Chapter 7

In-vitro Cell Line & In-vivo Efficacy Studies

TABLE OF CONTENTS

7.1 DETERMINATION OF SYNERGISM

Paclitaxel and Cyclophosphamide are widely used for treatment of Metastatic breast cancer [1]. However no literature exists which proves the synergism between Paclitaxel and Cyclophosphamide. Therefore, there arises a need to confirm the synergism between the two drugs. Literature review suggests that 200mg/m^2 dose of Paclitaxel and 1750mg/m^2 dose of Cyclophosphamide are suitable for the treatment of breast cancer [1]. Therefore, ratio of Paclitaxel to Cyclophosphamide as 1: 8.75 was considered for the determination synergism.

7.1.1 Methodology

The Response Additivity approach was adapted for the determination of synergism between Paclitaxel and Cyclophosphamide. The MTT assay was performed for plain drug Paclitaxel, plain drug Cyclophosphamide alone and Paclitaxel + Cyclophosphamide combination (1: 8.75) on MCF-7 cell line to determine the % viability and IC_{50} for all the three components [2-5].

MCF-7 Cells cultured in T-25 flasks were trypsinized and aspirated into a 5mL centrifuge tube and cell pellet was obtained by centrifugation at 300 x g [6-9]. The cell count was adjusted, using DMEM-HG medium, such that 200µL of suspension contained approximately 10,000 cells [6-9]. To each well of the 96 well microtitre plate, 200µL of the cell suspension was added and the plate was incubated at 37°C and 5% CO_2 atmosphere for 24 h [6-9]. After 24 h, the spent medium was aspirated and 200µL of different test concentrations of test drugs were added to the respective wells [6-9]. The plate was then incubated at 37°C and 5% CO_2 atmosphere for the specified time [6-9].

The plate was removed from the incubator and the drug containing media was aspirated. 200µL of medium containing10% MTT reagent was then added to each well to get a final concentration of 0.5mg/mL and the plate was incubated at 37°C and 5% CO_2 atmosphere for 3 h [6-9]. The culture medium was removed completely without disturbing the crystals formed. Then 100µL of solubilisation solution (DMSO) was added and the plate was gently shaken in a gyratory shaker to solubilise the formed formazan [6-9]. The absorbance was measured using a microplate reader at a wavelength of 570 nm and also at 630 nm [6-9]. The percentage growth inhibition was calculated, after subtracting the background and the blank [6-9].

7.1.2 Results and Discussion

Response Additivity is calculated as:

Expected effect = Effect of Drug 1+ Effect of Drug 2

If the response of the drug combination is higher than the expected effect, then the drug combination shows synergism whereas if the response is lower than the expected effect, the drug combination shows antagonism[2-5]. Based on the results obtained, the Response Additivity graph of Paclitaxel and Cyclophosphamide alone versus Paclitaxel and Cyclophosphamide combination was derived and is presented in Figure 7.1.

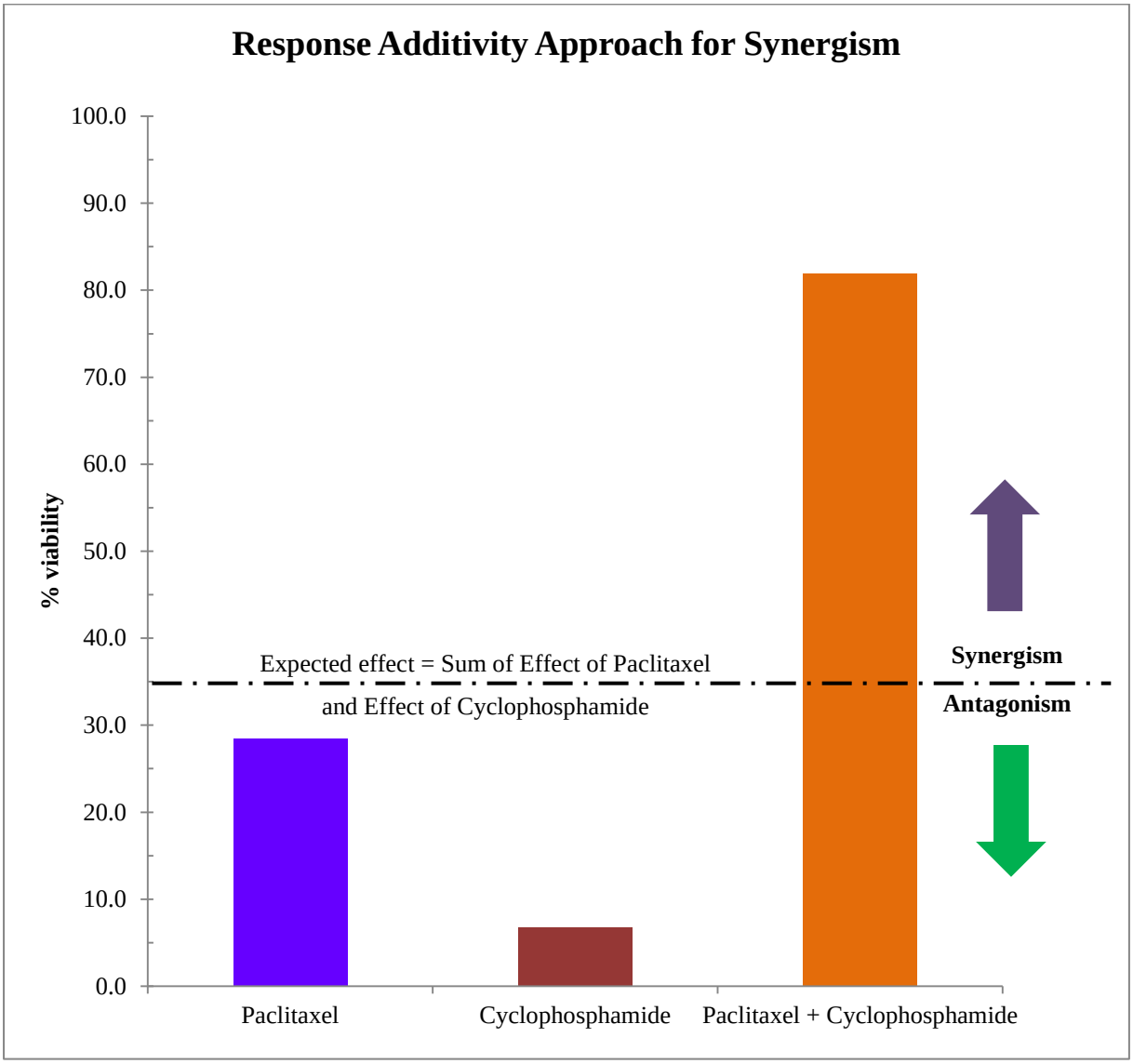


Figure 7.1: Response Additivity graph of Paclitaxel and Cyclophosphamide alone versus Paclitaxel and Cyclophosphamide combination (1: 8.75)

The analysis of Response Additivity reveals critical insights into pharmacological interactions between Paclitaxel and Cyclophosphamide. By comparing observed effects of drug combination against expected additive effects, it is evident that combination exhibits a synergistic effect, suggesting enhanced therapeutic efficacy when used together [10]. Figure 7.1 substantiates this finding, illustrating that combined treatment surpasses expected outcomes derived from individual drugs. This synergy not only underscores the potential for improved clinical outcomes but also supports the feasibility of employing this combination in novel drug delivery systems [11-13].

7.2 CELL VIABILITY ASSAY

The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation [6-9]. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH [6-9]. The resulting intracellular purple formazan can be solubilised and quantified by spectrophotometric means [6-9]. The assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability [6-9].

7.2.1 Methodology

MCF-7 Cells cultured in T-25 flasks were trypsinized and aspirated into a 5mL centrifuge tube and cell pellet was obtained by centrifugation at 300 x g [6-9]. The cell count was adjusted, using DMEM-HG medium, such that 200µL of suspension contained approximately 10,000 cells [6-9]. To each well of 96 wells microtitre plate, 200µL of cell suspension was added and plate was incubated at 37°C and 5% CO_2 atmosphere for 24 h [6-9]. After 24 h, spent medium was aspirated and 200µL of different test concentrations of test drugs were added to respective wells [6-9]. The plate was then incubated at 37°C and 5% CO_2 atmosphere for specified time [6-9].

The plate was removed from incubator and drug containing media was aspirated and 200µL of medium containing 10% MTT reagent was then added to each well to get a final concentration of 0.5mg/mL and plate was incubated at 37°C and 5% CO_2 atmosphere for 3 h [6-9]. The culture medium was removed completely without disturbing crystals formed and

then 100µL of solubilisation solution (DMSO) was added and plate was gently shaken in a gyratory shaker to solubilise formed formazan [6-9]. The absorbance was measured using a microplate reader at a wavelength of 570 nm and also at 630 nm [6-9]. The percentage growth inhibition was calculated, after subtracting background and blank, and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) was generated from dose-response curve for cell line [6-9].

7.2.2 Results and Discussion

To investigate therapeutic efficiency of formulations, MCF-7 cells were treated with pure Paclitaxel, pure Cyclophosphamide, Paclitaxel & Cyclophosphamide loaded NLCs and Paclitaxel & Cyclophosphamide loaded Microemulsion at different concentrations, and cell proliferation was measured by standard MTT colorimetric assay. Results are shown in Table 7.1 and In-vitro cytotoxicity analysis on MCF-7 cell lines at different time point is graphically described in Figure 7.2.

Pure Paclitaxel and pure Cyclophosphamide showed gradual, dose-dependent decrease in cell viability. At the highest concentration tested, cell viability decreased significantly, indicating strong cytotoxic effects. The combination of Paclitaxel and Cyclophosphamide within NLCs displays superior efficacy compared to either drug alone. This drastic reduction in cell viability suggests a potential synergy between Paclitaxel and Cyclophosphamide when encapsulated within NLCs. Microemulsions also show enhanced cytotoxic effects relative to pure drugs, though they are slightly less effective than NLCs.

The slight edge observed with NLCs over microemulsions can be attributed to several factors inherent to NLC technology. NLCs are known for their biocompatible and biodegradable lipid matrix, which not only enhances drug stability but also allows for controlled drug release, maintaining an effective concentration at the target site for longer periods [14]. This sustained release mechanism likely contributes to the enhanced cytotoxicity seen with NLCs. In contrast, while microemulsions improve drug solubility and bioavailability, they may have faster drug release kinetics, which could lead to a rapid peak in drug concentration followed by a quicker decline. This release profile might make microemulsions slightly less effective than NLCs in maintaining prolonged cytotoxic effects over time [14]. The in-vitro drug release studies indicate that PAC-CYC NLCs required 24 hours to achieve a release of over

90% of the drugs, whereas PAC-CYC microemulsion accomplished the same release within 6 hours.

Table 7.1: Results of In-vitro Cytotoxicity study

Pure Paclitaxel							
Conc. of PAC (µg/mL)	**Conc. of CYC (µg/mL)**	**Time Point**					
		24 Hours		**48 Hours**		**72 Hours**	
		% Viability	**% S.D.**	**% Viability**	**% S.D.**	**% Viability**	**% S.D.**
0	NA	100.00	0.67	100.00	1.63	100.00	1.01
2	NA	92.13	0.91	83.57	1.96	78.10	1.94
4	NA	83.25	2.21	74.66	2.63	69.80	1.49
6	NA	75.39	2.16	68.46	1.71	61.12	2.19
8	NA	67.16	1.19	63.94	2.62	54.45	2.79
10	NA	60.87	1.51	54.97	3.12	46.88	3.40
Pure Cyclophosphamide							
Conc. of PAC (µg/mL)	**Conc. of CYC (µg/mL)**	**Time Point**					
		24 Hours		**48 Hours**		**72 Hours**	
		% Viability	**% S.D.**	**% Viability**	**% S.D.**	**% Viability**	**% S.D.**
NA	**0**	100.00	0.67	100.00	1.63	100.00	2.43
NA	**17.5**	96.75	0.49	78.41	2.01	67.00	4.74
NA	**35**	87.66	2.11	72.40	5.75	58.54	2.89
NA	**52.5**	86.93	3.59	60.08	1.49	44.38	2.92
NA	**70**	77.62	2.99	45.60	1.06	15.28	0.43
NA	**87.5**	61.86	4.22	13.84	0.30	1.34	0.23
PAC-CYC NLCs							
Conc. of PAC (µg/mL)	**Conc. of CYC (µg/mL)**	**Time Point**					
		24 Hours		**48 Hours**		**72 Hours**	
		% Viability	**% S.D.**	**% Viability**	**% S.D.**	**% Viability**	**% S.D.**
0	**0**	100.00	0.67	100.00	1.63	100.00	2.43
2	**17.5**	84.94	6.02	69.31	2.91	54.52	6.56
4	**35**	75.24	2.24	63.35	2.60	43.15	2.96
6	**52.5**	70.91	3.44	54.21	1.19	32.90	1.44
8	**70**	67.04	1.51	45.70	1.89	19.63	1.10
10	**87.5**	60.93	6.22	25.29	2.19	4.18	0.54
PAC-CYC Microemulsion							
Conc. of PAC (µg/mL)	**Conc. of CYC (µg/mL)**	**Time Point**					
		24 Hours		**48 Hours**		**72 Hours**	
		% Viability	**% S.D.**	**% Viability**	**% S.D.**	**% Viability**	**% S.D.**
0	**0**	100.00	0.67	100.00	1.63	100.00	2.43
2	**17.5**	87.23	4.76	61.45	1.62	52.13	2.40
4	**35**	82.47	4.13	57.00	4.77	47.42	1.65
6	**52.5**	74.96	3.87	47.03	2.96	32.85	3.27
8	**70**	64.29	4.99	36.40	6.57	20.66	3.82
10	**87.5**	52.34	3.32	26.24	1.50	10.50	0.67

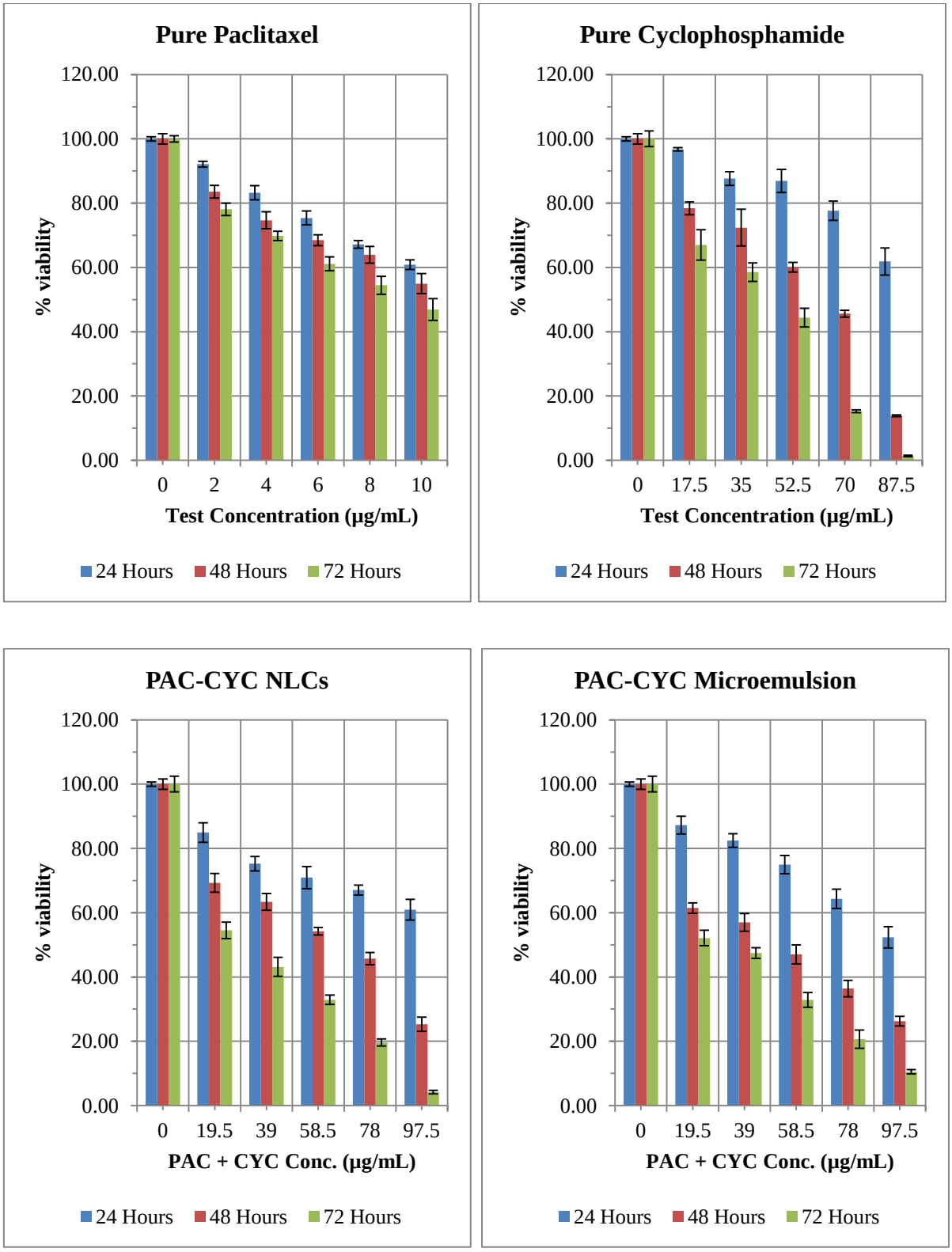


Figure 7.2: Results of In-vitro Cytotoxicity study

The combined use of Paclitaxel and Cyclophosphamide in both NLCs and microemulsions exemplifies the potential of synergistic drug formulations [15]. Each drug works through

distinct mechanisms: Paclitaxel targets cell division via microtubule disruption, while Cyclophosphamide targets DNA integrity. When used in tandem, these drugs can maximize cell death by simultaneously disrupting different cellular pathways essential for cancer cell survival [15, 16]. By encapsulating both drugs within NLCs or microemulsions, the study achieves a combined cytotoxic effect that is greater than either drug alone.

At 72 hours, pure Paclitaxel exhibited IC_{50} value of 9.10µg/mL whereas Paclitaxel in PAC-CYC NLCs exhibited IC_{50} value of 2.92µg/mL and Paclitaxel in PAC-CYC Microemulsion exhibited IC_{50} value of 2.86µg/mL. At 72 hours, pure Cyclophosphamide exhibited IC_{50} value of 39.80µg/mL whereas Cyclophosphamide in PAC-CYC NLCs exhibited IC_{50} value of 25.56µg/mL and Cyclophosphamide in PAC-CYC Microemulsion exhibited IC_{50} value of 24.99µg/mL. The observed differences in IC_{50} values for Paclitaxel and Cyclophosphamide when delivered via PAC-CYC NLCs and PAC-CYC Microemulsion compared to their pure forms was attributed to the enhanced drug delivery capabilities of nanotechnology-based formulations. Lower IC_{50} values for both Paclitaxel and Cyclophosphamide encapsulated in NLCs and microemulsion clearly confirm that low dose were sufficient to kill 50% of the MCF-7 cells in comparison to pure drugs. Both PAC-CYC NLCs and PAC-CYC Microemulsion exhibited low I_{C50} values in contrast to pure Paclitaxel and pure Cyclophosphamide.

To conclude, in-vitro cytotoxicity studies demonstrated that PAC-CYC loaded NLCs and microemulsion showed higher anti-proliferative activity than pure Paclitaxel and pure Cyclophosphamide. Even at very low dose, NLCs and Microemulsion therapy was much more effective than pure Paclitaxel and Cyclophosphamide alone [17]. PAC-CYC loaded NLCs and Microemulsion can thus increase the therapeutic efficiency of both drugs by suppressing the multidrug resistance phenomenon and exhibiting synergistic effect [18].

7.3 CELLULAR UPTAKE AND CELL APOPTOSIS BY FLUORESCENCE MICROSCOPY

The assessment of cellular uptake and apoptosis through fluorescence microscopy holds significant relevance in the realm of anticancer therapy. These methodologies assist in assessing the efficacy of anticancer therapeutics and elucidating their mechanisms of action at the cellular level. For an effective anticancer agent, it is essential that it penetrates the cancer cells at adequate concentrations. The drug uptake assay is instrumental in evaluating the efficiency of drug delivery and absorption by cancer cells [19, 20].

Apoptosis represents a regulated mechanism of cellular destruction that effectively reduces harm to adjacent tissues, in contrast to necrosis, which may lead to inflammatory responses. Apoptosis assays serve as essential tools for evaluating the synergistic effects of combination therapies [21, 22]. Fluorescence microscopy demonstrates increased apoptosis when multiple drugs are administered concurrently, indicating a potentially more effective therapeutic approach [19-22].

7.3.1 Methodology

7.3.1.1 Preparation of FITC tagged samples

FITC tagged pure Paclitaxel was prepared by dissolving Paclitaxel and FITC in mixture of ethanol and Cremophor EL (1:1). FITC tagged pure Cyclophosphamide was prepared by dissolving Cyclophosphamide and FITC in dehydrated ethanol. FITC tagged NLCs and microemulsions were prepared by dissolving FITC in lipid phase and further manufactured as per the optimized process [23-25].

7.3.1.2 Method for Cellular uptake and Apoptosis by fluorescence microscopy

Cellular uptake and Apoptosis study by fluorescence microscopy was performed for pure Paclitaxel, pure Cyclophosphamide and Paclitaxel & Cyclophosphamide combination loaded NLCs and Microemulsion.MCF-7 Human breast cancer cells were cultured in a 6-well plate at a density of 2 x 10^5 cells/2 ml and incubated in a CO_2 incubator overnight at 37°C for 24 hours and the spent medium was aspirated and washed with 1ml 1X PBS [26-29]. The cells were treated with FITC tagged NLCs/ Microemulsion in 2 ml of culture medium, incubated for 24 hours and then cells were washed two times with 1X PBS at end of treatment[26-29]. 500µL of mounting medium was added before imaging. Cells were observed under 10X &

20X objectives of fluorescence microscope using filter cube with Excitation 470/40 and Emission 525/50 for FITC. Images were analysed using ImageJ Software v1.48 [26-29].

7.3.2 Results and Discussion

The fluorescence microscopy images (Figure 7.3 and 7.4) of MCF-7 cells illustrate the uptake of FITC conjugated pure drug solutions, NLCs, and microemulsion. The bright green fluorescence contrasting with the dark background signifies the effective internalization of these drug-loaded NLCs and microemulsion by the cells.

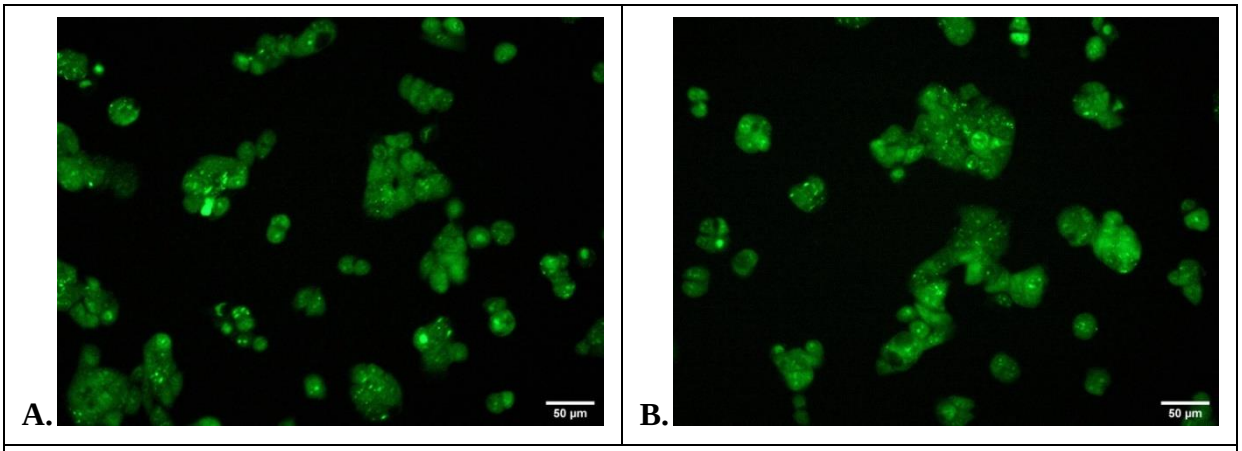


Figure 7.3: Cellular Uptake and Apoptosis study by Fluorescence Microscopy of [A] Pure Paclitaxel and [B] Pure Cyclophosphamide

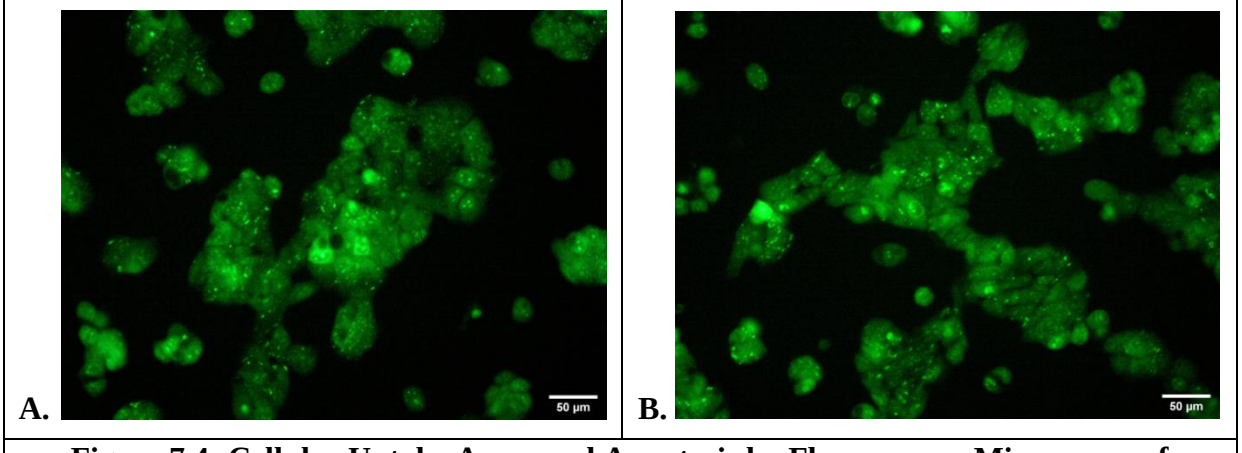


Figure 7.4: Cellular Uptake Assay and Apoptosis by Fluorescence Microscopy of [A] PAC-CYC loaded NLCs and [B] PAC-CYC loaded microemulsion

The observed bright green fluorescence in the cells indicated uptake of the pure Paclitaxel, pure Cyclophosphamide, PAC-CYC loaded NLCs, and PAC-CYC loaded Microemulsion by the cells. The Figure 7.3[A] and 7.3[B], depicts MCF-7 cells exhibiting extensive blebbing along with aggregation and cluster formation, a distinct morphological characteristic

associated with apoptosis in cell cycle. Blebbing, Aggregation and cluster formation was more in PAC-CYC loaded NLCs, and PAC-CYC loaded Microemulsion as compared to pure Paclitaxel and pure Cyclophosphamide. Blebs represent atypical growth or bulges of the plasma membrane, resulting from localized separation of the cytoskeleton from the plasma membrane. The observed blebs appear as small, spherical formations extending from the cell surface, indicative of cells in the process of apoptosis [30-34].

The increased aggregation, cluster formation, and blebbing observed in MCF-7 cells when treated with Paclitaxel and Cyclophosphamide-loaded NLCs and microemulsion as opposed to individual drug treatments was attributed to several synergistic and mechanistic factors. NLCs and microemulsion both facilitated the simultaneous delivery of both Paclitaxel and Cyclophosphamide, allowing them to work together at the cellular level. This combination therapy promoted synergistic effects, leading to enhanced cytotoxicity and more aggressive cellular responses such as increased aggregation and cluster formation [30-34]. Synergy between the drugs can amplify their impact on disrupting cellular structures, thereby inducing blebbing more than individual drug treatments would. NLCs and microemulsion improved drug availability and enable more efficient cellular uptake due to their nano-sized structure and lipid composition, which is similar to cellular membranes [33-36].

The NLCs and microemulsion can penetrate cell membranes more effectively, releasing the drugs in a controlled manner, which intensifies the stress on cancer cells and accelerates cytotoxic effects. This resulted in more pronounced physical changes, including blebbing and aggregation in the cell culture. Both Paclitaxel and Cyclophosphamide have mechanisms that target the cytoskeleton and mitochondria. Paclitaxel disrupts microtubule dynamics, while Cyclophosphamide causes DNA cross-linking. The dual effect of DNA damage and cytoskeletal disruption, when enhanced by the NLC and microemulsion delivery system, caused severe cellular stress, which manifested as cell membrane blebbing (a hallmark of apoptosis) and clustering as cells lost structural integrity [30-36].

Thus it is well evident that PAC-CYC NLCs and PAC-CYC microemulsion have better cellular uptake and apoptosis as compared to pure Paclitaxel and pure Cyclophosphamide.

7.4 CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

Cellular growth is considered as successive phases, characterized by specific biochemical processes known as cell cycle [37, 38]. Flow cytometry was used to determine the distribution of DNA in the cell replication state [37]. Quiescent and G1 cells will have one copy of DNA having 1X fluorescence intensity [37]. G2/M phase of the cell cycle will have two copies of DNA having 2X fluorescence intensity [37]. S phase which synthesizes DNA will have fluorescence values between the 1X and 2X populations [37, 38].

7.4.1 Methodology

MCF-7 cells were cultured in a 6-well plate at a density of 3 x 10^5 cells/2mL and incubated in a CO_2 incubator overnight at 37°C for 24 hours, spent medium was aspirated and washed with 1mL 1X PBS [39-42]. The cells were treated with PAC-CYC NLCs and PAC-CYC Microemulsion in 2 mL of culture medium and incubated for 24 hours. One of the wells was left as untreated to be used as negative control andfurther cells were fixed in 1ml cold 70% ethanol by adding 70% ethanol drop wise to cell pellet while vortexing to ensure fixation of all cells and minimise clumping[39-42].

Further the cells were fixed for at least 30 minutes on ice. Cells were washed with PBS twice and resuspended in 400 µL PI-RNase solution per million cells and taken into 12x75mm tubes and incubated for 5 to 10 minutes at room temperature [39-42]. Samples were mixed well and analysed by Cytomics FC500 Flow cytometer, Beckman Coulter, USA [39-42].

7.4.2 Results and Discussion

The results of cell cycle analysis are presented in Table 7.2.

Table 7.2: Results of Cell cycle distribution in MCF-7 cells using Flow cytometry

Cell Phase	Untreated Sample	Pure PAC	Pure CYC	PAC-CYC NLCs	PAC-CYC microemulsion
	% cells	% cells	% cells	% cells	% cells
SubG1	2.13	2.56	1.19	0.88	3.40
G0/G1	54.7	52.14	52.51	33.4	36.0
S	11.9	10.9	18.2	23.4	23.8
G2/M	30.4	33.4	30.4	40.6	35.2

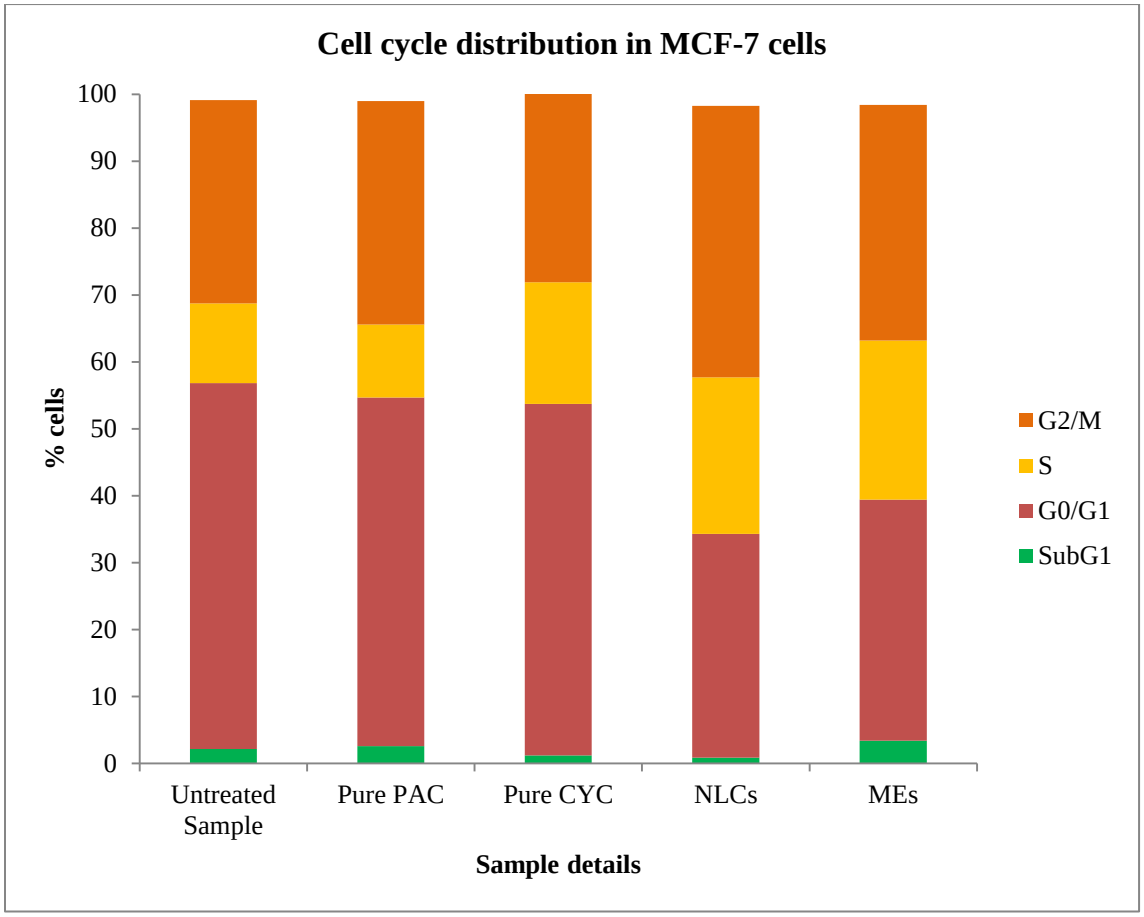


Figure 7.5: Cell cycle distribution in MCF-7 cells using Flow cytometry

Referring to Table 7.2 and Figure 7.5, pure Paclitaxel showed accumulation of cells in G2/M phase and pure Cyclophosphamide showed halting of cells in S phase. However, Paclitaxel loaded in PAC-CYC NLCs and microemulsion showed higher accumulation of cells in G2/M phase in comparison to pure Paclitaxel and Cyclophosphamide loaded in NLCs and microemulsion showed higher halting of cells in S Phase. The enhanced accumulation of cells in the G2/M phase by Paclitaxel and the increased halting of cells in the S phase by Cyclophosphamide when loaded in NLCs and microemulsions can be attributed to the improved delivery \of these nanocarrier systems [43, 44].

It is clear that there was reduction of % cells in G0/G1 phase. There was 2 folds increase in % cells at S phase, and there was significant increase in % cells at G2/M phase for both PAC-CYC loaded NLCs and PAC-CYC loaded microemulsion when compared to untreated sample. In untreated samples, cellular population was predominantly observed in the G0/G1 phase, indicating a state of quiescence or initiation of preparations for DNA replication [47].

Following administration of Paclitaxel and cyclophosphamide loaded NLCs and microemulsions, the cells transitioned from the G0/G1 phase as they advanced through the cell cycle, yet endured a blockade in the subsequent phases (S and G2/M) [48-57].

The integrated therapeutic approach prompted a greater number of cells to transition from the G0/G1 phase into cell cycle, thereby exposing them to the pharmacological effects of the treatment [25, 58]. The DNA damage response induced by cyclophosphamide and inhibition of mitotic spindle by Paclitaxel significantly decreased G0/G1 cell population by obstructing their advancement in cell cycle due to synergistic effect of combination loaded in NLCs and microemulsion [25, 58]. This led to more effective drug delivery to cancer cells, resulting in enhanced therapeutic effects. Thus, cyclophosphamide-induced DNA damage caused arrests of cancer cells in S phase and microtubule stabilization effect of Paclitaxel prevented proper mitotic spindle formation which arrested cells at the G2/M phase [25, 58-60].

Thus synergistic combination of Paclitaxel and Cyclophosphamide loaded in NLCs and microemulsion could demonstrate better cell arrest in in-vitro cell cycle analysis study in comparison to pure drugs.

7.5 IN-VIVO EFFICACY STUDY

7.5.1 Methodology

The in-vivo efficacy of Paclitaxel and Cyclophosphamide loaded Nanostructured Lipid Carriers (NLCs) and Microemulsion (ME) was evaluated using tumor regression studies in xenograft model in female Sprague Dawley rats.

Female Sprague Dawley rats from the animal facility of Department of Pharmacology, Institute of Pharmacy of Nirma University were used [60]. Animals were kept in a room with ventilation (16-18 air changes/hour) relative humidity (45-65%), controlled temperature (20-24 ºC) and light/ dark cycle of 12 hours [60, 61]. The rats were fed a normal diet and given tap water to drink ad libitum and all animal experimentations were carried out in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines, using Institutional Animal Ethics Committee (IAEC) approved protocols [60, 61].

Animals were divided randomly into 5 groups with each group containing 6 animals. The carcinogen 7, 12-dimethylbenzanthracene (DMBA) diluted in olive oil was used for induction of breast cancer [61]. In order to induce tumours in female rats, 35 mg/kg DMBA in 1 ml olive oil was injected subcutaneously beneath third mammary gland on right flank and animals were palpated twice per week beginning five weeks after DMBA administration to detect tumours [61]. Tumours were observed after 12 weeks after DMBA induction. Treatment was started after tumor size of 80mm^3 was reached. INTAXEL® of Fresenius Kabi (Paclitaxel Injection 30mg/5mL (6mg/mL) Reference Product was given at the dose of 2mg/kg for once a week for 6 weeks, ENDOXAN™ of Zydus Lifesciences (Cyclophosphamide for Injection, 200mg/vial) reference standard was given at the dose of 17.5mg/kg for once a week for 6 weeks, NLCs and Microemulsion were given at dose where Paclitaxel was equivalent to 2mg/kg and Cyclophosphamide was equivalent to 17.5mg/kg for once a week for 6 weeks. Animals were euthanized using thiopental sodium (120 mg/kg) after 6 weeks of dosing [61]. Necropsy was performed immediately after euthanasia. Tumors were collected for histological estimations. For histological examination, Formalin-fixed tissues were embedded in paraffin, cut into 5 µm thick sections, and stained with haematoxylin and eosin (H&E) and histopathological changes were photographed using a digital camera mounted on a BX51 Olympus optical microscope. [62-64]

7.5.2 Results and Discussion

To demonstrate the antitumor efficacy, INTAXEL®, ENDOXAN™, PAC-CYC NLCs and PAC-CYC Microemulsion were injected in breast cancer bearing rats via lateral tail vein after dilution with 0.9% sodium chloride Injection. The graph of Tumor volume versus drug sample dosed is presented in Figure 7.6. The representative images of tumor for disease control, INTAXEL®, ENDOXAN™, PAC-CYC NLCs and PAC-CYC Microemulsion are presented in Figure 7.7.

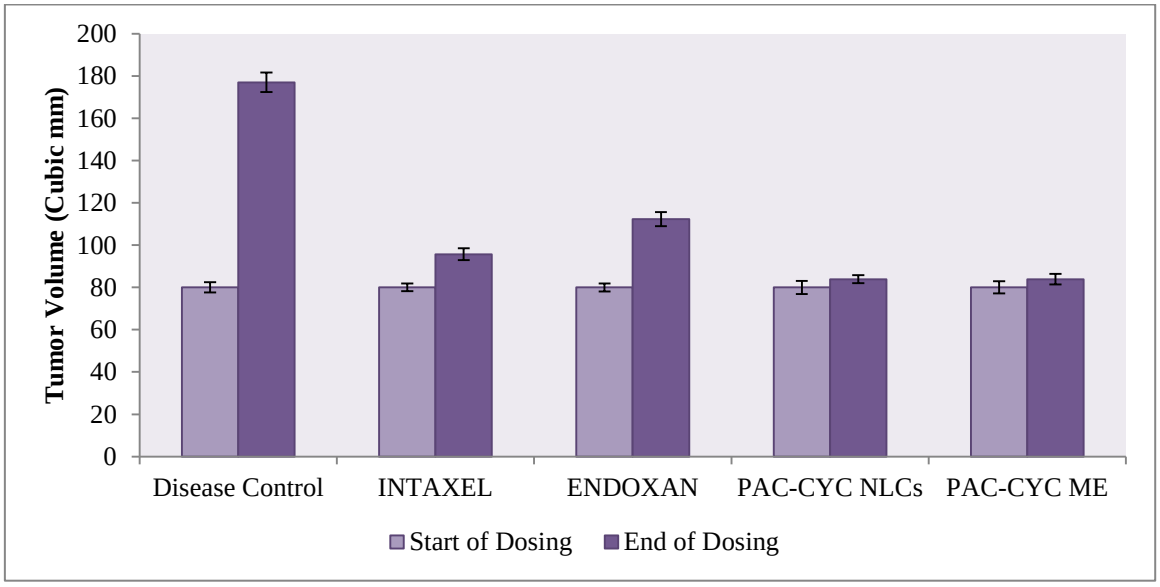
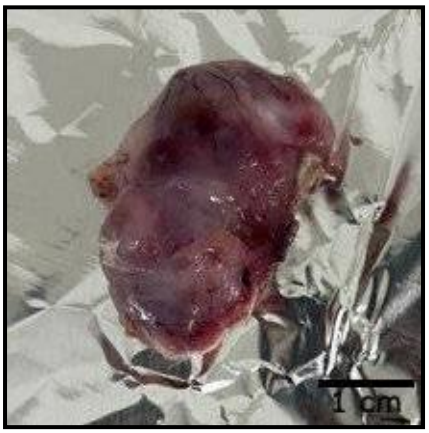


Figure 7.6: Observed Tumor volume for Disease control vs. Treatment groups

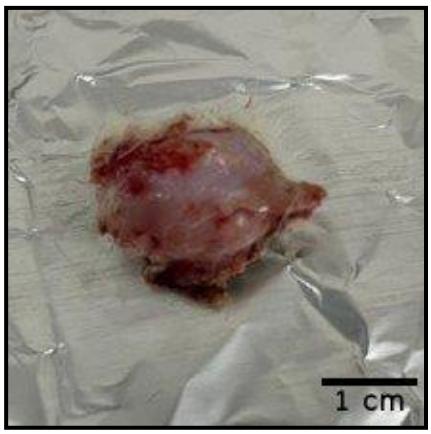
Dosing was started in rats when the tumor volume size of 80mm^3 was reached. In disease control group, the tumor volume reached to ~ 180 mm^3 size within 6 weeks, as no treatment was given to the rats. When compared to disease control group, tumor growth was retarded in INTAXEL and ENDOXAN group whereas groups treated with PAC-CYC NLCs and PAC-CYC microemulsion prevented the tumor from growing. The prevention of tumor growth in groups treated with PAC-CYC NLCs and PAC-CYC microemulsion can be attributed to the enhanced delivery of these formulations [65-67].

These advanced drug delivery systems improved the bioavailability and therapeutic efficacy of the chemotherapeutic agents, allowing for more effective targeting of tumor cells [65-67]. The combination of Paclitaxel and Cyclophosphamide in NLCs and microemulsions can provide synergistic effects, enhancing the overall anti-tumor activity [68]. These formulations

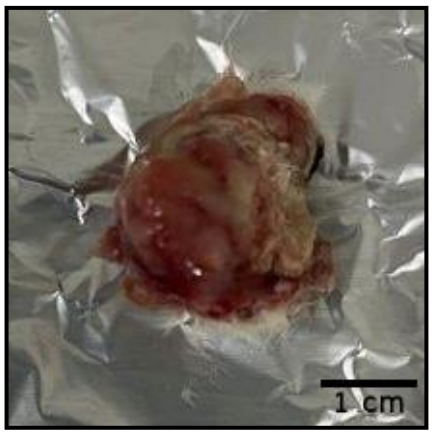
allow for the simultaneous delivery of both drugs, potentially improving their efficacy compared to single-agent treatments [69, 70]. The images of tumours collected from different groups are presented in Figure 7.7.



A. Disease Control Group

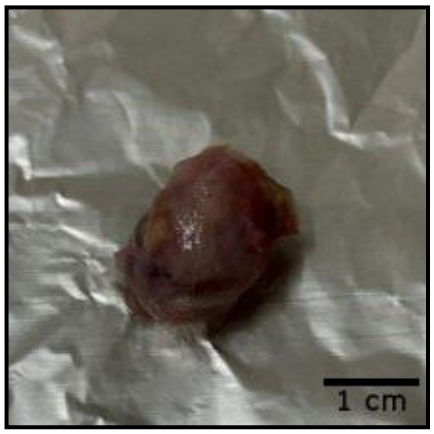


B. INTAXEL treated Group



C. ENDOXAN treated Group

D. PAC-CYC NLCs treated Group



E. PAC-CYC ME treated Group

Figure 7.7: Images of tumor for disease control, INTAXEL®, ENDOXAN™, PAC-CYC NLCs and PAC-CYC Microemulsion

Further, the histological examination of tumours was done to understand the impact of treatment. Histological examination of tumours collected after 6 weeks of treatment are presented in Figure 7.8 to Figure 7.13. Figure 7.8 shows histological micrographs of normal control group with no induction of breast cancer. Histological micrographs of normal control group showed normal adipose tissue, normal ducts and epithelial cells. There was no presence of any tumour cells in adipose tissue, epithelial cells, normal ducts and epithelial cells.

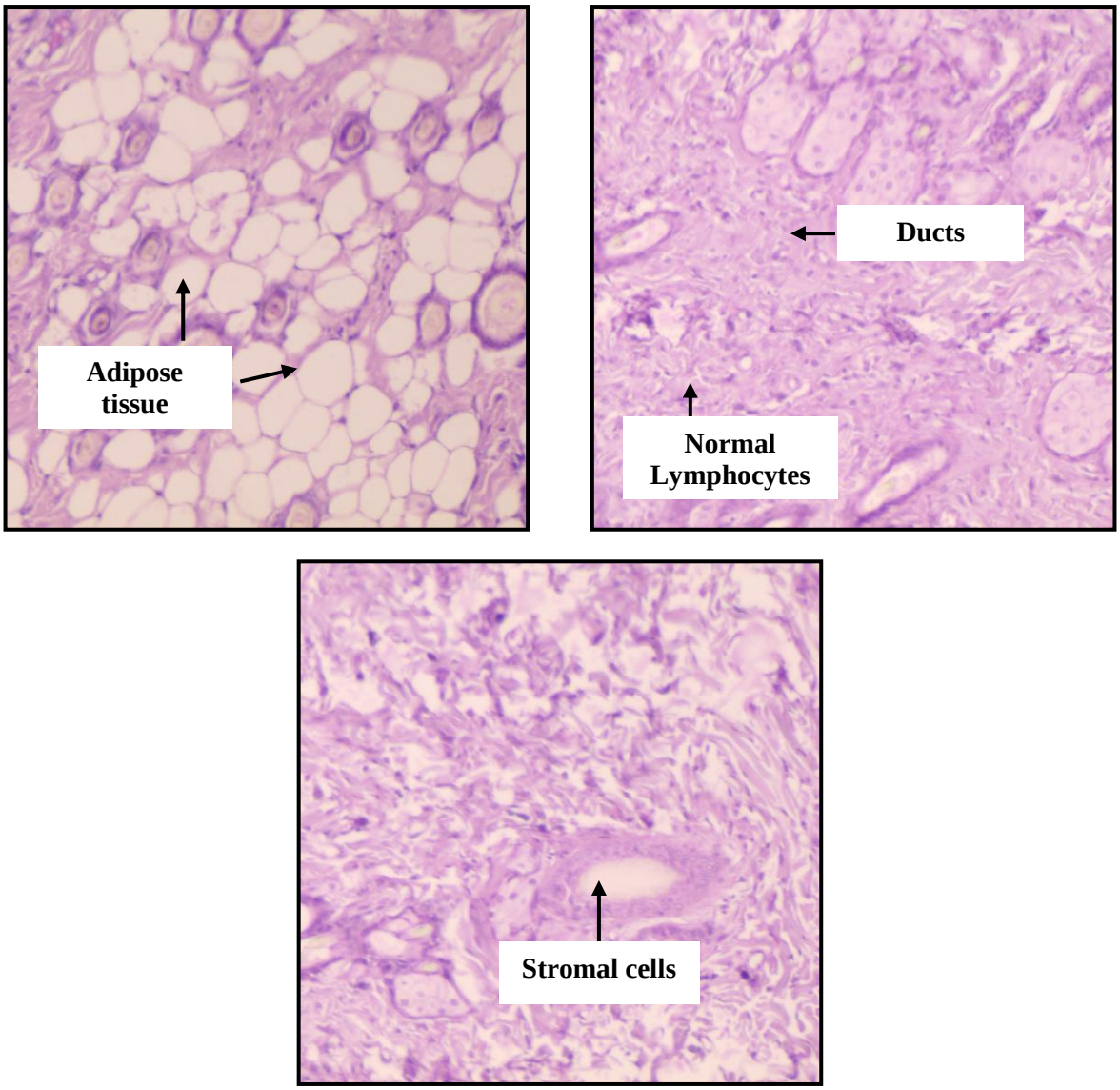


Figure 7.8: Histological micrographs of Normal control group showing normal adipose tissue, epithelial cells, normal ducts and epithelial cells

Figure 7.9 shows histological micrographs of disease control group where the rats induced with breast cancer were not given any treatment. Histological micrographs clearly depict infiltration of adipose tissue by tumor cells and increased lymphocytes. Calcification of ducts and infiltration of stromal cells by tumor cells is clearly visible in histological micrographs of disease control group. Based on the histological micrographs of disease control group, it is evident that breast cancer was induced in female rats.

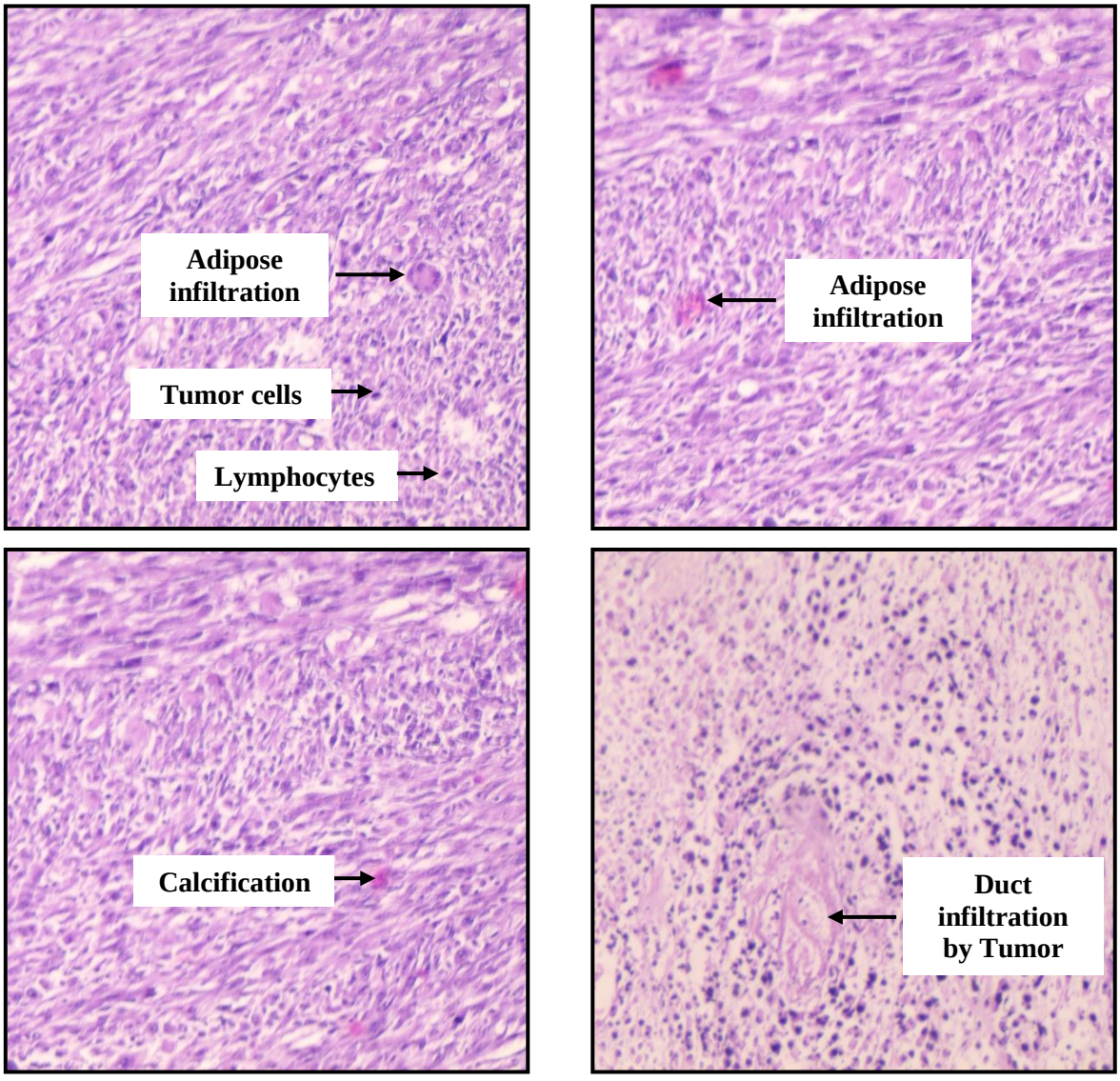


Figure 7.9: Histological micrographs of Disease control group

Figure 7.10 and Figure 7.11 presents the histological micrographs of groups treated with INTAXEL (Paclitaxel Injection) and ENDOXAN (Cyclophosphamide Injection). Individual treatment of Paclitaxel Injection was not completely effective in eradicating the tumour cells

across the breast tissues. There was only partial reduction of tumour cells in adipose tissue, epithelial cells, and other tissues however there was no calcification observed.

The partial reduction of tumor cells and the absence of complete eradication suggest the emergence of drug resistance mechanisms. These mechanisms can be attributed to various molecular and cellular factors that affect the drugs' effectiveness. One of the primary mechanisms of resistance to Paclitaxel involves the overexpression of ATP-binding cassette (ABC) transporters, which actively pump the drug out of cancer cells, reducing its intracellular concentration and efficacy [71, 72]. Changes in the molecular targets of Paclitaxel, such as microtubules, and associated proteins can lead to reduced drug binding and effectiveness [71].

In the case of Cyclophosphamide, resistance can develop gradually, as observed in murine models, where repeated drug exposure leads to decreased therapeutic response without the selection of highly resistant sub-populations [73]. Additionally, the weekly administration of Paclitaxel and Cyclophosphamide at moderate doses is designed to maintain sustained exposure to tumor cells, potentially improving efficacy. However, this approach may not be sufficient to overcome resistance or achieve complete tumor eradication in all cases [74]. Also, the absence of targeted delivery systems may contribute to the partial reduction of tumor cells. Targeted therapies, including those using nanoparticles, can improve drug accumulation in tumor tissues and reduce systemic toxicity, leading to better outcomes [72, 75].

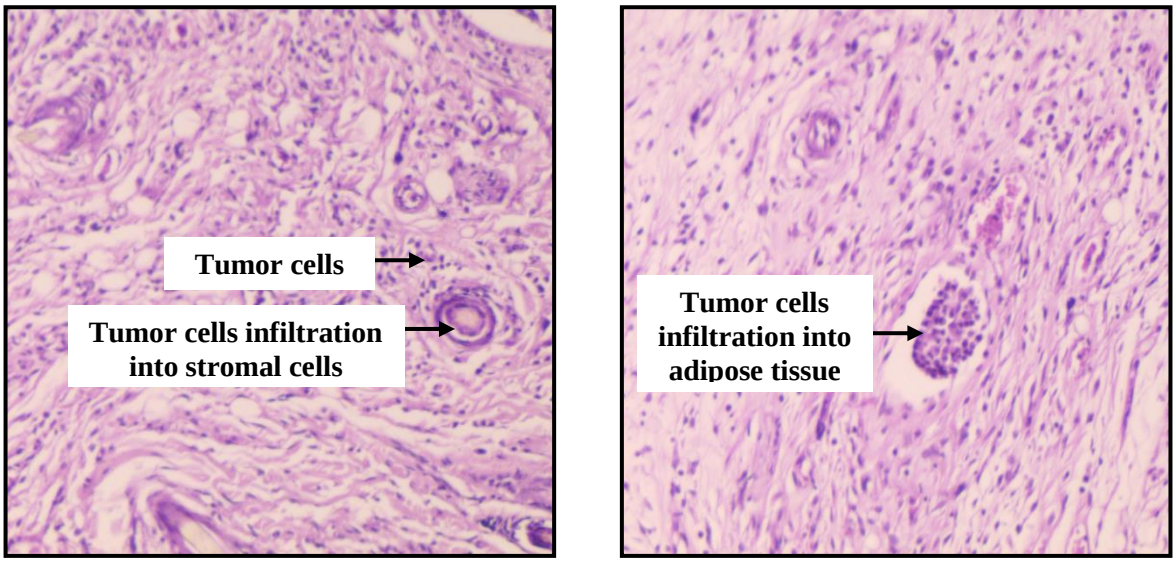


Figure 7.10: Histological micrographs of group treated with INTAXEL

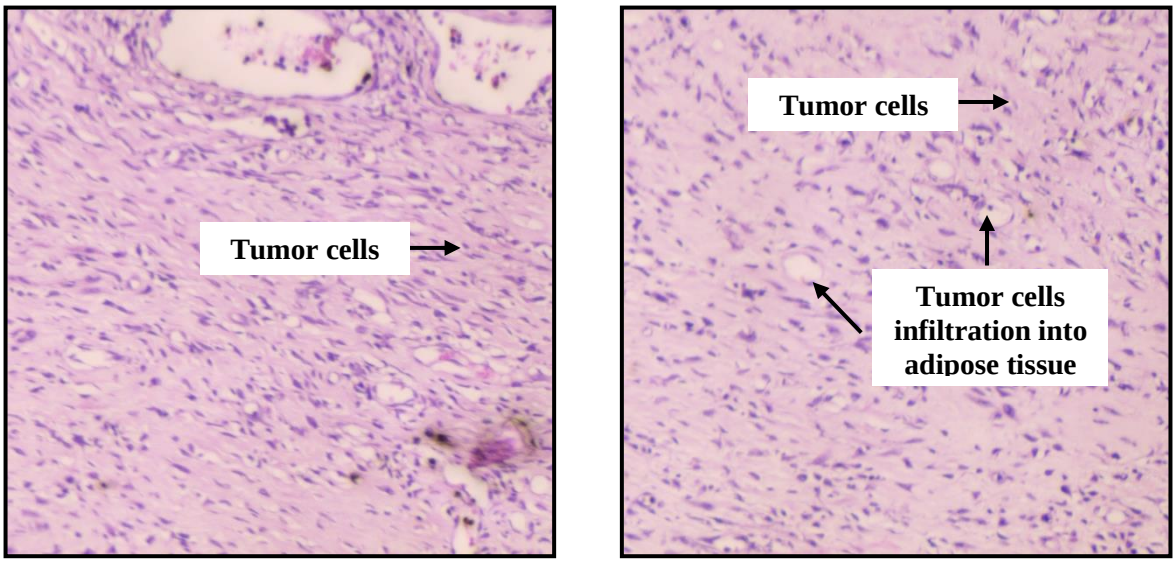


Figure 7.11: Histological micrographs of group treated with ENDOXAN

Figure 7.12 presents the histological micrographs of group treated with Paclitaxel and Cyclophosphamide loaded NLCs which showed no infiltration of tumor cells within the adipose tissue, and normal ducts. Few tumor cells were observed in the epithelial cells; however the number was negligible as compared to micrographs of disease control group, INTAXEL group and ENDOXAN group. There was no calcification observed and the tissues lacked the abundance of lymphocytes which is typical in cancerous condition.

The use of Paclitaxel and Cyclophosphamide loaded in nanostructured lipid carriers resulted in significant tumor regression, as evidenced by the absence of tumor cell infiltration in adipose tissue and normal ductal structures. The negligible presence of tumor cells in epithelial tissues, compared to control groups, highlights the efficacy of this treatment [76]. The absence of calcification and reduced lymphocyte abundance further indicate a reduction in cancerous conditions, suggesting that the NLCs effectively delivered the drugs to the tumor site, enhancing their therapeutic action [77].

NLCs provide a promising platform for the co-delivery of synergistic drug combinations, improving pharmacokinetics and enhancing intracellular drug delivery [78]. NLCs exhibited improved delivery and efficacy of chemotherapeutic agents like Paclitaxel and Cyclophosphamide by targeting delivery to tumor sites [79]. The combination of Paclitaxel and Cyclophosphamide exploited their non-overlapping mechanisms of action and enhancing therapeutic outcomes [79]. Thus Paclitaxel and Cyclophosphamide combination loaded in NLCs and microemulsion was effective even at the moderate doses of both the drugs which were not evident in individualized and conventional formulations of INTAXEL and CYTOXAN.

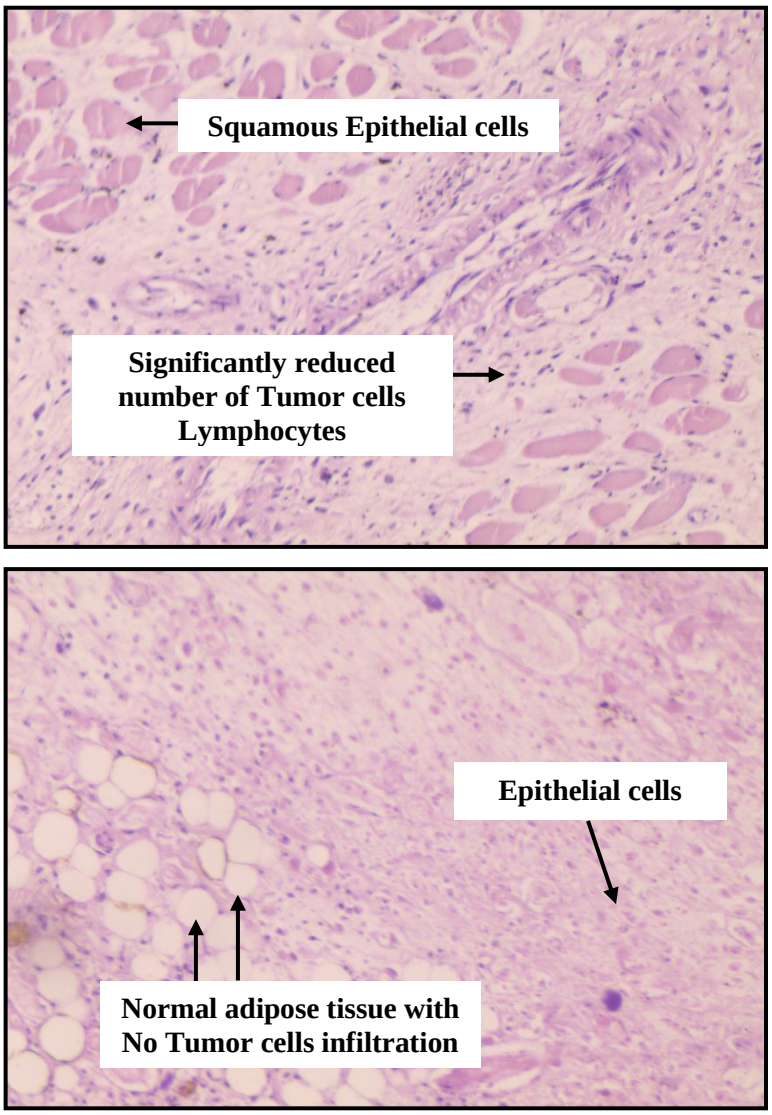


Figure 7.12: Histological micrographs of group treated with PAC-CYC NLCs

Figure 7.13 presents the histological micrographs of group treated with Paclitaxel and Cyclophosphamide loaded microemulsions which exhibited complete absence of tumour cells from the breast tissues. Histological micrographs show normal adipose tissues, ducts, normal lymphocytes, and stromal cells with no tumor cell infiltration. Micrographs of group treated with PAC-CYC Microemulsion were comparable to normal control group and strongly represented effective therapy for the treatment of breast cancer.

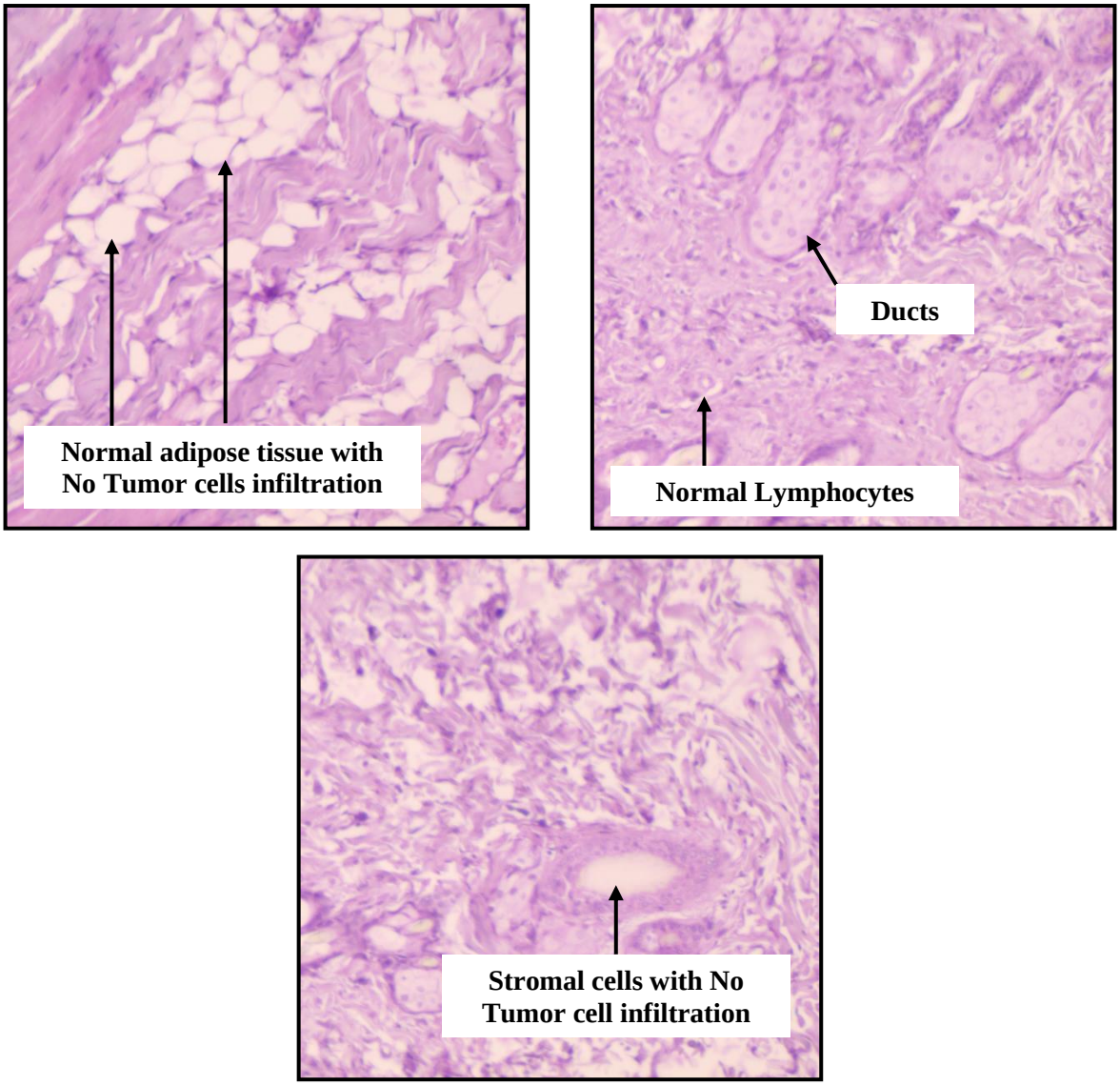


Figure 7.13: Histological micrographs of group treated with PAC-CYC Microemulsion

Thus, the use of Paclitaxel and Cyclophosphamide loaded microemulsions for treating DMBA-induced breast cancer in rats showed promising results, with complete eradication of tumor cells observed in treated group. This approach leveraged the benefits of microemulsions as drug delivery systems, enhancing the therapeutic efficacy of the chemotherapeutic agents [80]. This combination, when delivered via microemulsion, showed superior outcomes compared to conventional formulations, highlighting the importance of delivery systems in enhancing drug efficacy [81].

REFERENCES

1. Pagani O, Sessa C, Martinelli G, Cerny T, de Jong J, Goldhirsch A, Zimatore M, Cavalli F. Dose-finding study of paclitaxel and cyclophosphamide in advanced breast cancer. Ann Oncol. 1997 Jul;8(7):655-61. doi: 10.1023/a:1008211629858. PMID: 9296218.

2. Duarte D, Vale N. Evaluation of synergism in drug combinations and reference models for future orientations in oncology. Curr Res Pharmacol Drug Discov. 2022 May 12;3:100110. doi: 10.1016/j.crphar.2022.100110. PMID: 35620200; PMCID: PMC9127325.

3. Tallarida RJ. Quantitative methods for assessing drug synergism. Genes Cancer. 2011 Nov;2(11):1003-8. doi: 10.1177/1947601912440575. PMID: 22737266; PMCID: PMC3379564.

4. Foucquier J, Guedj M. Analysis of drug combinations: current methodological landscape. Pharmacol Res Perspect. 2015 Jun;3(3):e00149. doi: 10.1002/prp2.149. Epub 2015 May 20. Erratum in: Pharmacol Res Perspect. 2019 Dec;7(6):e00549. PMID: 26171228; PMCID: PMC4492765.

5. Simone, Lederer.,Tjeerd, M., H., Dijkstra., Tom, Heskes. (2018). 2. Additive Dose Response Models: Defining Synergy. bioRxiv, doi: 10.1101/480608

6. MTT Cell Proliferation Assay Instruction Guide – ATCC, VA, USA www.atcc.org

7. Carla, Luís.,Yuselis, Castaño-Guerrero., Raquel, Soares., Goreti, F., Sales., Rúben, Fernandes. (2019). 2. Avoiding the Interference of Doxorubicin with MTT Measurements on the MCF-7 Breast Cancer Cell Line.. doi: 10.3390/MPS2020029

8. Somaieh, Ahmadian., Jaleh, Barar., Amir, Ata, Saei., Mohammad, Amin, Abolghassemi, Fakhree., Yadollah, Omidi. (2009). 4. Cellular toxicity of nanogenomedicine in MCF-7 cell line: MTT assay.. Journal of Visualized Experiments, doi: 10.3791/1191

9. Wan, Yong, Ho., Swee, Keong, Yeap., Chai, Ling, Ho., Raha, Abdul, Rahim., Noorjahan, Banu, Alitheen. (2012). 3. Development of Multicellular Tumor Spheroid (MCTS) Culture from Breast Cancer Cell and a High Throughput Screening Method Using the MTT Assay. PLOS ONE, doi: 10.1371/JOURNAL.PONE.0044640

10. Rocchetti, M., Del Bene, F., Germani, M., Fiorentini, F., Poggesi, I., Pesenti, E., Magni, P., & De Nicolao, G. (2009). Testing additivity of anticancer agents in pre-clinical studies: a PK/PD modelling approach. European Journal of Cancer. https://doi.org/10.1016/J.EJCA.2009.09.025

11. Whitehead, K. A., Karr, N., &Mitragotri, S. (2008). Discovery of synergistic permeation enhancers for oral drug delivery. Journal of Controlled Release. https://doi.org/10.1016/J.JCONREL.2008.03.005

12. He, C., Tang, Z., Tian, H., & Chen, X. (2016). Co-delivery of chemotherapeutics and proteins for synergistic therapy. Advanced Drug Delivery Reviews. https://doi.org/10.1016/J.ADDR.2015.10.021

13. Hwangbo, H., Patterson, S. C., Dai, A.-X., Plana, D., & Palmer, A. C. (2023). Abstract 5718: Additivity predicts the clinical efficacy of most approved combination therapies for advanced cancer. Cancer Research. https://doi.org/10.1158/1538-7445.am2023-5718

14. Ali, Sabouri, Shirazi.,Reyhaneh, Varshochian., Reyhaneh, Varshochian., Mahsa, Rezaei., Yalda, Hosseinzadeh, Ardakani., Rassoul, Dinarvand. (2021). 1. SN38 loaded nanostructured lipid carriers (NLCs); preparation and in vitro evaluations against glioblastoma. Journal of Materials Science: Materials in Medicine, doi: 10.1007/S10856-021-06538-2

15. Thu, Thi, Ninh., Tuan, Hiep, Tran., Chi, Ying, F., Huang., Chien, Ngoc, Nguyen. (2022). 5. Application of computational screening tools and nanotechnology for enhanced drug synergism in cancer therapy.. Current Drug Delivery, doi: 10.2174/1567201819666220426092538

16. Martin, Peifer., Jonathan, Weiss., Martin, L., Sos., Mirjam, Koker., Stefanie, Heynck., Christian, Netzer., Stefanie, Fischer., Haridas, B., Rode., Daniel, Rauh., Jörg, Rahnenführer., Roman, K., Thomas. (2010). 9. Analysis of compound synergy in high-throughput cellular screens by population-based lifetime modeling. PLOS ONE, doi: 10.1371/JOURNAL.PONE.0008919

17. Razan, B., Alhumaidi., Bahgat, Fayed., Sarra, B., Shakartalla., Jayalakshmi, Jagal., Manju, Jayakumar., Zainab, Al, Shareef., Suleiman, I., Sharif., Ayman, M., Noreddin., Mohammad, H., Semreen., Hany, M., Omar., Mohamed, Haider., Sameh, S., M., Soliman. (2022). 6. Optimum inhibition of MCF-7 breast cancer cells by efficient targeting of the macropinocytosis using optimized paclitaxel-loaded nanoparticles.. Life sciences, doi: 10.1016/j.lfs.2022.120778

18. Qingmei, Yuan.,Yadong, Song., Qinming, Xu., Xiaocui, Deng. (2023). 1. Controllable release of self-assembled reduction-sensitive paclitaxel dimer prodrug and tetrandrine nanoparticles promotes synergistic therapy against multidrug-resistant

cancer..Biochimica Et Biophysica Acta - General Subjects, doi: 10.1016/j.bbagen.2023.130362Chuandong, Ge.,Zhe, Chen., Heming, Sun., Ping, Sun., Jiayin, Zhao., Yanjuan, Wu., Jing, Xu., Mingyang, Zhou., Mingming, Luan. (2024). 1. Visually evaluating drug efficacy in living cells using COF-based fluorescent nanoprobe via CHA amplified detection of miRNA and simultaneous apoptosis imaging. Analytica Chimica Acta, doi: 10.1016/j.aca.2024.342502

19. Nicole, D., Barth., Lorena, Mendive-Tapia., Ramon, Subiros-Funosas., Ouldouz, Ghashghaei., Rodolfo, Lavilla., Laura, Maiorino., Xue, Y., He., Ian, Dransfield., Mikala, Egeblad., M., Vendrell. (2021). 4. A Bivalent Activatable Fluorescent Probe for Screening and Intravital Imaging of Chemotherapy-Induced Cancer Cell Death. doi: 10.1002/ange.202113020

20. Robert, Mandelkow., Denis, Gümbel., Hannes, Ahrend., Anne, Kaul., Uwe, Zimmermann., Martin, Burchardt., Matthias, B., Stope. (2017). 3. Detection and Quantification of Nuclear Morphology Changes in Apoptotic Cells by Fluorescence Microscopy and Subsequent Analysis of Visualized Fluorescent Signals.. Anticancer Research, doi: 10.21873/ANTICANRES.11560

21. Verena, Richter., Petra, Weber., Michael, Wagner., Sarah, Schickinger., Thomas, Bruns., Herbert, Schneckenburger. (2013). 5. Visualizing a cytostatic drug and probing apoptosis of cancer cells. Biomedical spectroscopy and imaging, doi: 10.1117/12.2032461

22. Luana, Almeida, Fiel., Renata, Vidor, Contri., Juliane, Freitas, Bica., Fabrício, Figueiró., Ana, Maria, Oliveira, Battastini., Silvia, Stanisçuaski, Guterres., Adriana, Raffin, Pohlmann. (2014). 7. Labeling the oily core of nanocapsules and lipid-core nanocapsules with a triglyceride conjugated to a fluorescent dye as a strategy to particle tracking in biological studies. Nanoscale Research Letters, doi: 10.1186/1556-276X-9-233

23. Leng, Xi-gang. (2008). 8. Optimization of the assay for labeling chitosan-DNA nanoparticles with fluorescein isothiocyanate. Biomedical Engineering and Clinical Medicine

24. Medha, D., Joshi., Rashmi, H., Prabhu., Vandana, B., Patravale. (2019). 3. Fabrication of Nanostructured Lipid Carriers (NLC)-Based Gels from Microemulsion Template for Delivery Through Skin. Methods of Molecular Biology, doi: 10.1007/978-1-4939-9516-5_19

25. Pawley JB. (1995). Handbook of Biological Confocal Microscopy (2nd Edition). Plenum Publishing Corporation, New York.

26. Avrum, Jacobson.,Jozo, Delic., Henri, Magdelenat. (2020). 1. Apoptosis in Response to Anti-estrogens in MCF-7 Human Mammary Adenocarcinoma Cells. McGill Journal of Medicine, doi: 10.26443/MJM.V1I2.726

27. Koanhoi, Kim.,Hyok-rae, Cho., Yonghae, Son. (2024). 4. Astaxanthin Induces Apoptosis in MCF-7 Cells through a p53-Dependent Pathway. International Journal of Molecular Sciences, doi: 10.3390/ijms25137111

28. Yubin, Ji., G, S, Xin., Zhongyuan, Qu., Xiang, Zou., Miao, Yu. (2016). 5. Mechanism of juglone-induced apoptosis of MCF-7 cells by the mitochondrial pathway.. Genetics and Molecular Research, doi: 10.4238/GMR.15038785

29. Harshita.,Abul, Barkat., Rizwanullah., Sarwar, Beg., Faheem, Hyder, Pottoo., Sahabjada, Siddiqui., Farhan, Jalees, Ahmad. (2019). 1. Paclitaxel-loaded Nanolipidic Carriers with Improved Oral Bioavailability and Anticancer Activity against Human Liver Carcinoma. AapsPharmscitech, doi: 10.1208/S12249-019-1304-4

30. Sandip, B., Tiwari.,Mansoor, M., Amiji. (2006). 8. Improved oral delivery of paclitaxel following administration in nanoemulsion formulations.. Journal of Nanoscience and Nanotechnology, doi: 10.1166/JNN.2006.440

31. Sumit, K., Dey., Raman, K., Singh., Shyamtanu, Chattoraj., Shekhar, Saha., Alakesh, Das., Kankan, Bhattacharyya., Kaushik, Sengupta., Shamik, Sen., Siddhartha, S., Jana. (2017). 1. Differential role of nonmuscle myosin II isoforms during blebbing of MCF-7 cells. Molecular Biology of the Cell, doi: 10.1091/MBC.E16-07-0524

32. Riyo, Morimoto-Kamata., Sei-ichiro, Mizoguchi., Takeo, Ichisugi., Satoru, Yui. (2012). 2. Cathepsin G Induces Cell Aggregation of Human Breast Cancer MCF-7 Cells via a 2-Step Mechanism: Catalytic Site-Independent Binding to the Cell Surface and Enzymatic Activity-Dependent Induction of the Cell Aggregation. Mediators of Inflammation, doi: 10.1155/2012/456462

33. Yuki, Fujii., Junichi, Ikenouchi. (2023). 8. Cytoplasmic zoning in membrane blebs.. Journal of Biochemistry, doi: 10.1093/jb/mvad084

34. Sorin, Mitran., Jennifer, L., Young. (2010). 10. Multiscale Computation of Cytoskeletal Mechanics During Blebbing. doi: 10.1007/8415_2010_18

35. Samuel, V., Mussi., Samuel, V., Mussi., Rupa, R., Sawant., Federico, Perche., Mônica, Cristina, de, Oliveira., Ricardo, B., Azevedo., Lucas, Antônio, Miranda, Ferreira., Vladimir, P., Torchilin. (2014). 9. Novel Nanostructured Lipid Carrier Co-Loaded with Doxorubicin and Docosahexaenoic Acid Demonstrates Enhanced in Vitro Activity and

Overcomes Drug Resistance in MCF-7/Adr Cells. Pharmaceutical Research, doi: 10.1007/S11095-013-1290-2

36. Lisa, C., Crowley., Grace, Chojnowski., Nigel, J., Waterhouse. (2016). 6. Measuring the DNA Content of Cells in Apoptosis and at Different Cell-Cycle Stages by Propidium Iodide Staining and Flow Cytometry.. CSH Protocols, doi: 10.1101/PDB.PROT087247

37. Pedro, Pereira., Ana, Serra-Caetano., Marisa, Cabrita., Evguenia, Bekman., José, Braga., José, Pedro, Rino., René, Santus., Paulo, Filipe., Ana, E., Sousa., João, A., Ferreira. (2017). 8. Quantification of cell cycle kinetics by EdU (5-ethynyl-2′-deoxyuridine)-coupled-fluorescence-intensity analysis. Oncotarget, doi: 10.18632/ONCOTARGET.17121

38. Byron, H., Long., Craig, R., Fairchild. (1994). 5. Paclitaxel Inhibits Progression of Mitotic Cells to G1 Phase by Interference with Spindle Formation without Affecting Other Microtubule Functions during Anaphase and Telephase. Cancer Research

39. Ormerod et al., Consensus Report of the Task Force on Standardisation of DNA Flow Cytometry in Clinical Pathology. Anal Cell Pathol. 1998; 17(2): 103–110.

40. Ramy, Rahmé. (2021). 1. Assaying Cell Cycle Status Using Flow Cytometry. Methods of Molecular Biology, doi: 10.1007/978-1-0716-1217-0_11

41. Zbigniew, Darzynkiewicz., Hong, Zhao. (2001). 8. Cell Cycle Analysis by Flow Cytometry. doi: 10.1002/9780470015902.A0002571.PUB2

42. (2022). 3. Application of nanocarriers for paclitaxel delivery and chemotherapy of cancer. doi: 10.1016/b978-0-323-90951-8.00004-7

43. Saloni, Malla., Rabin, Neupane., Sai, H.S., Boddu., Mariam, Sami, Abou-Dahech., Mariah, A., Pasternak., Noor, Hussein., Charles, R., Ashby., Yuan, Tang., R., Jayachandra, Babu., Amit, K., Tiwari. (2022). 2. Application of nanocarriers for paclitaxel delivery and chemotherapy of cancer. doi: 10.1016/B978-0-323-90951-8.00004-7

44. Masoumeh, Kaveh, Zenjanab., Sajjad, Alimohammadvand., Abolfazl, Doustmihan., Sepideh, Kianian., Behnaz, Sadeghzadeh, Oskouei., MirAhmad, Mazloomi., Morteza, Akbari., rana, jahanban-esfahlan. (2024). 1. Paclitaxel for breast cancer therapy: A review on effective drug combination modalities and nano drug delivery platforms. Journal of Drug Delivery Science and Technology, doi: 10.1016/j.jddst.2024.105567

45. Ki, Hyun, Bang., Young-Guk, Na., Hyun, Wook, Huh., Sung, Joo, Hwang., Min-Soo, Kim., Min-Ki, Kim., Hong-Ki, Lee., Cheong-Weon, Cho. (2019). 5. The Delivery Strategy of Paclitaxel Nanostructured Lipid Carrier Coated with Platelet Membrane. Cancers, doi: 10.3390/CANCERS11060807

46. Toshihiko, Oki., Koutarou, Nishimura., Jiro, Kitaura., Katsuhiro, Togami., Akie, Maehara., Kumi, Izawa., Asako, Sakaue-Sawano., Atsushi, Niida., Satoru, Miyano., Hiroyuki, Aburatani., Hiroshi, Kiyonari., Atsushi, Miyawaki., Toshio, Kitamura. (2015). 3. A novel cell-cycle-indicator, mVenus-p27K − , identifies quiescent cells and visualizes G0–G1 transition. Scientific Reports, doi: 10.1038/SREP04012

47. Elwira, Lasoń.,Elżbieta, Sikora., Małgorzata, Miastkowska., Elvira, Escribano., María, José, García-Celma., Conxita, Solans., Meritxell, Llinàs., Jan, Ogonowski. (2018). 5. NLCs as a potential carrier system for transdermal delivery of forskolin. Acta BiochimicaPolonica, doi: 10.18388/ABP.2018_2554

48. Shinji, Yasuhira., Masahiko, Shibazaki., Masao, Nishiya., Chihaya, Maesawa. (2016). 6. Paclitaxel-induced aberrant mitosis and mitotic slippage efficiently lead to proliferative death irrespective of canonical apoptosis and p53.. Cell Cycle, doi: 10.1080/15384101.2016.1242537

49. Philip, C., Mack., Christopher, M., Mahaffey., Paul, H., Gumerlock., David, R., Gandara. (2004). 1. Enhancing the anticancer activity of paclitaxel through cyclin-dependent kinase inhibition. Cancer Research

50. Lisong, Lin., Guochu, Lin., Wantao, Chen., Wei, Guo., Xü, Lin. (2002). 8. [Paclitaxel-induced apoptosis in ACC-2 cells is associated with the arrest of G(2)/M].. Chinese journal of stomatology

51. Giovanni, Sena., Carlo, Onado., Paolo, Cappella., Francesco, Montalenti., Paolo, Ubezio. (1999). 5. Measuring the complexity of cell cycle arrest and killing of drugs: kinetics of phase-specific effects induced by taxol.. Cytometry, doi: 10.1002/(SICI)1097-0320(19991001)37:2<113::AID-CYTO4>3.0.CO;2-M

52. (2022). 1. Cyclophosphamide. doi: 10.1201/9781003016786-16

53. Jerry, Z., Finklestein., Robert, E., Hittle., G., Denman, Hammond. (1969). 3. Evaluation of a high dose cyclophosphamide regimen in childhood tumors. Cancer, doi: 10.1002/1097-0142(196905)23:5<1239::AID-CNCR2820230535>3.0.CO;2-F

54. Ekaterina, A., Alyamkina., Evgenia, V., Dolgova., Anastasia, Sergeevna, Likhacheva., Vladimir, A., Rogachev., Tamara, E., Sebeleva., Valeriy, P., Nikolin., Nelly, A., Popova., Nelly, A., Popova., Konstantin, E., Orishchenko., Dmitriy, N., Strunkin., Elena, R., Chernykh., Stanislav, N., Zagrebelniy., S., S., Bogachev., Mikhail, A., Shurdov. (2009). 9. Combined therapy with cyclophosphamide and DNA preparation inhibits the tumor growth in mice. Genetic Vaccines and Therapy, doi: 10.1186/1479-0556-7-12

55. Victor, C., Wong., Haley, L, Cash., Jessica, L., Morse., Shan, Lu., Anatoly, Zhitkovich. (2012). 1. S-phase sensing of DNA-protein crosslinks triggers TopBP1-independent ATR activation and p53-mediated cell death by formaldehyde. Cell Cycle, doi: 10.4161/CC.20905

56. Rebecca, M., Jones., Eva, Petermann. (2012). 2. Replication fork dynamics and the DNA damage response.. Biochemical Journal, doi: 10.1042/BJ20112100

57. Pozarowski P, Darzynkiewicz Z, Analysis of cell cycle by flow cytometry. Methods Mol Biol. 2004; 281:301-11.

58. Saeideh, Abdolahpour., Nejat, Mahdieh., Zahra, Jamali., Abolfazl, Akbarzadeh., Tayebeh, Toliyat., Maliheh, Paknejad. (2017). 4. Development of Doxorubicin-Loaded Nanostructured Lipid Carriers: Preparation, Characterization, and In Vitro Evaluation on MCF-7 Cell Line. Journal of Bionanoscience, doi: 10.1007/S12668-016-0391-X

59. Praveen, Sharma.,Brahmanand, Dube., Krutika, K., Sawant. (2011). 5. Development and evaluation of nanostructured lipid carriers of cytarabine for treatment of meningeal leukemia.. Journal of Nanoscience and Nanotechnology, doi: 10.1166/JNN.2011.4235

60. Animal Ethics Committee Protocol No.: IP/PCOL/FAC/32/2022/24

61. Pankti Patel, Jigna Shah, Protective effects of hesperidin through attenuation of Ki67 expression against DMBA-induced breast cancer in female rats, Life Sciences, Volume 285, 2021, 119957, ISSN 0024-3205, https://doi.org/10.1016/j.lfs.2021.119957.

62. Sejben A, Kószó R, Kahán Z, Cserni G, Zombori T. Examination of Tumor Regression Grading Systems in Breast Cancer Patients Who Received Neoadjuvant Therapy. Pathol Oncol Res. 2020 Oct; 26 (4): 2747-2754. doi: 10.1007/s12253-020-00867-3. Epub 2020 Jul 20. PMID: 32691390; PMCID: PMC7471177.

63. Zhao, H., Wu, L., Yan, G. et al. Inflammation, and tumor progression: signaling pathways and targeted intervention. Sig Transduct Target Ther 6, 263 (2021). https://doi.org/10.1038/s41392-021-00658-5

64. Sahar A. Helmy, Saif El-Mofty, Amal M. El Gayar, Ibrahim M. El-Sherbiny, Yousra M. El-Far,Novel doxorubicin / folate-targeted trans-ferulic acid-loaded PLGA nanoparticles combination: In-vivo superiority over standard chemotherapeutic regimen for breast cancer treatment,Biomedicine & Pharmacotherapy,Volume 145,2022,112376,ISSN 0753-3322,https://doi.org/10.1016/j.biopha.2021.112376.

65. Stephen, K., Dordunoo., William, C., Vincek., Randall, Hoover., Wenbin, Dang. (2005). 6. Sustained release of paclitaxel from PACLIMER® Microspheres. Cancer Research

66. Kim, A., Selting., Sandra, M., Bechtel., Jahna, C., Espinosa., Carolyn, J., Henry., Deborah, J., Tate., Jeffrey, N., Bryan., Lian, G., Rajewski., Brian, K., Flesner., Charles, J., Decedue., Michael, Baltezor. (2018). 2. Evaluation of intravenous and subcutaneous administration of a novel, excipient-free, nanoparticulate formulation of paclitaxel in dogs with spontaneously occurring neoplasia. Veterinary and Comparative Oncology, doi: 10.1111/VCO.12435

67. Po-Chang, Chiang., Stephen, E., Gould., Michelle, Nannini., Ann, Qin., Yuzhong, Deng., Alfonso, Arrazate., Kimberly, R, Kam., Yingqing, Ran., Harvey, Wong. (2014). 8. Nanosuspension delivery of paclitaxel to xenograft mice can alter drug disposition and anti-tumor activity. Nanoscale Research Letters, doi: 10.1186/1556-276X-9-156

68. Zabezhinskiĭ, Ma., Irina, G., Popovich., Shtylik, Av., Kovalenko, Al., Vladimir, N., Anisimov. (1998). 10. The effect of cycloferon administration on the growth of transplantable tumors in rats and mice.

69. A.S., Moore., A.E., Frimberger., Catherine, M., Chan. (2018). 7. Dosage escalation of intravenous cyclophosphamide in cats with cancer.. Veterinary Journal, doi: 10.1016/J.TVJL.2018.10.003

70. Rena, G., Lapidus.,Wenbin, Dang., David, Marc, Rosen., Alyssa, M., Gady., Yelena, Zabelinka., Robert, N., O'Meally., Theodore, L., DeWeese., Samuel, R., Denmeade. (2004). 9. Anti-tumor effect of combination therapy with intratumoral controlled-release paclitaxel (PACLIMER® microspheres) and radiation. The Prostate, doi: 10.1002/PROS.10331

71. Alicja, Zajdel., Daniel, Wolny., Magdalena, Kałucka-Janik., Adam, Wilczok. (2019). 4. Paclitaxel in breast cancer – drug resistance and strategies to counteract it. Postępyhigieny i medycynydoświadczalnej, doi: 10.5604/01.3001.0013.5251

72. Tala, M., Abu, Samaan., Marek, Samec., Alena, Liskova., Peter, Kubatka., Dietrich, Büsselberg. (2019). 5. Paclitaxel's Mechanistic and Clinical Effects on Breast Cancer. doi: 10.3390/BIOM9120789

73. T, J, McMillan., T, C, Stephens., G., Gordon, Steel. (1985). 9. Development of drug resistance in a murine mammary tumour.. British Journal of Cancer, doi: 10.1038/BJC.1985.265

74. Paolo, Marchetti.,Saïk, Urien., Giancarlo, Antonini, Cappellini., Graziana, Ronzino., Corrado, Ficorella. (2002). 6. Weekly administration of paclitaxel: theoretical and clinical basis.. Critical Reviews in Oncology Hematology, doi: 10.1016/S1040-8428(02)00109-9

75. Laura, Cabeza.,Mazen, M., El-Hammadi., Raúl, Ortiz., Maria, Dolores, Cayero-Otero., Julia, Jiménez-López., Gloria, Perazzoli., Lucía, Martín-Banderas., José, M., Baeyens., Consolación, Melguizo., Jose, Prados. (2022). 2. Evaluation of poly (lactic-co-glycolic acid) nanoparticles to improve the therapeutic efficacy of paclitaxel in breast cancer. Bioimpacts, doi: 10.34172/bi.2022.23433

76. Marina, Franco., Marjorie, Coimbra, Roque., André, Luís, Branco, de, Barros., Juliana, de, Oliveira, Silva., Geovanni, Dantas, Cassali., Mônica, Cristina, de, Oliveira. (2019). 5. Investigation of the antitumor activity and toxicity of long-circulating and fusogenic liposomes co-encapsulating paclitaxel and doxorubicin in a murine breast cancer animal model.. Biomedicine & Pharmacotherapy, doi: 10.1016/J.BIOPHA.2018.11.011

77. Sifeng, Zhu., Chao, Sun., Yunyan, Li., Wendian, Liu., Yun, Luan., Cheng, Wang. (2024). 6. Effective therapy of advanced breast cancer through synergistic anticancer by paclitaxel and P-glycoprotein inhibitor. Materials today bio, doi: 10.1016/j.mtbio.2024.101029

78. Rui, Xue, Zhang. (2016). 2. Polymer and Lipid-based Nanomedicine of Synergistic Drug Combination for Improving Chemotherapy of Multidrug Resistant Breast Cancer.

79. Zeenat, Iqbal. (2022). 1. Nanomedicine-Based Combinational Therapy for Breast Cancer. doi: 10.1007/978-981-19-5558-7_9

80. Pavan, Kumar, Yadav., Ravi, Saklani., Amrendra, Kumar, Tiwari., Saurabh, Verma., Divya, Chauhan., Pooja, Yadav., Rafquat, Rana., Navodayam, Kalleti., Jiaur, R., Gayen., Wahajuddin., Srikanta, Kumar, Rath., Madhav, Nilakanth, Mugale., Kalyan, Mitra., Manish, K., Chourasia. (2023). 3. Ratiometric Codelivery of Paclitaxel and Baicalein Loaded Nanoemulsion for Enhancement of Breast Cancer Treatment.. International Journal of Pharmaceutics, doi: 10.1016/j.ijpharm.2023.123209

81. Jun, Ye., Lin, Li., Jiye, Yin., Hongliang, Wang., Renjie, Li., Yanfang, Yang., Yongbiao, Guan., Xuejun, Xia., Yuling, Liu. (2022). 5. Tumor-targeting intravenous lipid emulsion of paclitaxel: Characteristics, stability, toxicity, and toxicokinetics. Journal of Pharmaceutical Analysis, doi: 10.1016/j.jpha.2022.08.002