line therapy in breast cancer is Paclitaxel (PTX), that was initially isolated from the bark of Pacific yew tree (*Taxus brevifolia*) and reported in 1960s[10]. PTX is known as broad-spectrum anticancer drug that is a microtubule-targeted agent. Its primary cellular effect is to cause abnormal stabilization of the dynamic microtubule polymerization, leading to the failure of mitosis. The main mechanism of PTX action is binding to microtubules and inhibiting their disassembly. In addition, PTX also alters other cellular functions that involve microtubules, such as intracellular signaling and organelle transport and locomotion[11]. Recent studies showed that PTX is able to induce early reactive oxygen species (ROS) production in cancer cells, and reactive nitrogen species (RNS) was found to be involved in paclitaxel-induced cancer cell death *in vitro* and *in vivo*. The aim of the study was to evaluate the ability of PTX to induce ROS and RNS production in MCF-7 cells.

II. MATERIAL AND METHODS:

Chemical reagents: MCF-7 cell line was procured from NCCS pune, PTX were purchased from Sigma-Aldrich. MTT [(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], was procured from Sigma Chemical Co., (St. Louis, MO, United States). Ethanol, Glutaraldehyde (25% v/v), and Dimethyl sulphoxide (DMSO) were obtained from SRL Pvt Ltd. India. Dulbecco's modified Eagle's medium (DMEM), Antibiotic cocktail containing streptomycin and penicillin, Trypsin were procured from Hi Media Pvt Ltd. Fetal bovine serum (FBS) was purchased from GIBCO. Proteinase K, 1-chloro, 2, 4-dinitrobenzene (CDNB), and sodium bicarbonate were purchased from Sigma-Aldrich (Bengaluru, India) and paraformaldehyde was purchased from Merck India Ltd., (Mumbai, India). N-1-naphthyl ethylenediamine dihydrochloride (NEDD), ethylenediaminetetraacetic acid (EDTA), nitro-blue tetrazolium (NBT), trichloroacetic acid (TCA), and dinitrobenzoic acid (DTNB) were procured from SRL, India. All other chemicals were of analytical grade and obtained from Merck India Ltd. (Mumbai, India).

- **Maintenance of cell line:** MCF-7 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin antibiotic in CO_2 incubator maintained at 37°C and 5% CO_2 with media replenished every 1 day. The MCF-7 cells were cultured in the culture plate, and the experiment was carried out when the cells entered the logarithmic growth period.

- **Preparation of stock solutions:** Stock solutions of PTX were prepared in DMSO, HEX solutions were prepared in distilled water. The working solution of PTX, HEX and DMSO were in the range of 12.5 µg/mL, 25 µg/mL, 50µg/mL and 100µg/mL. The cytotoxicity of the drugs was determined using ELISA reader. Untreated cells were used as control. All untreated and treated cells were incubated for 24, 48 and 72 hours as per the protocol.

- **Estimation of IC_{50} :** Protocol adapted with slight modifications from Moin et al. 2018. The effect of PTX and HEX on cell viability was assessed by the colorimetric assay, in which MTT yellow crystals gets converted into formazan crystals in presence of live cell mitochondrial enzyme, succinate dehydrogenase (a NADPH dependent cellular oxido-reductase). Briefly, 1×10^5 cells/well were seeded in a 96-well plate for overnight in a CO_2 incubator at 37°C. Next day cells were treated with different concentrations of PTX and/or HEX (12.5 µg/mL, 25 µg/mL, 50 µg/mL and 100µg/mL) for 48 and 72 hrs. Afterwards, 20µL of MTT solution (5mg/mL) has been added in each well and incubated for 4 hrs. This step leads to formation of formazan crystals which dissolve using 200 µL of DMSO for 30 min. The organic solvent causing dissolution of formazan crystals and intensity was recorded at 562nm using ELISA reader.

The % cytotoxicity was calculated as

$$\frac{\boldsymbol{Absorbance\ of\ the\ control\ sample\ (A)c - absorbance\ of\ the\ sample\ to\ be\ tested(A)t}}{\boldsymbol{Absorbance\ of\ the\ control\ sample}} \times \mathbf{100}$$

Determination of total ROS production in MCF-7 cells:

DCFDA assay: To detect the ROS generation, $1x10^6$ cells were seeded in 6-well plate and kept in incubator for overnight in DMEM supplemented media with 10% FBS and streptomycin/penicillin antibiotic and treated with PTX and HEX with their respective IC_{50} concentrations. Cells were scrapped after 24 hours treatment using scrapper and incubated with 25 µM of DCFDA in the dark for 30 min. at 37°C. Fluorescence intensity was recorded by fluorescence spectrophotometer having emission and excitation at 529 nm, 485 nm respectively, slit width adjusted at 5.0[12].

Cell lysate preparation: For biochemical estimation of the cells after PTX treatment was calculated using the following method: cells were seeded in 6-well plate at 5 $X10^6$ for the overnight in DMEM media supplemented with 10 % FBS. Next days the cells were treated with PTX, HEX and untreated as control. After that, adherent cells were trypsinized and centrifuged for few min. then collect supernatant and pellet in different Eppendorf to processed for NO, LDH, and SOD, GSH, GST respectively[13].

Measurement of antioxidant parameters

LDH (Lactate dehydrogenase) release assay: Briefly, 20µL of the supernatant from each sample was mixed with LDH reaction mixture of 34 mM sodium pyruvate (160 µL) which was prepared in 100µM PBS (pH 7.5) and 0.28 mM β-NADH (20 µL). The OD was recorded at 340 nm using ELISA reader (USCN Life Sciences, Wuhan). The LDH release was calculated on the basis of reduction of NADH over time[15]. The final result of LDH was represented in terms of nM/mg protein.

2.6.1.2 NO (Nitric oxide) assay: The protocol of NO assay was adopted from Griess 1879 with slight modifications. First of all, 0.1 % NEDD was prepared using 30 mg in 30 mL of OPA as the same 1% sulfanilamide was prepared using 300 mg in 30 mL of OPA. Both reagents mixed together to form Griess reagent in ratio 1:1. This cocktail mixture was used in 100 µL with 100 µL of the samples. OD was recorded at 540 nm along with blank sample having PBS only. The final result of NO was represented as µM/mg of protein.

2.6.1.3 SOD (Superoxide dismutase) assay: This protocol is adopted from Kakkar et al. 1984 in which SOD cocktail was prepared using 300µM NBT (Nitroblue-tetrazolium), 180 µM PMS and 0.025 M sodium pyrophosphate (pH 8.3). The reaction mixture contains 60 µL of SOD cocktail, 20 µL of CFE added into 20 µL of 780 µM NADH compound. The OD was recorded at 560 nm. A blank was measured having PBS instead of CFE to record the chemicals absorbance. The final result was represented in the form of ng/mg of protein.

2.6.1.4 GSH (Reduced Glutathione): CFE were used for GSH activity which developed by Moron et. al 1979 involving DTNB, 0.2 µM of PBS (pH 8) with protein free CFE. Briefly, 100 µL of CFE was added with 20µL of 5% TCA which centrifuged at 3000 rpm at 4 ℃ for 10 minutes to remove any protein traces from the CFE. After that, 45 µL of 5 % TCA added CFE supernatant was added in the reaction mixture was added with 20 µL DTNB and 45 µL of PBS to complete volume of 110 µL. The OD was recorded after 10 min. to activate the process and recorded continuously for 5 min. The final concentration of GSH represented in the form of nM/min/mg of protein.

Statistical analysis : All experimental results are shown as the mean ± SD. The one-way analysis of variance (ANOVA) was employed to analyze statistical differences between the groups. $p < 0.05$ represents the experimental results with statistical significance.

III. RESULT AND DISCUSSION:

Cytotoxicity assay: After 48 and 72 hours, the untreated and treated cells were shown significant colorimetric difference. The control cells had more formazan crystals whereas the HEX treated cells had low number of crystals followed by PTX was minimal crystal formation. The IC_{50} has been calculated for PTX from 48 and 72 hrs as 5.56 µg/mL and 4.56 µg/mL respectively. HEX $IC5_0$ has been found as 18.36 µg/mL and 14.56 µg/mL for 48 and 72 hours respectively.

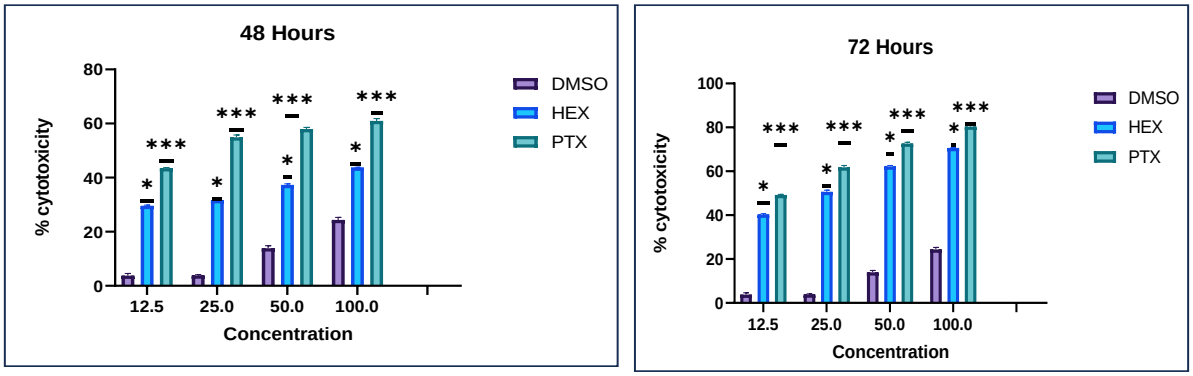

Figure 1: The % cytotoxicity and % viability of the drug in MCF-7 cells post 24-hours of PTX and HEX treatment. (Here DMSO used as solvent to dissolved cycloheximide and paclitaxel due to hydrophobic nature of the drug.)

Table 1: IC50 value of PTX and/or HEX cells post 48, and 72 hrs of treatment.

IC50 Dose µg/mL		
	PTX	**HEX**
48 HOURS	**5.56**	**18.36**
72 HOURS	**4.56**	**14.56**

ROS generation via DCFDA: Oxidants and anti-oxidants release plays important role in cell viability, growth and progression. Anti-oxidants have been further involved in cellular functions, signalling pathways and many more. ROS generation at cellular levels has been quantified using live cells staining technique which oxidises DCFDA into DCF (a fluorescent compound) which directly proportional to quantity of DCFDA oxidises to DCF. PTX causes maximum H_2O_2 generation as compared to untreated control. Although cancer cells have been reported for the generation of high ROS levels but during the treatment, ROS level further increase leads to cell death. Same set of results obtained by anthraquinone obtained by oyster extract in MCF-7 cells[19].

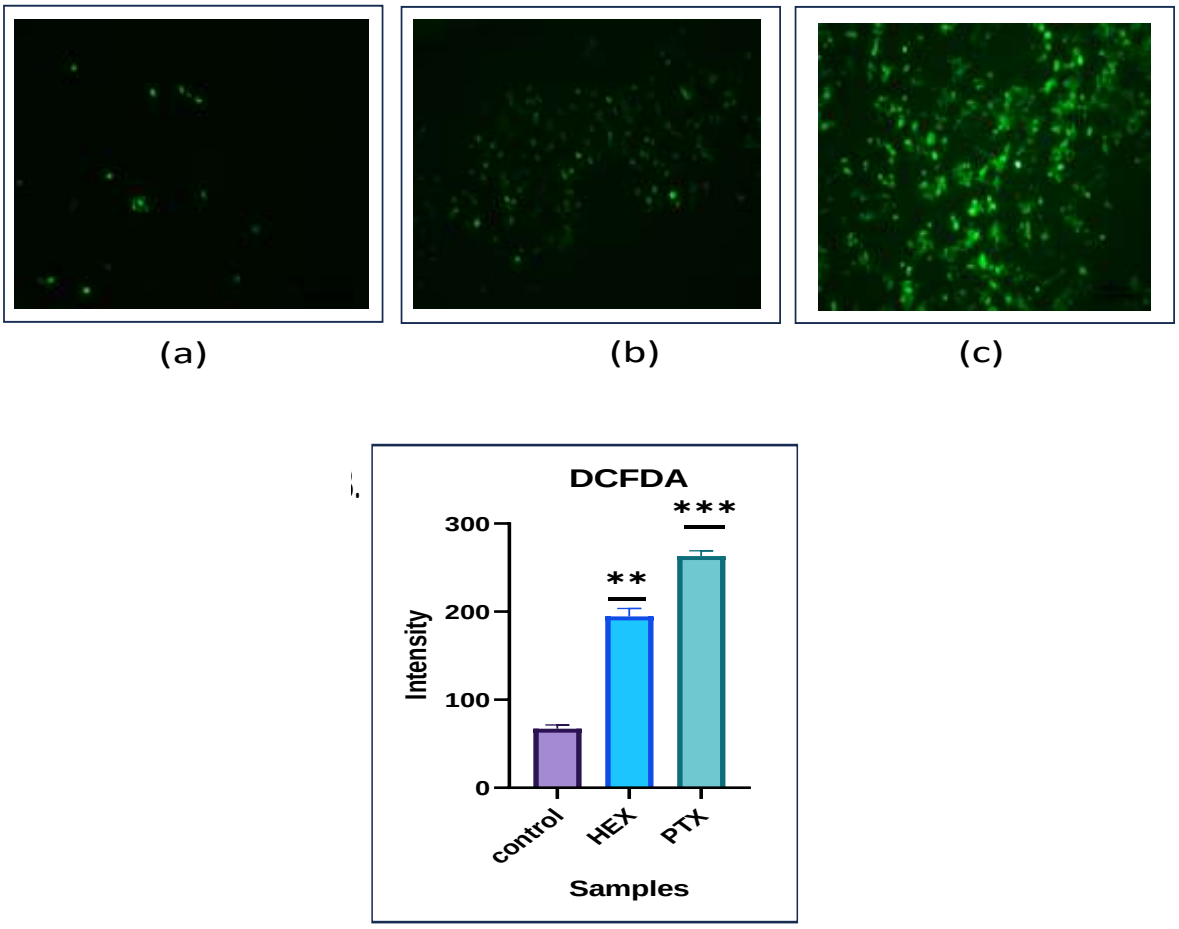

Figure 3: A) ROS generated due to the stress caused by PTX and/or HEX after 24-hours of exposure to the MCF-7 cells. a) control (untreated cells) b) HEX treated c) PTX treated cells. Initially cells were stained with DAPI and fluorescence was recorded at 459 emission spectra and 520 of absorption spectrum. The more amount of ROS, more stained were absorbed by the cells.

B) The intensity of the cells was recorded and graph was plotted between intensity and samples. Here, n = 3; the significance of the experiment was $p < 0.001$.

The effects of PTX on ROS and RNS activities: LDH, SOD, GSH and NO in MCF-7 cells: Cell lysate was processed for the oxidative assays which known as ROS subclass involves NO radicals and various superoxide products e.g. nitrous oxide and peroxynitrite radicals. The tumor killing properties of NO has been already reported in some cancers. Results obtained from LDH, NO assay depicted that, RNS generation were significantly enhanced with cells treated with PTX and/or HEX as in compared with untreated control cells. The growth inhibition of therapy calculated by LDH and NO assay were supported by the bio-cytotoxicity experiment. An important detoxifying intracellular enzyme which catalyses the electrophile binding to GSH which further leads to *S*-thiolated protein restoration causes elimination of ROS. To reduce the oxidative stress effects inside the cell environment, the mitochondrial membrane possesses GSH, SOD which exist on both side of the membrane.

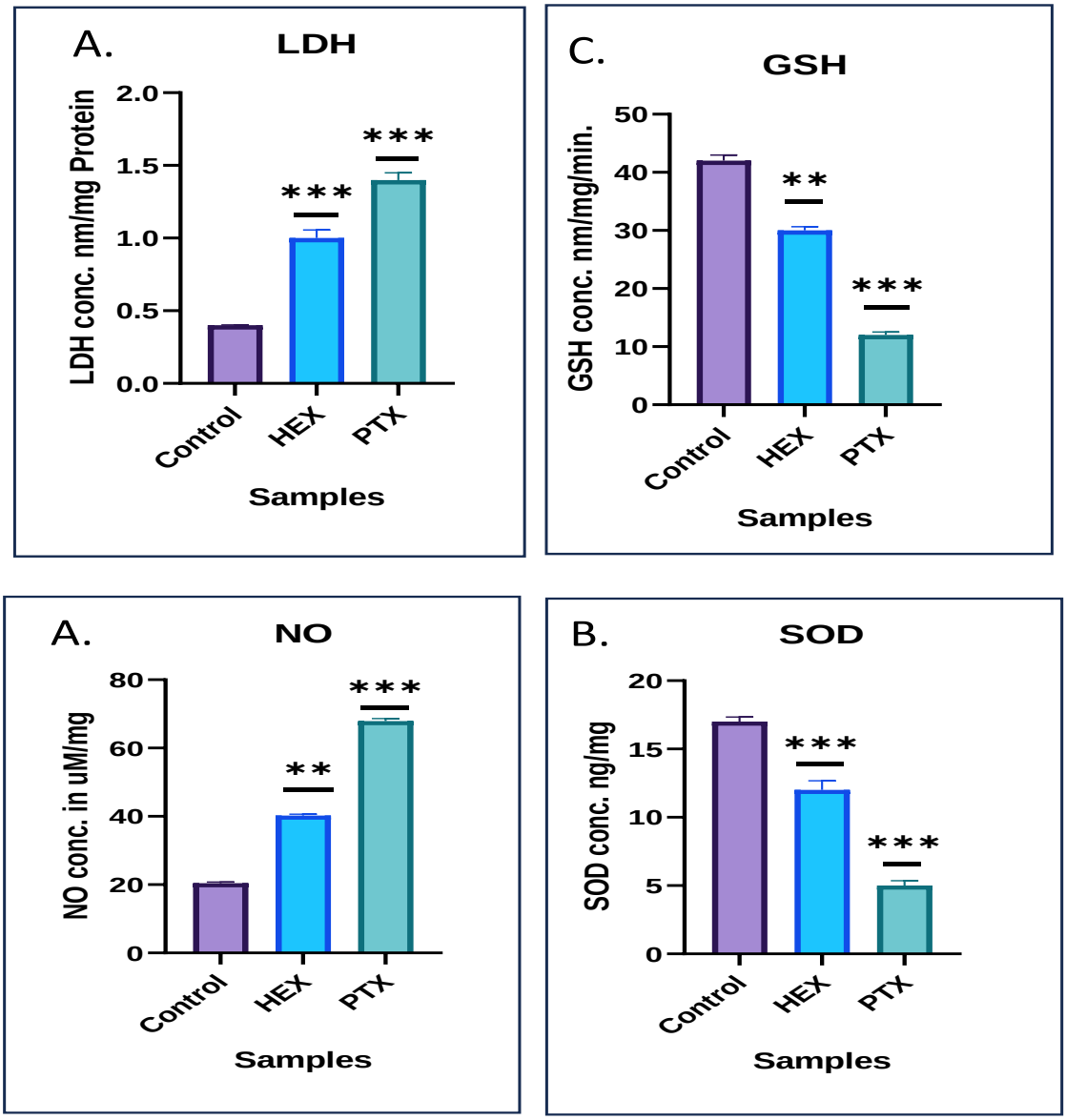

Figure 4: The biochemical estimation of MCF-7 cells after 24-hours of PTX and HEX treatment. LDH, NO and SOD assay was done using the supernatant, whereas GSH was performed using the cell pellet. **n=3 were used for the experiment.**
$p < 0.001$ was used for the significance of the data.

Redox-controlled signal transduction occurs through the reverse oxidation of thiol proteins. Thoil oxidation by PTX implicated that GSH gets oxidised directly by PTX and protein SH. Both endogenous and exogenous stimuli may generate ROS in cells. The LDH assay shows integrity of cell membrane and its release enhanced in treated cells. The LDH end points are 1± 0.056 and 1.4 ± 0.05 for HEX and PTX respectively. GST is also one of the important enzymes responsible for innate defence mechanism which works on the clearance of ROS and found in many human tissues. GST also involved in generation of various cancer via redox balance. GSH nucleophile also balance by GST enzymes. The GST levels increased by treatment with the drugs such as 340 ± 3.5, 690 ± 3.2 for HEX and PTX respectively. GSH was only non-protein sulhydryl antioxidant present in all kind of cells. RNS and ROS causing disbalance was shielded by GSH. The ROS enhanced due to stress causes reduction in GSH levels after the drug treatment such as 30 ±0.65, 12 ± 0.02 for HEX and PTX as in compared to high level of GSH (42) in untreated cells. SOD catalyses the superoxide dismutation and turns them into less harmful H_2O_2 which further processed through some other enzymes which finally leads to reduced SOD levels after drug treatment. The SOD levels in untreated cells are 17 ± 0.34 and 12 ± 0.67, 5 ± 0.35 for HEX and PTX respectively. Same set of experiments done by Moin et. al 2021 on MCF-7 cell line using etoposide gelatin nanoparticle (EtopNPs) where the highest activity of LDH was observed in Etop and all other assays followed the same trend as below. The enhanced intracellular ROS pushes the cancer cells in the state of oxidative stress. Although tumor cells easily adapt to this increased oxidative state to a certain extent by upregulating the antioxidant systems, the further ROS insults disrupt the transient intracellular redox balance, eventually leading to apoptosis and necrosis. PTX increased the intracellular ROS levels that effectively amplified the oxidative stress and triggered apoptosis in MCF-7 cells. ROS has a threshold value up to which helps in disease progression and above that level causes disease regression. This strategy can be used for effective tumor therapy. Hence, it has been concluded that, ROS has worked as 'double-edge weapon' showing potential to stimulate the disease and also, worked as potential therapeutic tool against cancer cells.

IV. CONCLUSION:

From above experimental research data, it has been concluded that paclitaxel cytotoxicity increases with dose and time dependent manner. The oxidative panel shows reduced levels of GSH and SOD post treatment with PTX coupled with enhanced levels of NO by PTX indicates an effective treatment for breast cancer treatment. Therefore, PTX treatment as antioxidants can suppress cancer initiation or progression.

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