RESEARCH PAPER

Targeting TNBC: Fisetin and Cabazitaxel NLCs synergistic effects on cell lines and tumor suppression in mice

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ABSTRACT

Objective(s): Although various treatments for Triple-Negative Breast Cancer (TNBC) and its associated diseases exist, the Nanostructured Lipid Carrier (NLC) system has garnered significant attention in recent years due to its advantageous properties. However, drugs administered in combination therapy tend to have a more potent effect than those administered in monotherapy. This study evaluated the anticancer efficacy of Fis-NLCs and CBZ-NLCs on TNBC cell lines, MDA-MB-468 and MCF-7, by assessing cytotoxicity, cell migration, and apoptosis induced by both Fis-NLCs and CBZ-NLCs, both individually and in combination.

Materials and Methods: In vivo, experiments on Swiss albino mice were conducted to evaluate the efficacy of drug-loaded NLCs in reducing tumor volume and hemotoxicity.

Results: Cytotoxicity assays revealed that, against MDA-MB-468 cells, the IC50 values at 24 hours were 10.85±1.39 µM for Fis-NLCs and 4.25±0.78 µM for CBZ-NLCs, while at 48 hours, the IC50 values were 3.48±0.74 µM for Fis-NLCs and 1.36±0.3 µM for CBZ-NLCs. For MDA-MB-231 cells, IC50 values at 24 hours were 10.84±0.97 µM for Fis-NLCs and 5.07±1.46 µM for CBZ-NLCs, and at 48 hours, 3.18±0.67 µM for Fis-NLCs and 3.38±1.05 µM for CBZ-NLCs. Additionally, CBZ-NLCs decreased the inhibitory concentration of Fis-loaded NLCs, inhibiting 50% of the cancer cell population at concentrations of 2.52 ± 0.57 µM, 2.17 ± 0.24 µM, and 2.12 ± 0.45 µM after 48 hours.

Conclusion: Furthermore, in vivo studies have demonstrated that NLC-based drug delivery effectively reduces tumor size in mice compared to pure drugs. When Fis-NLCs were combined with CBZ-NLCs, a synergistic effect was observed, leading to enhanced tumor growth inhibition, improved pharmacokinetics, and reduced hemotoxicity in mice.

Keywords: *Lipid nanoparticles, Triple negative breast neoplasms, Drug therapy, Combination, Tumor suppression*

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Abbreviation
(TNBC) Triple-negative breast cancer, (NLC) Nanostructure lipid carrier system, (Fis) Fisetin, (CBZ) Cabazitaxel, (CBZ-loaded NLCs) Cabazitaxel-loaded nanostructured lipid carriers, (Fis-loaded NLCs) Fisetin loaded nanostructured lipid carriers, (BC) Breast cancer, (BL1) Basal-like1, (BL2) Basal-like 2, (LAR) Luminal androgen receptor, (MSL) Mesenchymal stem-like, (M), Mesenchymal, (IM) Immune modulatory, (EMT) Epithelial-to-mesenchymal transition, (EGFR) Endothelial growth receptor factor, (SRB assay) Sulforhodamine-B assay, (DMSO) Dimethyl sulfoxide, (DADS) Diallyl disulfide, (PBS) Phosphate-buffered saline, (EAC) Ehrlich ascites carcinoma, (TV) Tumor volume, (H & E) Hematoxylin and eosin, (CI) Combination index, (WBC) White blood cell, (RBC) Red blood cell, (HgB) Hemoglobin, (HCT) Hematocrit, (MCV) Mean corpuscular volume, (MCH) Mean corpuscular hemoglobin, (MCHC) Mean corpuscular hemoglobin concentration, (PLT) Platelet count.

INTRODUCTION

Females are more likely than males to develop breast cancer (BC), which has contributed to a higher mortality rate among women. Approximately 2 to 2.5 million women are diagnosed with breast cancer each year, leading to approximately 627,000 global deaths. According to a WHO study, BC disproportionately affects women, with an estimated 170,000 cases, reflecting a 14% increase in the country's overall cancer incidence [1]. Chemotherapy, surgery, and radiotherapy are commonly used to treat BC patients. However, these treatments are less effective for patients who lack HER2 receptors, leading to a lack of response and benefit [2]. Triple-Negative Breast Cancer (TNBC) is known for its higher responsiveness to chemotherapy compared to other breast cancer subtypes and is recognized as the most aggressive variant of the disease [3,4]. Despite considerable progress in developing alternative therapies for BC and TNBC, clinical success has been limited [5]. This study aligns with several Sustainable Development Goals (SDGs). It

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supports SDG 3: Good Health & Well-Being by contributing to the reduction of cancer-related mortality and promoting universal health coverage. It also advances SDG 9: Industry, Innovation, and Infrastructure by fostering innovation in cancer treatment [6,7].

The molecular diversity of tumor cells plays a significant role in defining the unique characteristics of Triple-Negative Breast Cancer (TNBC). Its increased aggressiveness and distinctive differentiation set TNBC apart from other breast cancer subtypes. TNBC presents unique treatment challenges because it lacks estrogen and progesterone receptors, as well as the HER2 protein.

Research has identified six distinct subtypes of TNBC, each with unique characteristics. Basal-like 1 (BL1) is characterized by high proliferation rates and robust immune responses, which can often result in poorer outcomes. Basal-like 2 (BL2) shares similarities with BL1 but differs in differentiation and growth factor expression profiles. The Mesenchymal Stem-like (MSL) subtype exhibits stem cell-like properties and demonstrates greater resistance to conventional treatments. The Luminal Androgen Receptor (LAR) subtype expresses androgen receptors and may respond effectively to androgen-targeted therapies. The Mesenchymal (M) subtype is associated with increased invasiveness due to epithelial-to-mesenchymal transition. Finally, the Immune Modulatory (IM) subtype is marked by heightened immune activity, which could be beneficial in response to immunotherapies [8].

Understanding these subtypes is crucial for the development of targeted treatments and for improving patient outcomes in Triple-Negative Breast Cancer (TNBC). Researchers are actively investigating these variations to enhance affected individuals' survival rates and quality of life [9,10]. Specific subtypes of TNBC are highly associated with epithelial-to-mesenchymal transition (EMT) processes, which contribute to tumor development, poor prognosis, and chemotherapeutic resistance [11]. Previous studies on TNBC have revealed similar clinical and pathological features between TNBC and Basal-like 1/2 (BL1/2) BC, though they are not identical. In general, 70% of TNBC tumors lack estrogen (ER) and progesterone (PR) receptors, but are enriched in endothelial growth factor receptor (EGFR), vimentin, cytokeratin 8/18, and cytokeratin 5/6. The expression of BRCA1/2 in TNBC is also higher, approximately 80-90%, compared to other breast cancer subtypes [12].

Breast cancer (BC) cells are sensitive to chemotherapy drugs, such as anthracyclines and taxanes [4]. However, the combination of anthracyclines with taxanes leads to higher hematological, cardiovascular, and gastrointestinal toxicities compared to single taxane-based therapy [13]. This highlights the need to develop appropriate formulations to improve therapeutic effectiveness. Mitigating dose-related toxicity is essential for enhancing the safety profile of promising drug candidates, such as CBZ.

CBZ, a semi-synthetic derivative of 10-deacetylbaccatin-3, represents a significant advancement in the taxane family. It is recognized for its effectiveness as an anti-microtubule agent in treating various types of cancer [14]. Drug resistance presents a significant challenge, requiring the use of additional therapies to slow disease progression and minimize the side effects associated with treatment. Combining two or more chemotherapy drugs is often necessary to overcome resistance to single-drug therapies, with the potential to act via either synergistic or additive mechanisms [15,16]. Recent reports have shown that CBZ is effective in treating HER2-negative metastatic breast cancer (BC) and in cases of taxane-resistant variants [17,18]. However, formulating CBZ remains challenging due to its poor permeability and low solubility [19].

Herbal medicines, particularly those containing polyphenolic compounds, are increasingly being recognized as potential alternative treatment options for managing various cancers, including Triple-Negative Breast Cancer (TNBC) [20,21]. Flavonoids exhibit significant anticancer properties, and Fisetin (Fis), a naturally occurring flavonoid, is known for its strong antiproliferative, apoptotic, neuroprotective, and antioxidative effects [22,23]. Research indicates that Fisetin effectively inhibits the growth of human melanoma cells through various mechanisms, including the suppression of the Res, MAPK, and NF-κB signaling pathways [24]. However, Fisetin faces significant challenges in terms of bioavailability due to its poor water solubility, high lipophilicity, and rapid metabolism in the body. These issues are attributed to the 4-hydroxy groups on the catechol moiety of its B-ring structure, necessitating higher doses to achieve the desired therapeutic or anticancer effects. Unfortunately, increasing the dosage can lead to notable adverse effects, such as liver toxicity (evidenced by elevated ALT and AST levels), neutropenia (low neutrophil counts), and thrombocytopenia (low platelet count), which increases the risk of bleeding. Despite its potential

benefits, these factors highlight the need for careful research and consideration in its clinical use [25–27]. The delivery of Fisetin remains a significant challenge due to its low water solubility. Therefore, developing effective delivery systems to overcome these solubility-related obstacles is essential [28,29].

Recently, Nanostructured Lipid Carriers (NLCs) have emerged as promising vehicles for drug delivery [30,31]. NLCs are advanced lipid nanocarrier systems composed of both solid and liquid lipids. This unique formulation effectively prevents drug-lipid polymorphisms and enhances the stability of the formulations, allowing for extended storage periods [32,33]. The core of NLCs is made up of liquid medium-chain triglycerides capable of encapsulating both lipophilic and hydrophilic pharmaceuticals. The outer surfaces consist of solid triglycerides, offering opportunities for surface modification to enhance functionality and performance. This design improves the versatility and functional application of NLCs in drug delivery systems [34,35]. Surface-modified NLCs offer several advantages, including ensuring effective drug action in the body, enabling a steady and controlled release of the medication, and providing a safer means of administering drugs to living organisms [36].

As previously reported in our research article on fisetin (Fis) and CBZ-optimized formulation parameters, which were determined through Design of Experiments (DoE) software and characterized accordingly [33], this study evaluates the effectiveness of NLCs in both monotherapy and combination therapy against Triple-Negative Breast Cancer (TNBC) cells (MDA-MB-468), compared to hormone-positive breast cancer (BC) cells (MCF-7). We investigate the combination of fisetin-loaded NLCs and CBZ-loaded NLCs to assess their effectiveness against BC cell lines. In vivo studies were also performed using drug-loaded NLCs for fisetin and CBZ in Swiss albino mice. The fisetin-NLCs were combined with CBZ-NLCs to evaluate their synergistic effect on mouse tumor growth inhibition and enhance drug pharmacokinetics.

MATERIALS AND METHODS

Material

The ingredients used in the formulation of lipid-based nanoparticles were as follows: Fisetin (obtained from Cayman Chemical, USA); Cabazitaxel (obtained from Shilpa Medicare, India); Precirol ATO5, Labrafac, and Labrasol (obtained from Gattefose, India); Tween 80 (obtained from Merck Millipore, Mumbai, India); Ethanol and Methanol (obtained from Merck, India); Glyceryl monostearate (obtained from Henan Eshine Chemicals Co., Ltd.); Pluronic F127, Coumarin-6, and Ethylene diamine tetraacetic acid (EDTA) (obtained from Sigma Aldrich, India); and Dimethyl sulfoxide (obtained from Sisco Research Laboratories, India).

Methods

Method of preparation of NLCs

The preparation of NLCs for Fisetin (Fis) and Cabazitaxel (CBZ) using the hot melt homogenization method. Briefly, to prepare Fis-loaded NLCs, solid and liquid lipids were heated to a temperature above the melting point (70°C), and the drug was added. The surfactant-containing aqueous phase was maintained at the same temperature. Once both phases reached the desired temperature, the lipid phase was progressively mixed with the aqueous phase using high-speed homogenization. Afterward, the NLCs were cooled to room temperature and lyophilized for long-term preservation [37,38].

In vitro evaluations (CBZ-loaded NLCs and Fis-loaded NLCs against BC cells)

All the cell culture studies were carried out using specific types of cells, such as MDA-MB-231, MDA-MB-468 (TNBCs), and MCF-7 (ER+, PR+ & Her2/neu negative) cells, with passage numbers of 60-90.

Cell viability studies

Cell viability in breast cancer (BC) cell lines was evaluated using the sulforhodamine B (SRB) assay. The cells were pre-cultured in 96-well plates for 36 hours to ensure proper adherence and confluency. Following this, increasing concentrations of Cabazitaxel (CBZ) and its nanostructured lipid carriers (CBZ-NLCs), as well as Fisetin (Fis) and its nanostructured lipid carriers (Fis-NLCs), were added, ranging from 0 to 100 µM. Diallyl disulfide (DADS) at 100 µM was used as a positive control, while dimethyl sulfoxide (DMSO) at 0.1% v/v was a vehicle control. The cells were then incubated in a CO2 incubator for 24 to 48 hours, allowing the compounds to exert their effects. After incubation, cell viability was measured using the SRB assay, providing insights into the potential of CBZ and Fis in inhibiting BC cell growth [39].

Cellular uptake studies

Breast cancer (BC) cells were cultured in 6-well plates, ensuring a precise density of 4 × 10⁵ cells per well. This careful approach supports robust growth and ensures reliable experimental outcomes. The cells were

supplemented with growth media and incubated at 37 °C for 24 hours. Once they reached sufficient confluence, the cells were treated with fluorescent coumarin-6 (C6)-labeled nanostructured lipid carriers (NLCs) containing Cabazitaxel (CBZ) and Fisetin (Fis) formulations, using amounts equivalent to sub-IC50 values. The cells were then incubated with these samples for various time points: 8, 12, 24, and 48 hours at 37 °C. After the treatment, the growth media were carefully removed, and the cells were washed three times with phosphate-buffered saline (PBS, pH 7.4, 1X) to eliminate residual particles and cell debris. The cells were then scraped off using a scraper and collected into 1.5 mL vials containing PBS. The cells were centrifuged at 3000 rpm, and the supernatants were discarded. An analysis buffer consisting of 0.5% Triton® in 0.2 N NaOH solution was added to completely lyse the cells. Coumarin-6 associated with the cells was quantified using a fluorescent microplate reader (PerkinElmer, USA) with excitation at 515 nm and emission at 558 nm [40].

Cell migration assay

Breast cancer (BC) cells were pre-cultured in 12-well plates for 36 hours to ensure optimal adherence and sufficient confluency. A scratch assay was then performed using a sterile 20 µL pipette tip to create a wound in the cell monolayer. After aspirating the growth media, the wells were washed with phosphate-buffered saline (PBS) to remove residual cell debris. Fresh media containing varying concentrations of Cabazitaxel (CBZ), CBZ-loaded nanostructured lipid carriers (CBZ-NLCs), Fisetin (Fis), and Fisetin-loaded nanostructured lipid carriers (Fis-NLCs) at 5, 10, and 15 µM were added. The cells were incubated for 48 hours, and cell migration was monitored using an inverted microscope at regular 24-hour intervals. The relative migration was quantified using ImageJ software (NIH, USA). A control group remained untreated, while the vehicle control (VC) and positive control (PC) groups received DMSO (0.1% v/v) and itraconazole (3.5 mM), respectively. The effects of the treatments on cell migration were assessed and presented by comparing the treatment conditions with the corresponding percentage inhibition of cell migration [41].

Cell apoptosis assay

Breast cancer (BC) cells were cultured in 6-well plates at a density of 4.0 × 10⁵ cells per well and incubated for 24 hours to reach confluency. Once confluency was achieved, the cells were treated with Cabazitaxel (CBZ), its nanoparticle formulation (CBZ-NLCs), Fisetin (Fis), Fis-NLCs, and combinations of Fis with CBZ and Fis-NLCs with CBZ-NLCs, using concentrations below the IC50 value. The cells were then incubated for 24 and 48 hours at 37°C. After treatment, the cells were trypsinized, collected, and centrifuged at 3780 RCF for at least 5 minutes to form cell pellets. The pellets were stained with acridine orange and ethidium bromide (10 µL, 100 µg/mL) for 5 minutes to distinguish live and dead cells. The stained cells were placed on glass slides, covered with coverslips, and observed under a fluorescence microscope using green and red filters for FITC and TRITC channels. Images were captured, and the number of live and dead cells was quantified using ImageJ software, enabling the calculation of the apoptosis ratio. This process provides insights into the effects of various treatments on BC cells [42].

Assessment of apoptotic and antioxidant gene expression levels

Breast cancer (BC) cells were cultured in 6-well plates and treated with samples at 1, 5, and 10 µM concentrations for 48 hours. For comparison, a group treated with dimethyl sulfoxide (DMSO) and an untreated group were included as controls. After treatment, the cells were lysed, and RNA was extracted using Trizol [43]. This process involved the addition of chloroform and isopropanol, followed by purification with 70% ethanol to enhance sample quality. The quantity and quality of the extracted RNA were assessed using a NanoDrop spectrophotometer (DeNovix, Wilmington, USA). Integrity was verified by running a 1.5% agarose gel, ensuring the RNA was suitable for further experiments [44].

cDNA synthesis and RT-PCR analysis

Total RNA was quantified and used to synthesize complementary DNA (cDNA) using Verso cDNA kits. Each reaction contained 4 µl of cDNA synthesis buffer, 2 µl of dNTP mix, 1 µl of hexamer, 1 µl of RT enhancer, 1 µl of Verso enzyme, 1 µg of RNA template, and nuclease-free water, totaling 20 µl. To amplify the cDNA, 10 µl of SYBR Green (2x), 1 µl each of forward and reverse primers, 4 µl of nuclease-free water, and 5 µl of cDNA templates were added to 200 µl PCR tubes. These tubes were placed in the RT-PCR instrument, which was programmed to run for 40 cycles with specific conditions for each gene. Relative fold changes in gene expression were calculated using the comparative 2^(-ΔCt) method, with normalization against β-actin to ensure accurate results. This approach provided a clear understanding of the expression levels of the targeted genes.

In vitro evaluations for combinatorial effects of Fis-

loaded NLCs and CBZ-loaded NLCs

The combinatorial effects of Fisetin (Fis) and Cabazitaxel (CBZ) and their drug-loaded NLC formulations were assessed. The combinatorial treatment was performed to evaluate cell viability, cell migration, cell apoptosis, and gene expression in breast cancer (BC) cells. The cell culture techniques were the same as those described above. However, different combinatorial patterns were used for the cell viability assay to determine the optimal combination effect of these drugs and their NLC formulations. The treatments involved sequential and simultaneous combinations of Fis and CBZ, including: (1) varying concentrations of Fis and a fixed concentration of CBZ at the IC50 value, and (2) varying concentrations of CBZ and Fis at the IC50 value. Based on the cell viability results, the combinatorial therapeutic effect (CI) was calculated mathematically using the equation provided below, with CompuSyn Version 1.0 software (Cambridge, MA, USA). The fixed optimal concentration doses for Fis, CBZ, and their NLC formulations were selected for further studies, such as cell migration and apoptosis assessments [45].

$$\boldsymbol{CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2}}$$

Whereas, (D)1 and (D)2 represent the combination doses of Fisetin (Fis) and Cabazitaxel (CBZ) or their NLC formulations, respectively, which result in 50% growth inhibition. Similarly, (Dx)1 and (Dx)2 correspond to the single doses of Fis and CBZ or their NLC formulations, which have a similar effect. The suggested results obtained by applying this mathematical equation could be as follows, if:

CI=1 additive effect, CI>1 synergistic effect, or CI<1 antagonist effect.

In vivo studies

Animal model

Healthy female Swiss albino mice weighing 25 and 30 grams were used as animal models for the in vivo experiments. The Institutional Ethical Committee of JSS College of Pharmacy, JSS Academy of Higher Education and Research, Mysuru (JSSSHER / CPT / IAEC / 061 / 2021) approved the experimental protocols.

Animal grouping

The mice were systematically assigned to six distinct groups (n = 6), and treatment was scheduled accordingly. The groups included: a Normal group, in which no disease was induced; a Positive group, with no treatment; and groups where the disease was caused and treated with pure Fisetin (Fis) and Fis-NLCs, respectively (equivalent to 25 mg/kg of pure drug). The same treatment approach was used for Cabazitaxel (CBZ) and CBZ-NLCs, respectively (equivalent to 5 mg/kg of pure drug). Additionally, the disease was treated with combination doses of the pure drugs Fis + CBZ and Fis-NLCs + CBZ-NLCs, respectively, at concentrations of 5 mg/kg for Fis and 2 mg/kg for CBZ, as shown in Table 1 [46].

In vivo bio-distribution studies

The single dose was administered using various treatments, such as monotherapy through the intraperitoneal route. After 24 hours, blood samples were collected and transferred into centrifuge tubes. The samples were subjected to high-speed centrifugation at 2500 rpm for a minimum of 10 minutes and stored at -20°C for further analysis. Following this, the mice were euthanized, and several organs, including the brain, heart, lungs, liver, spleen, and kidneys, were carefully excised. These organs were homogenized to facilitate evaluation, and the resulting homogenates were preserved in acetonitrile. The homogenates were centrifuged, and the drug concentrations were determined using high-performance liquid chromatography (HPLC) analysis [47].

Table 1. Group, treatment, and evaluations

GROUP	No. of animals	Treatment Dose duration and time	Evaluation
Group I (Negative Control)	6 (micewithout cancer)	-	Tumor volume measurement, Body weight measurement, Hematological parameter, and Histopathological investigations
Group II (Positive Control)	6 (mice with cancer)	Normal saline; 150 µL once in a three-day (IV)	
Group III (a)	6	Fisetin (25 mg/kg); once in a three day	
(b)	6	Fisetin- NLCs (≈25mg/kg) once in a three day (IV)	
Group IV (a)	6	Cabazitaxel (5mg/kg); once in a three-day	
(b)	6	Cabazitaxel -NLCs (≈ 5mg/kg) once in a three day (IV)	
Group V (a)	6	Combination Fisetin + Cabazitaxel; once in a three-day	
(b)	6	Drug loaded NLCs of Fisetin + Cabazitaxel (IV) once in a three-day	
Group VI	6	Blank NLCs (Placebo control) (IV)	

Fig. 1. EAC cells injected in mice to form a liquid tumor

Tumor induction

The mice were initially acclimatized in the animal house to ensure they adapted to their environment. After acclimatization, Ehrlich ascites carcinoma (EAC) cells were injected intraperitoneally into two mice to facilitate liquid tumor development, as illustrated in Figure 1. Over the following 14 days, the liquid tumors formed and grew. At the end of this period, the mice were humanely sacrificed, and the liquid tumors were carefully collected for further analysis. On day 0 of the study, solid tumors were induced in the left thigh pad of all the animals, except for the standard control group. This was done using a 26-gauge syringe to ensure a precise and controlled injection of the tumor cells. Solid tumors began to develop approximately 15 days post-inoculation, marking the transition from liquid to solid tumor formation. Throughout this developmental phase, tumor volume and body weight were measured regularly to monitor the mice's health and the progression of tumor growth. Measurements were taken at consistent intervals to provide accurate data for analysis. Once the tumor volume reached a threshold of 100 mm^3, various treatment protocols were initiated for the different groups of animals. This systematic approach allowed for a thorough investigation of the effects of the treatments on tumor growth and overall health [48].

Tumor regression and body weight analysis

The tumor developed approximately two weeks after induction. Tumor volume (TV) and body weight were measured from day 0. After 15 days, the tumor volume reached about 100 mm^3. The treatments were administered as described in section 4.2.g.(II). In each group, animals received the respective treatment for 21 days with a fixed dose. On day 22, 50% (n=3) of the animals from each group were euthanized, and the remaining 50% were observed for tumor recurrence and survival over the next 14 days. Tumors and other organs were collected for histopathological studies, including the lungs, brain, heart, liver, spleen, and kidneys.

Histopathology study

A histopathological analysis was performed on the tumor and other organs to study their morphological and toxicological changes. The tumor and all other organs were excised from the mice and preserved in a 10% formalin solution in a tightly sealed container, stored in a dry location. They were then dehydrated in ethanol and embedded in paraffin. The paraffin blocks were sectioned into 5 µm slices and stained with hematoxylin and eosin (H&E) for histopathological examination [49].

Hematological parameters

Approximately 2 mL of blood was withdrawn from each mouse and collected in EDTA-coated tubes during the sacrifice process. Subsequently, hematological analysis was performed to assess parameters including white blood cells (WBCs), hemoglobin, red blood cells (RBCs), and platelet count [50].

RESULTS

Physiological and morphological characterization

Scanning Electron Microscopy (SEM)

Morphological studies were conducted using scanning electron microscopy (SEM) to analyze both optimized Fisetin (Fis)-loaded nanostructured lipid carriers (NLCs) and blank NLCs. The SEM analysis revealed that both samples exhibited a uniform distribution of spherical NLCs. However, aggregation of the lipid nanoparticles was observed in the images due to the lyophilization process (Figure 2 A & B). SEM was also performed to examine the morphology of the prepared Cabazitaxel (CBZ)-loaded NLCs. Figure 2 C & D shows that the prepared NLCs were not homogeneous and uniform. SEM analysis was performed at 20,000× magnification and 15.0 kV [37,38].

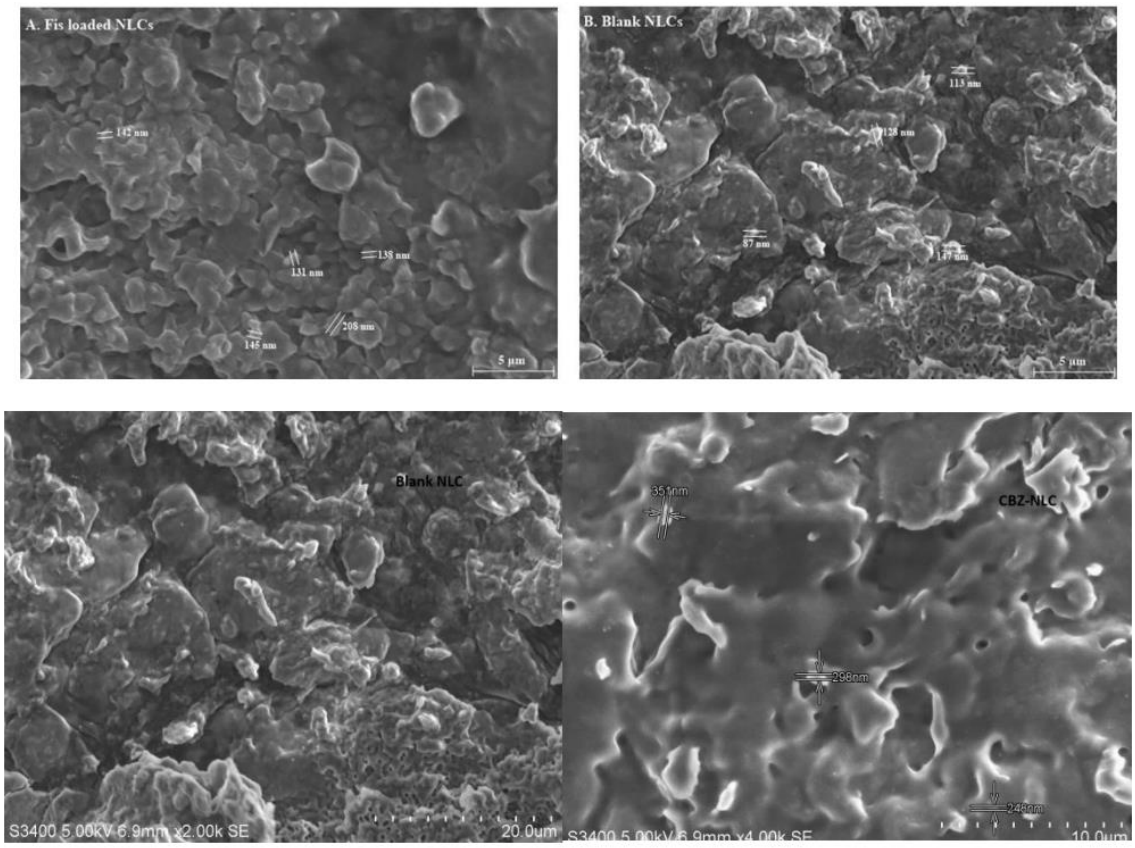

Fig. 2. SEM image of optimized (A) FisNLCs, (B) blank NLCs, (C) CBZ-loaded NLCs, and (D) blank NLCs.

Powder X-ray diffraction (PXRD) analysis

The PXRD results indicated that the pure drug Fisetin (Fis) is crystalline (see Figure 2A). The XRD spectra for Fis-loaded NLCs, blank NLCs, and the physical mixture of formulation components (see Figures 3B, 3C, and 3D) displayed diffuse peaks. This observation suggests a reduction in crystallinity, likely due to the lipids in the formulation. These results confirm that the drug is effectively encapsulated within the NLCs [37,38].

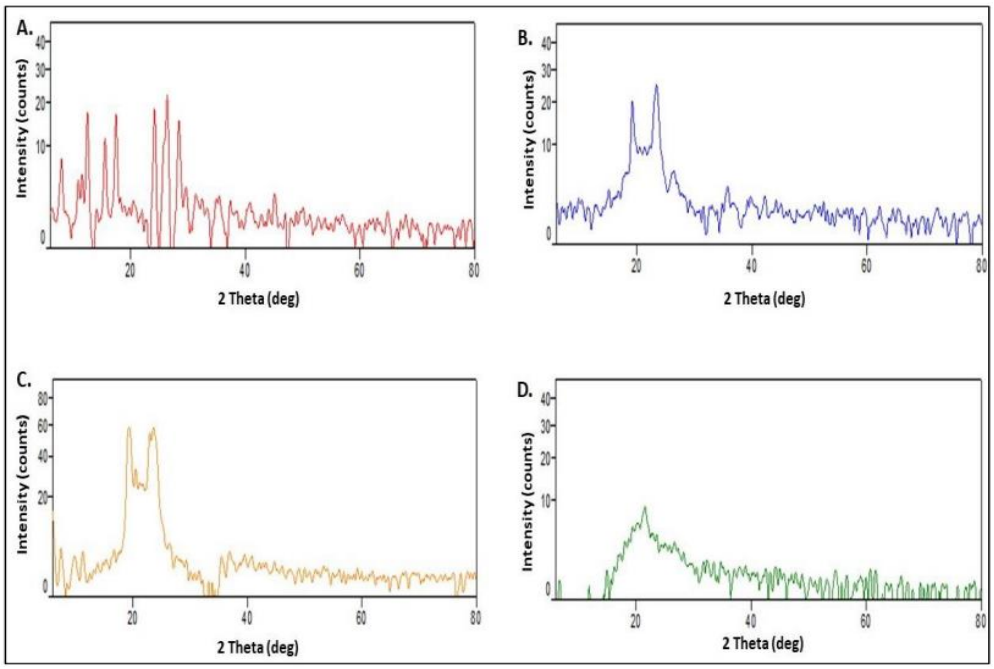

Fig. 3. PXRD: spectra of the following samples: (A) Fis, (B) NLCs of Fis, (C) Blank NLCs, and (D) the physical mixture.

In vitro evaluations against BC cells

In vitro cytotoxicity assay

The cytotoxicity assays of Fisetin (Fis) and Fis-loaded nanostructured lipid carriers (Fis-NLCs) were carried out in human breast carcinoma cell lines MDA-MB-468, MDA-MB-231, and MCF-7. As illustrated in Figure 4, the cytotoxicity graphs demonstrate the effects against MDA-MB-468. The IC50 values of Fis were 25.29±1.92 and 11.48±0.87 µM at 24 and 48 hours, respectively. Fis-NLCs exhibited IC50 values of 10.85±1.39 and 3.48±0.74 µM at 24 and 48 hours, respectively. Similarly, against MDA-MB-231 cells, the IC50 values of Fis were 21.73±0.54 and 10.85±0.98 µM at 24 and 48 hours, respectively. Fis-NLCs displayed IC50 values of 10.85±0.98 and 3.19±0.68 µM at 24 and 48 hours, respectively. Likewise, against MCF-7 cells, the IC50 values of Fis were 23.83±0.67 and 13.49±0.75 µM at 24 and 48 hours, respectively. Fis-NLCs showed IC50 values of 9.45±0.88 and 3.89±0.48 µM at 24 and 48 hours, respectively.

A cytotoxicity test was performed to evaluate the impact of Cabazitaxel (CBZ) and CBZ-loaded nanostructured lipid carriers (CBZ-NLCs) on MDA-MB-468, MDA-MB-231, and MCF-7 cell lines. As depicted in Figure 5, the IC50 values for CBZ were 16.2±1.52 and 9.93±0.88 µM for MDA-MB-468 cells, and 4.25±0.78 and 1.36±0.30 µM for CBZ-NLCs at 24 and 48 hours, respectively. Moreover, the IC50 values for CBZ in MDA-MB-231 cells were 19.31±2.41 and 7.25±1.62 µM, while those for CBZ-NLCs were 5.08±1.46 and 3.38±1.05 µM at 24 and 48 hours, respectively. The IC50 value of CBZ on the MCF-7 cell line was 18.98±1.80 and 15.74±1.24 µM at 24 and 48 hours, respectively. The optimized CBZ-NLCs exhibited IC50 values of 12.35±1.21 and 9.60±1.17 µM at 24 and 48 hours, respectively.

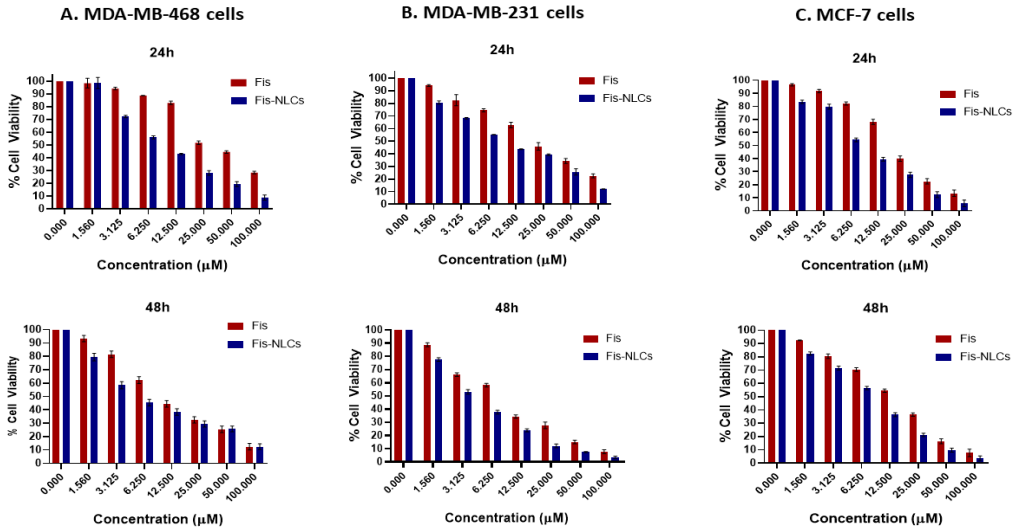

Fig. 4. Cell viability of Fis and Fis-NLCs at 24 and 48 h.

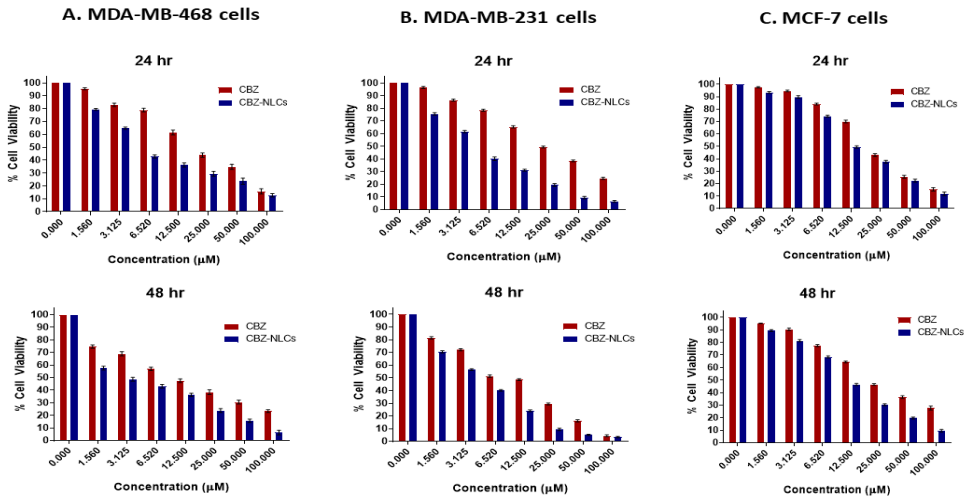

Fig. 5. CBZ- and CBZ-loaded NLCs cell viability at 24 and 48 h.

In vitro cytotoxicity assay for combination therapy

Based on the individual IC50 values obtained for Fisetin (Fis), Fis-loaded NLCs, Cabazitaxel (CBZ), and CBZ-loaded NLCs (for CBZ, as previously reported by Chand et al., 2021), different treatment patterns (sequential and simultaneous) were selected to achieve better outcomes through combination therapy. The best results were observed with the concurrent treatment of Fis/Fis-loaded NLCs at different concentrations (0-100 µM) combined with fixed CBZ/CBZ-NLCs at a concentration of 5 µM. CBZ-NLCs reduced the inhibitory concentration of Fis-loaded NLCs, inhibiting the growth of 50% of the population by 2.52±0.578, 2.17±0.24, and 2.12±0.45 µM against all the cancerous cells at 48 hours (Figure 6).

The combination index (CI) was calculated and reported in Table 2 for all treatment concentrations. The computed isobologram graphs were also plotted to normalize the values and predict the effect of drug-loaded NLCs in combination therapy, as shown in Figure 7. The concentrations selected to assess the combinatorial impact in further studies, such as migration, apoptosis, and RT-PCR, were Fis/Fis-loaded NLCs (5 and 10 µM) and CBZ/CBZ-loaded NLCs (5 or 10 µM).

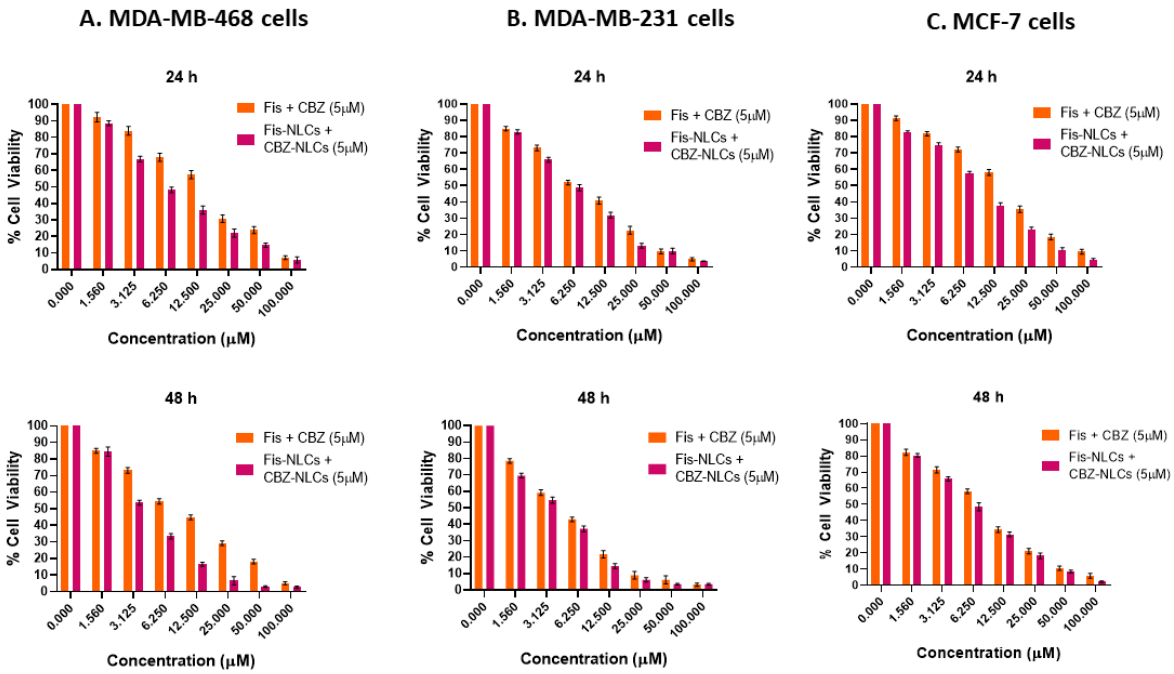

Fig. 6. Fis cell viability, Fis-NLCs with a combination of CBZ and CBZ-loaded NLCs at 24 and 48 h.

Table 2. Combination index (CI) data for drug combination experiments.

Dose of Fis/Fis-NLCs (µM)	Dose of CBZ/CBZ-NLCs (µM)	Total Dose (µM)	Default effect (Fa) levels	CI Value
1.56	5.0	6.56	0.84	4.35
3.12	5.0	8.12	0.53	0.79
6.25	5.0	11.25	0.33	0.41
12.5	5.0	17.5	0.16	0.22
25.0	5.0	30.0	0.06	0.12
50.0	5.0	55.0	0.03	0.08
100.0	5.0	105.0	0.01	0.07

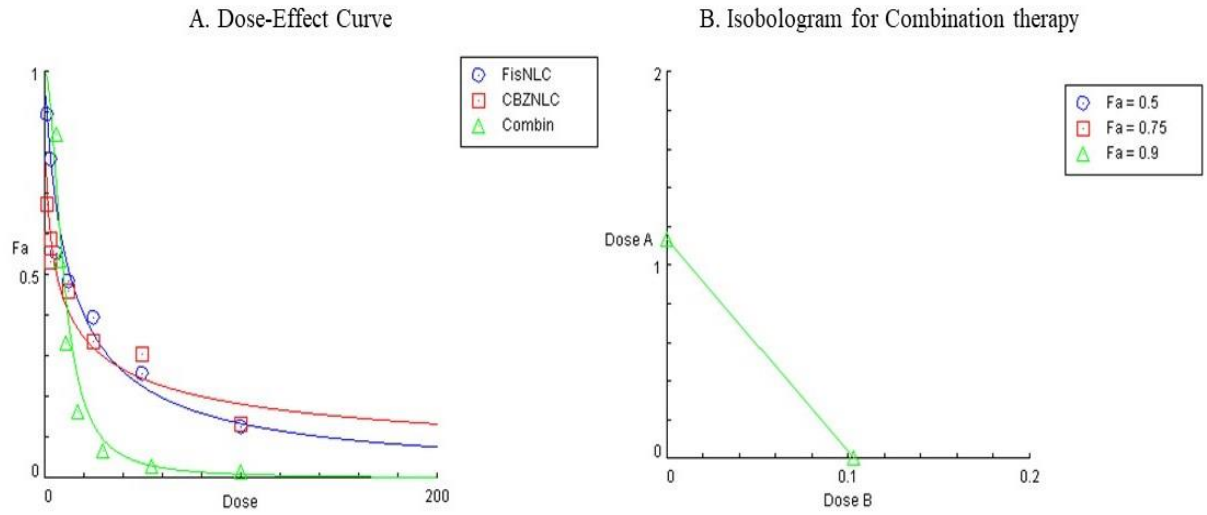

Fig. 7. Prediction of the computed combination index (CI) for combination therapy. (A) Dose-effect Curve and (B) Computed isobologram graphs. where Fa = default effect generated by the computed software CompuSyn.

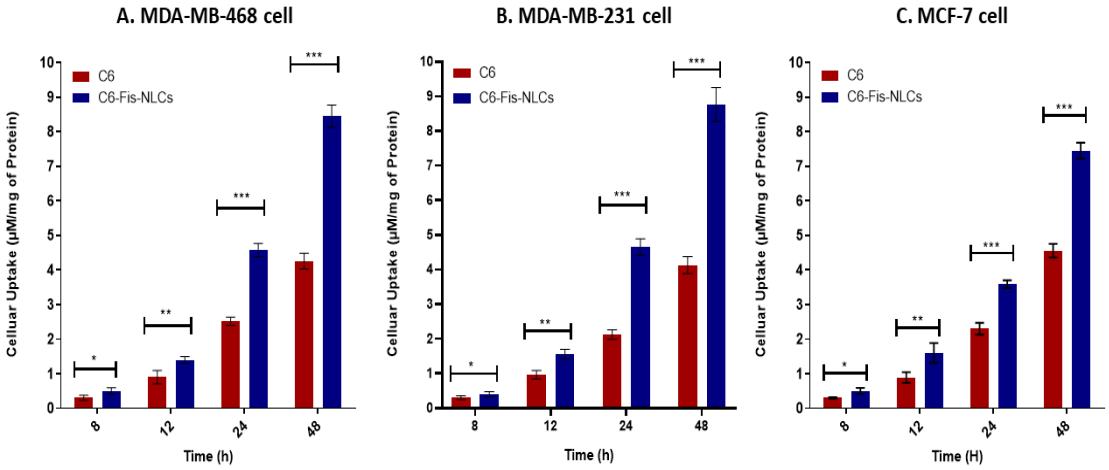

Fig. 8. Depicts the cellular uptake of C6 and C6-Fis-NLCs at several time intervals: 8, 12, 24, and 48 hours.

Cellular uptake assay

Please find below the details to be noted:

Cellular drug uptake in vitro was performed using coumarin-6 (C6), which was tagged to both the drug and NLCs to target the NLCs. As illustrated in Figure 8, the maximum cellular uptake at 48 hours was 4.26±0.23 and 8.5±0.3 µM/mg of protein for C6 and C6-Fis-NLCs, respectively, in MDA-MB-468 cell lines. In MDA-MB-231 cells, the cellular uptake was 4.13±0.25 and 8.76±0.48 µM/mg of protein for C6 and C6-Fis-NLCs, respectively. In MCF-7 cells, the maximum uptake observed was 4.56±0.20 and 7.45±0.23 µM/mg of protein for C6 and C6-Fis-NLCs, respectively.

The cellular uptake of CBZ formulations was assessed in vitro in MDA-MB-468, MDA-MB-231, and MCF-7 cell lines. The maximum observed cellular uptake in MDA-MB-468 cells was 1.61±0.014 and 3.82±2.24 µM/mg of protein for C6 and C6-CBZ-NLCs, respectively, at 48 hours. In MDA-MB-231 cells, the uptake was 1.84±0.044 and 4.12±0.25 µM/mg of protein for C6 and C6-CBZ-NLCs at 48 hours. In MCF-7 cells, the uptake was 1.52±0.025 and 3.48±0.12 µM/mg of protein for C6 and C6-CBZ-NLCs at 48 hours (see Figure 9).

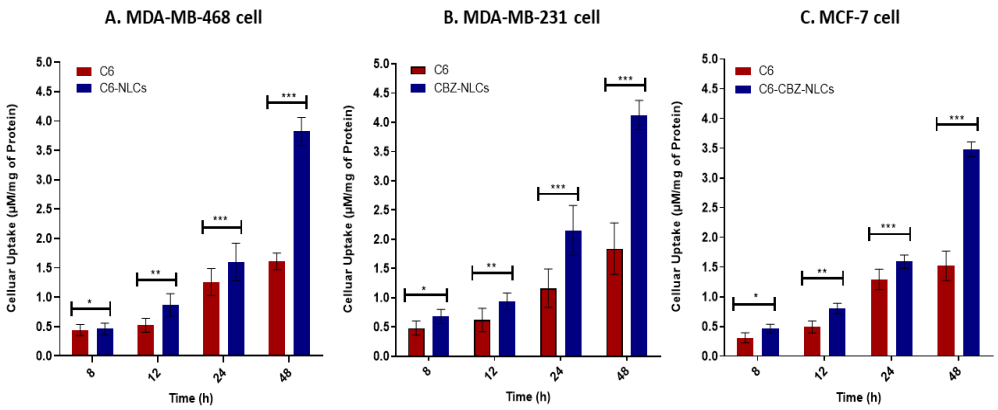

Fig. 9. Evaluation of cellular uptake of C6 and C6-CBZ-NLCs at specified time intervals (8, 12, 24, and 48 hours).

Cell migration assay

The cell migration assay evaluated the migratory capabilities of MDA-MB-468, MDA-MB-231, and MCF-7 breast cancer (BC) cell lines. We compared the percentage of cell migration inhibition across various experimental groups against the control group (no treatment) and the vehicle control group, as shown in Figure 10. The results indicated that Fis-NLCs (Fis-loaded nanostructured lipid carriers) significantly inhibited cell migration, with a p-value less than 0.0001. This finding demonstrates that Fis-NLCs are more effective at reducing cell migration than Fis alone and the vehicle control groups at their respective concentrations. These results suggest that Fis-NLCs could be a promising therapeutic strategy against BC cell migration, warranting further research into their mechanisms and efficacy.

The study assessed the antimigration efficacy of Cabazitaxel (CBZ) and CBZ-loaded nanostructured lipid carriers (CBZ-NLCs) using MDA-MB-468, MDA-MB-231, and MCF-7 cell lines. As illustrated in Figure 8, various concentrations of CBZ-NLCs were evaluated for their impact on cell migration inhibition. When comparing the cell migration of treated samples to the control group (which received no drug treatment), it was observed that CBZ exhibited a significant inhibitory effect on cell migration ($p<0.01$). Furthermore, CBZ-NLCs also demonstrated a substantial impact on the inhibition of cell migration ($p<0.001$). The results from the cell migration assay further confirmed that the vehicle control did not show any cytotoxic or inhibitory effects on the assessed cell lines.

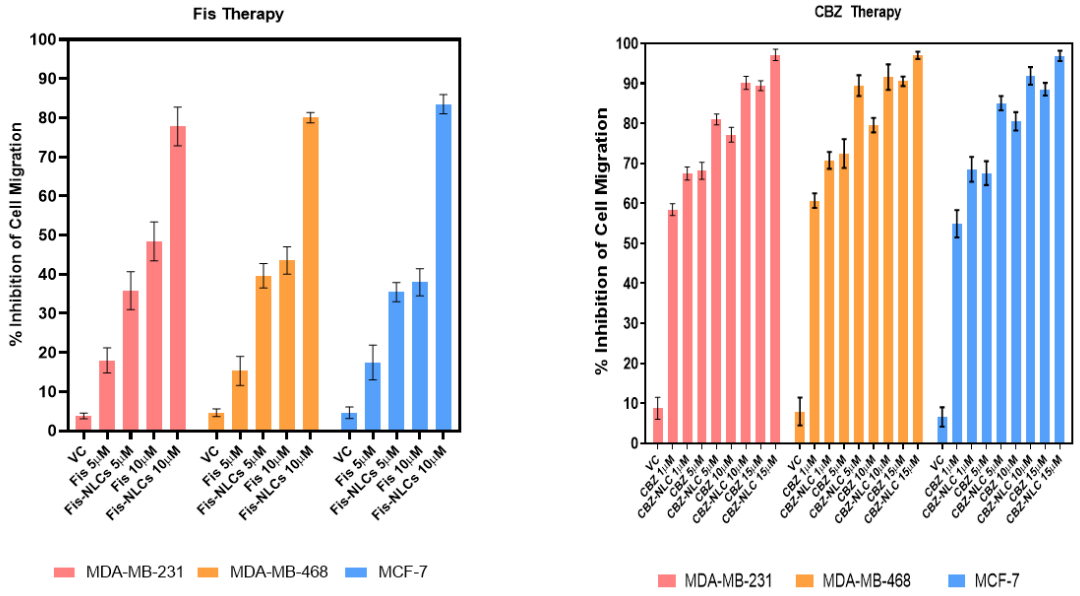

Fig. 10. Cell migration assay on BC cell lines. Fis therapy of Fis and Fis- NLCs. CBZ therapy of CBZ and CBZ-NLCs. VC- Vehicle Control (0.2% DMSO in DMEM).

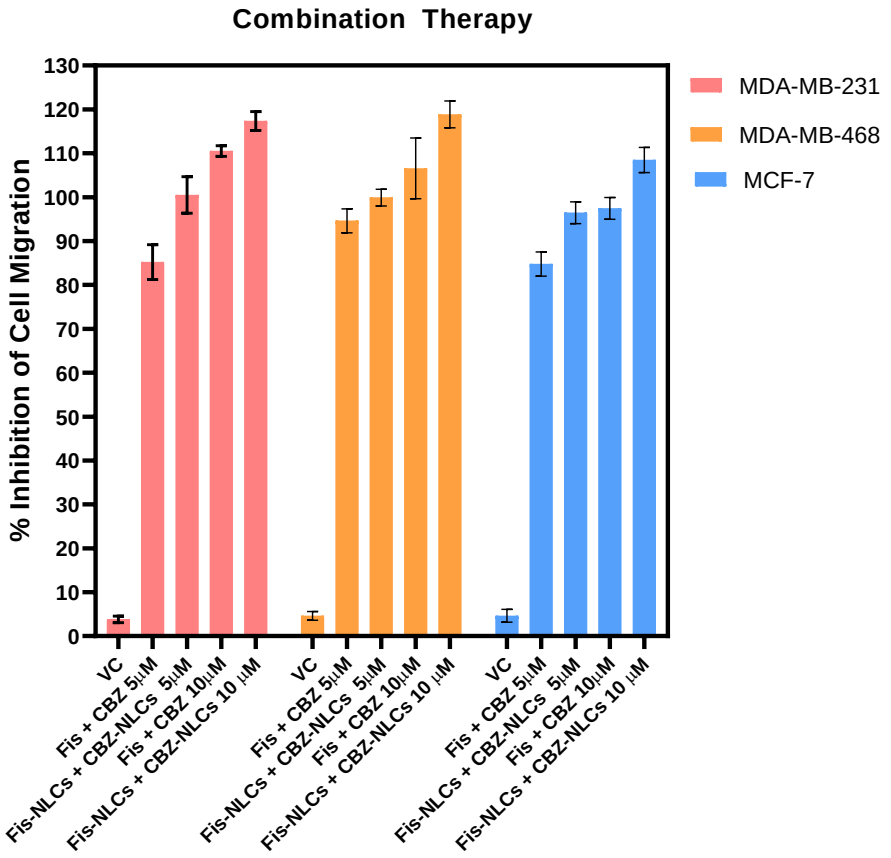

Fig. 11. Illustrates the cell migration assay conducted on BC cell lines. This assay evaluates the effects of combination therapy involving Fis and Fis-NLCs in conjunction with CBZ and CBZ-NLCs. The Vehicle Control (VC) is represented by a solution of 0.2% DMSO in DMEM.

Furthermore, when Fisetin (Fis) and Fis-NLCs were combined with Cabazitaxel (CBZ) and CBZ-NLCs (5 and 10 µM), combination therapy significantly inhibited cell migration in all three cell lines compared to single treatments ($p<0.0001$; Figure 11).

Apoptosis assay

The apoptosis assay for Fisetin (Fis) and Fis-NLCs was performed using dye-based identification of live and dead cells through fluorescence microscopy. Figure 12 shows that Fis-NLCs induced a significantly higher apoptosis rate in all three cell lines—MDA-MB-468, MDA-MB-231, and MCF-7—compared to the Fis-treated cell lines ($p>0.001$).

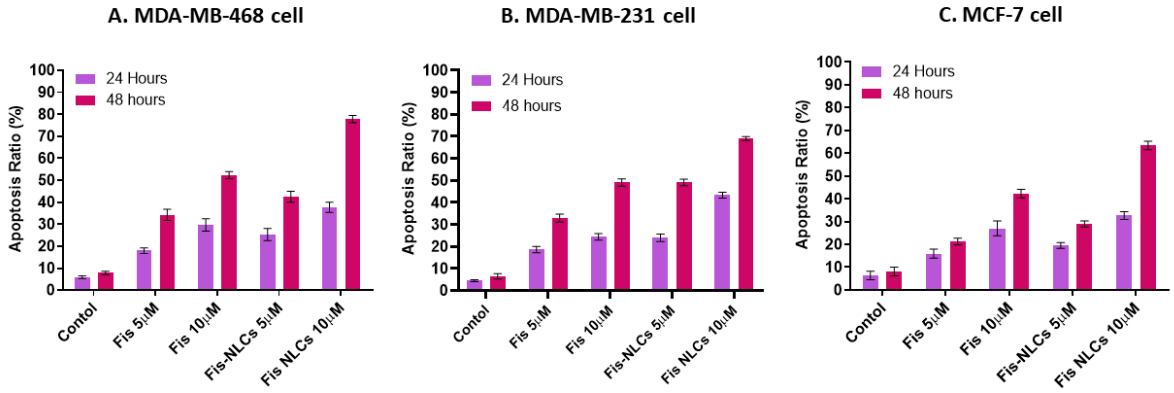

Fig. 12. Apoptosis assay conducted on BC cell lines treated with Fis and Fis-NLCs.

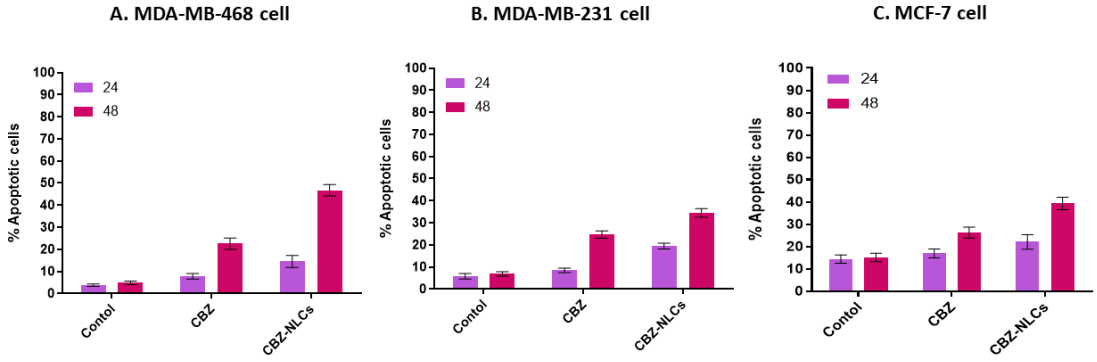

Fig. 13. Cell apoptosis assay performed on BC cell lines for CBZ and CBZ-NLCs.

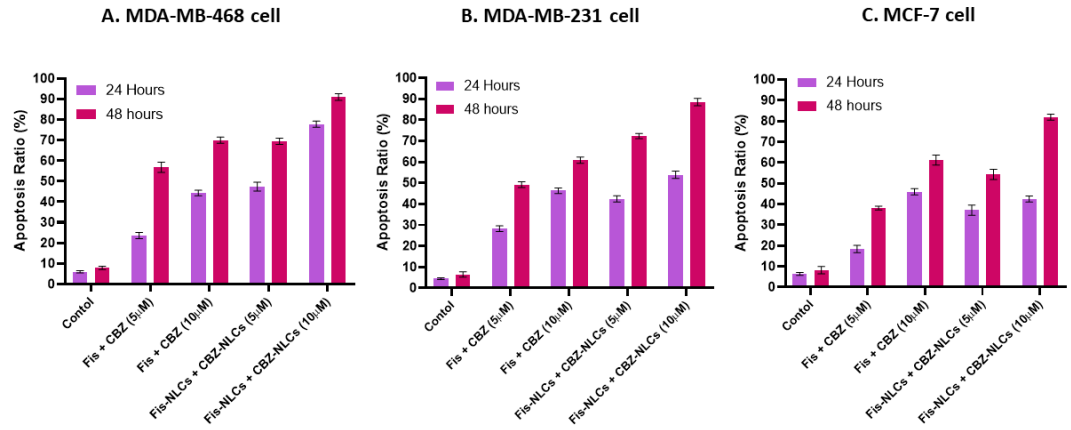

Fig. 14. Combined Effects of Fisetin (Fis) and Carboplatin (CBZ) and Fisetin-Loaded NLCs and Carboplatin-Loaded NLCs on Apoptosis in BC Cell Lines.

Apoptosis assays for Cabazitaxel (CBZ) and its formulations were conducted in vitro using the MDA-MB-468, MDA-MB-231, and MCF-7 cell lines. The evaluation of the apoptosis assays, as shown in Figure 13, indicates that CBZ-loaded nanostructured lipid carriers (NLCs) were more effective in inducing cell apoptosis than the pure drug ($p > 0.001$).

Similar combination therapies were also used in a migration assay to evaluate apoptosis in BC cell lines in vitro. The combination effect of Fisetin (Fis) + CBZ and Fis-loaded NLCs + CBZ-loaded NLCs was significantly higher ($p<0.0001$) than that of the single treatments (Figure 14).

Effect of Fis NLCs on antioxidant and apoptosis regulatory genes in A-375 cells

The RT-PCR analysis results (Figure 15) indicated that combination therapy using Fisetin (Fis)-loaded nanostructured lipid carriers (NLCs) and Cabazitaxel (CBZ)-loaded NLCs significantly increased the mRNA expression of the pro-apoptotic gene Bax. This increase suggests the effective promotion of apoptosis in the targeted cells. In contrast, the expression levels of anti-apoptotic genes were notably reduced compared to pure drug administration and single therapies, with p-values illustrating high significance ($p < 0.001$ for pure drugs and $p < 0.0001$ for single therapies). Additionally, the mRNA expression of antioxidant genes NRF-2 and NQO1 was assessed across various treatments. While NRF-2 mRNA did not significantly increase, NQO1 levels were significantly elevated in the NLC-treated group, indicating enhanced antioxidant activity. The combination therapy increased the mRNA expression of both antioxidant genes ($p < 0.001$), suggesting a robust cellular response to oxidative stress. Importantly, mRNA expression levels of the target genes in the vehicle control and blank NLC groups were minimal, highlighting the specific effectiveness of the combination therapy.

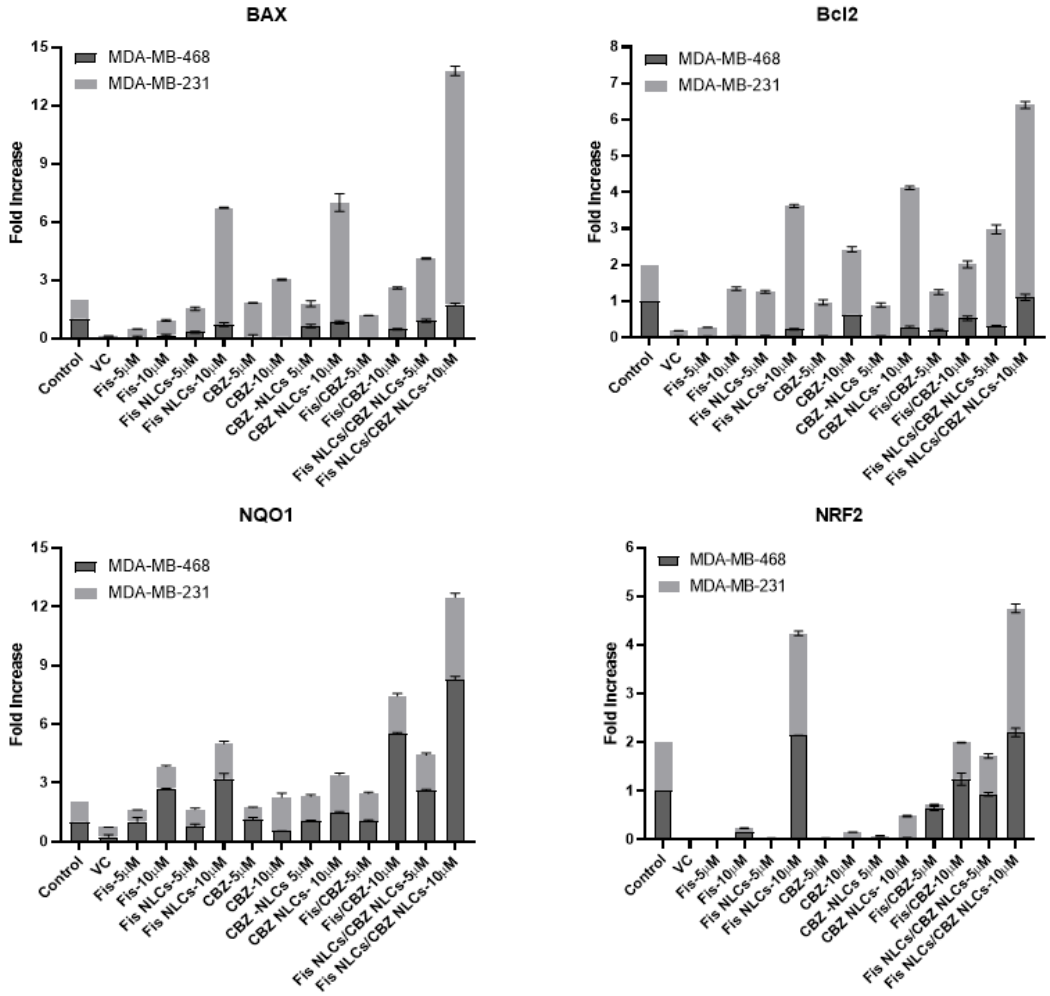

Fig. 15. Detection of mRNA expression levels of the genes (A) Bax, (B) Bcl2, (C) Nrf2, and (D) NOQ1 in BC cell lines using real-time RT–RT-PCR.

In vivo studies

In vivo biodistribution studies

The drug concentrations of Fisetin (Fis) and Cabazitaxel (CBZ), along with their nanostructured lipid carriers (NLCs), were measured in various mouse organs, including the brain, heart, lungs, liver, spleen, kidneys, and blood. The results shown in Figure 16 depict the drug and NLC concentrations 24 hours post-treatment with single therapy. The results demonstrated that the spleen and liver had higher drug concentrations; however, the blood exhibited the highest concentrations. The drug amounts were measured as ng (X103)/mg of organ or mL of blood. These findings indicate that the liver accumulates the most drugs, followed by the spleen.

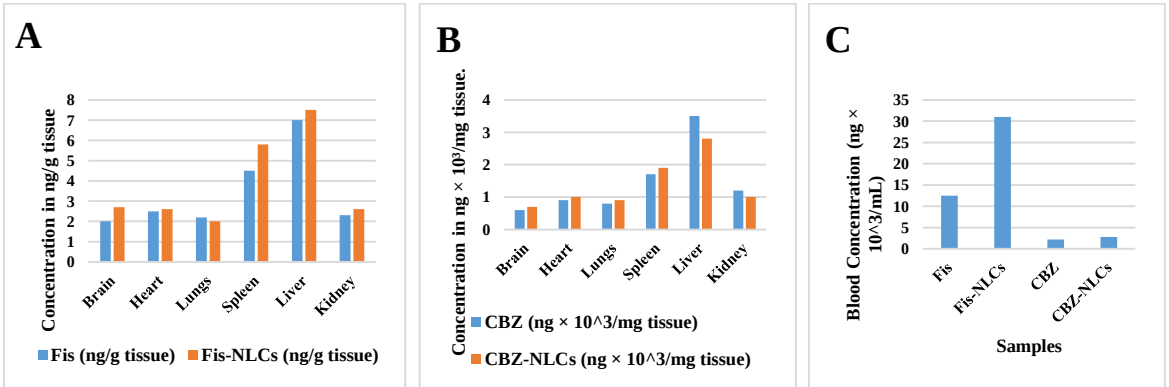

Fig. 16. Biodistribution study for (A) Fis and Fis-NLCs, (B) CBZ and CBZ-NLCs, and (C) a combination study on various organs.

Body weight and tumor regression analysis

During tumor inoculation, development, and treatment, the mice's body weight and tumor volumes were routinely measured (Figure 17). As shown in Figure 18, the body weights of the normal, positive control (PC), Fisetin (Fis), and Fis-NLC groups consistently increased. After treatment with Cabazitaxel (CBZ) and its NLC formulations, the body weight initially increased, but decreased once the treatments began. This finding indicates that CBZ may have some toxicity in mice. The combination treatments, however, did not significantly affect the mice's body weight.

Figure 18, which shows the tumor volume graphs, demonstrates that the tumor volume in the positive control (PC) group increased, as this group did not receive any drug or NLCs. The treatment groups of Fisetin (Fis) and Cabazitaxel (CBZ) as single therapies showed lower tumor volumes than the PC group. In addition, the targeted NLCs for both drugs significantly ($p<0.001$) reduced the tumor volume compared to the respective drugs and PC. Furthermore, the combination treatments decreased the tumor volume ($p<0.01$) compared to the respective single therapies. The maximum effect was observed in the targeted combination therapy ($p<0.0001$).

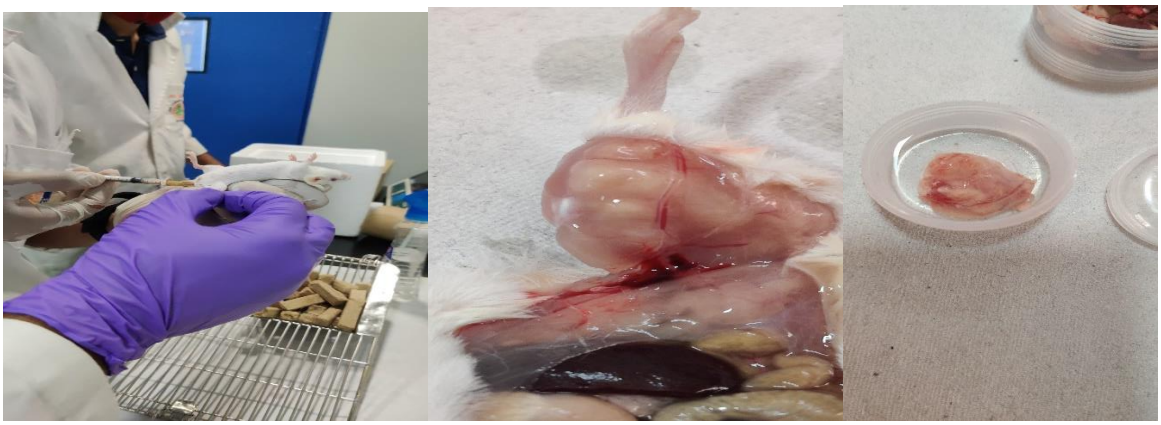

Fig. 17. Image depicts tumor inoculation, development, and treatment,

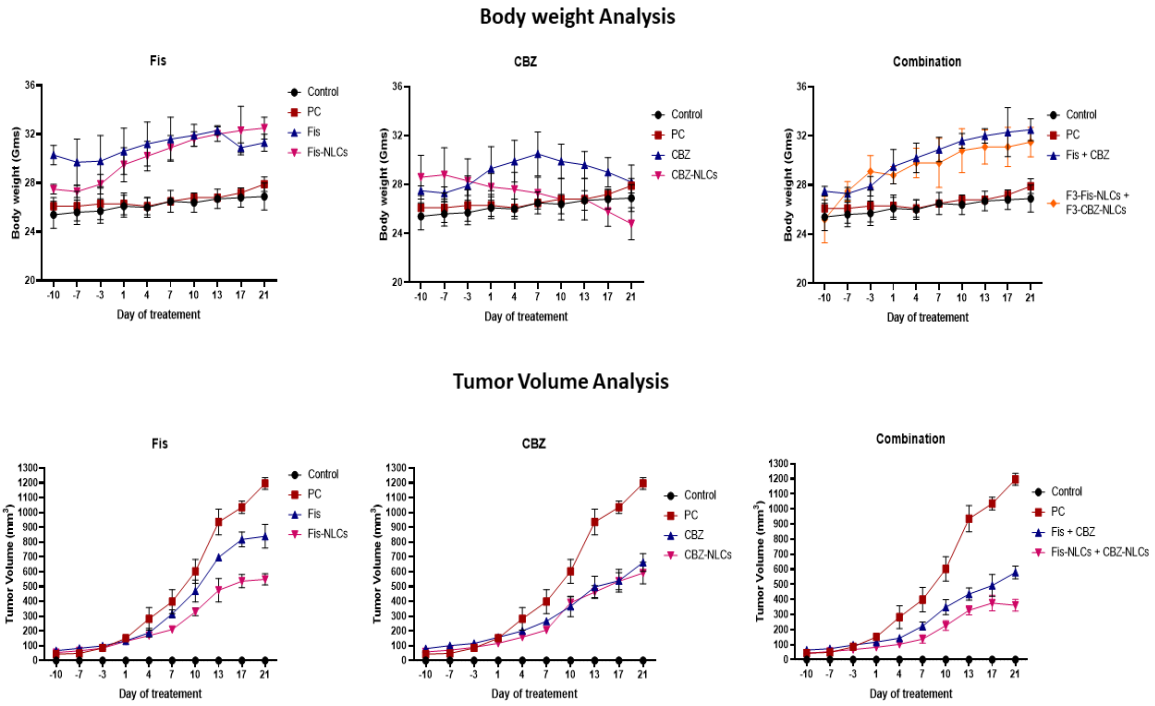

Fig. 18. Body weight and tumor volume of mice for each treatment group, i.e., Fis, CBZ, and their combination.

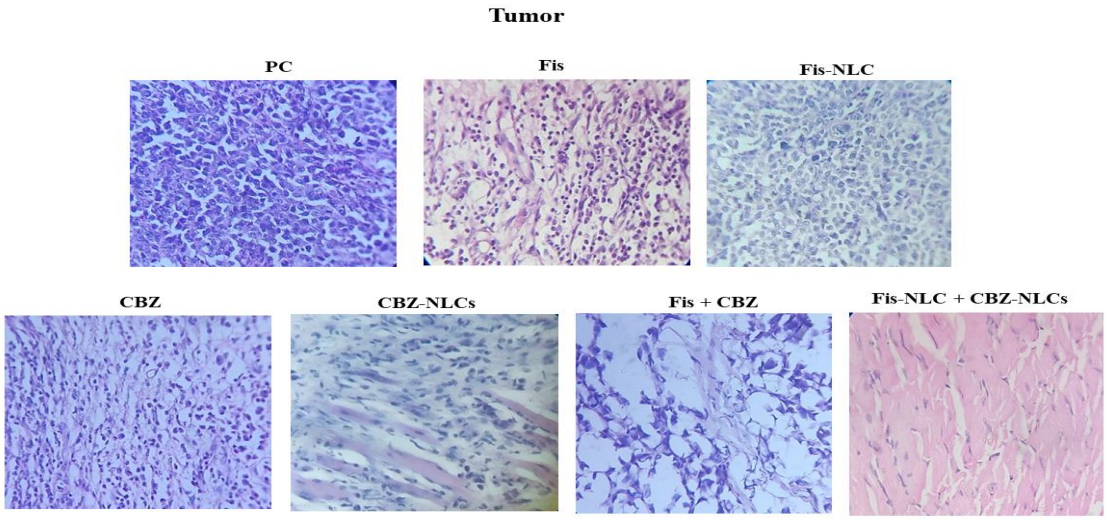

Fig. 19. Histological slides of tumor tissues after implantation of various formulations.

Histopathology studies

Histopathological parameters for tumors in different groups of animals

Histopathological studies investigated the morphological changes in tumors and other organs. As shown in Figure 19, histopathological images of the tumor mass revealed a complete loss of muscle fascicle architecture, vacuolation, and severe cellular infiltration in the positive control (PC) group. The Fisetin (Fis) group exhibited vacuolations with severe cellular infiltration, complete loss of muscle tissue architecture, diffuse necrotic changes, and congestion with perivascular degeneration of tissues. Fis-NLCs showed tissue hemorrhages with multifocal degenerative changes and necrotic swelling of the cells.

In the CBZ group, intact muscle fascicles with a high infiltration of inflammatory cells were observed. CBZ-NLCs showed degeneration of muscle fascicles with cellular infiltration due to degenerative changes and disorganized muscle architecture.

Next, the combination of Fisetin (Fis) and Cabazitaxel (CBZ) resulted in complete necrotic changes, degenerated cells, and loss of architecture. The combination of Fis-NLCs and CBZ-NLCs revealed a complete loss of cell architecture and necrotic fascicles with cell infiltration.

Histopathological Parameters for Brain in different groups of animals

Slight astrogliosis with diffuse hemorrhages and vacuolations, along with moderate architectural changes, was observed in the brain tissues of the positive control (PC) group. Diffusion of red and white pulp architecture was also observed. Normal tissue with intact neurons, focal vacuolations, mild gliosis, and degenerative changes was seen in animals from the Fisetin (Fis) treated groups (Figure 20).

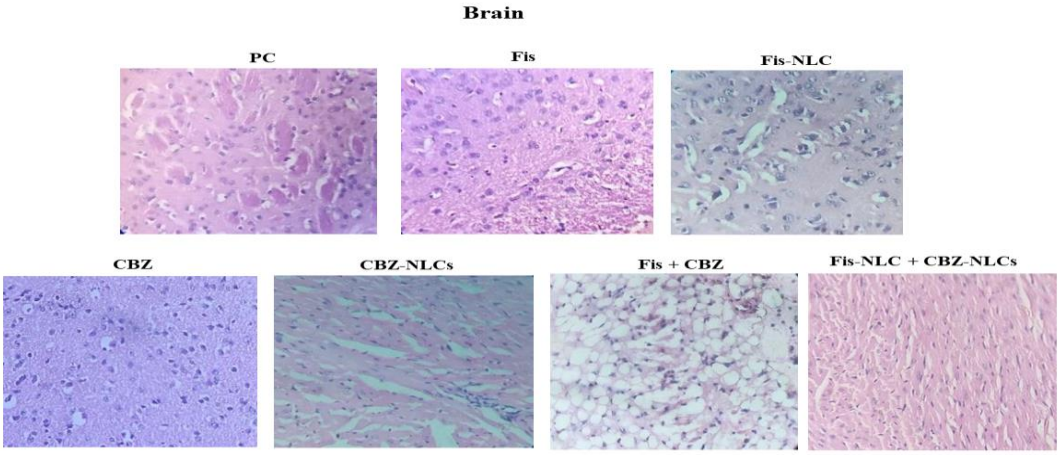

Fig. 20. Histological slides of brain tissues after implantation of various formulations.

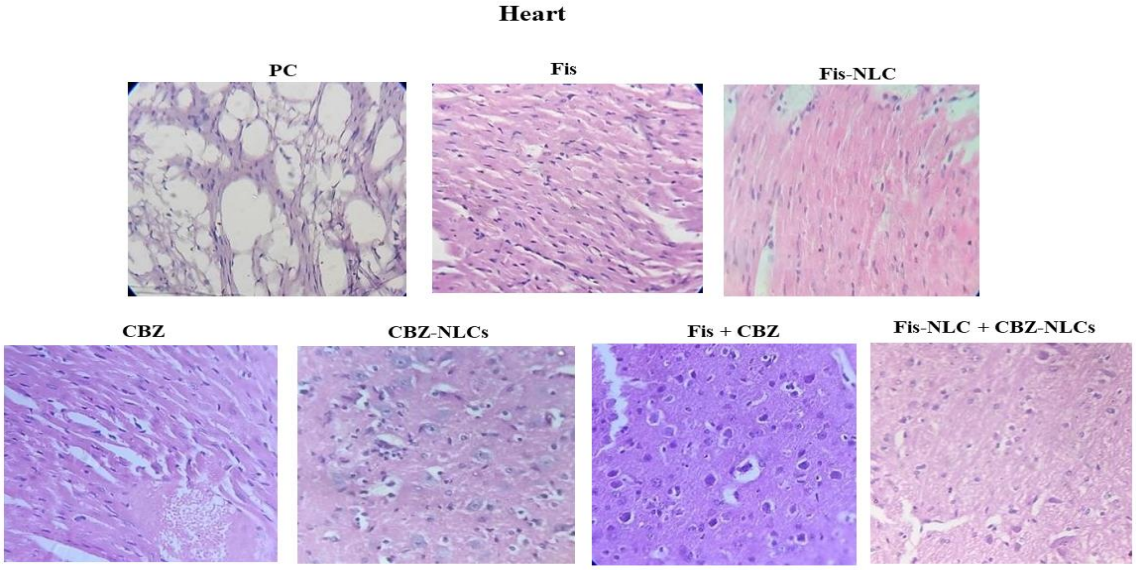

Fig. 21. Histological slides of heart tissues after implantation of various formulations.

In the CBZ group, a regular tissue pattern with intact neurons and mild gliosis was observed. The combination of Fis and CBZ resulted in swollen neurons with diffuse vacuolations, necrotic changes, severe architectural changes, and moderate astrogliosis was noted. The group treated with Fis-NLCs showed severe astrogliosis with diffuse necrosis and vacuolations.

On the other hand, CBZ-NLCs showed swollen neurons with diffuse necrotic changes, severe astrogliosis, and moderate architectural alterations. The combination of Fis-NLCs and CBZ-NLCs resulted in the complete loss of architecture, accompanied by vacuoles and severe gliosis.

Histopathological parameters of the heart in different groups of animals

The heart tissues of the positive control (PC) group showed completely disfigured architecture and degenerated pyknotic nuclei. In animals treated with Fisetin (Fis) (Figure 21), the architecture of cardiac myocytes and their centrally located nuclei were preserved. In the Cabazitaxel (CBZ) group, normal tissue architecture was observed, with maintained muscle fiber integrity, hemorrhage, and cellular infiltration. The combination of Fis and CBZ resulted in the loss of muscle architecture, diffuse necrotic muscle fascicles, and cellular infiltration with a complete loss of structure.

The group treated with Fis-NLCs exhibited severe necrotic changes, a disorganized arrangement of muscle fibers, and cellular infiltration with a significant loss of architecture. On the other hand, in the CBZ-NLCs group, necrotic muscle fibers with vacuolations and a complete loss of architecture with cellular infiltration were observed. The combination of Fis-NLCs and CBZ-NLCs revealed complete necrotic muscle fibers and a loss of architecture with cellular infiltration.

Histopathological parameters for kidneys in different groups of animals

Moderate architectural changes with swollen cells, diffuse necrotic changes, and cellular infiltration were observed in the kidney tissues of the positive control (PC) group. Slight changes in tissue architecture with mild cellular infiltration, swollen cells, and mild degenerative changes were observed in the animals treated with Fisetin (Fis).

In the Cabazitaxel (CBZ) group, architectural integrity was maintained, with normal cellular density and cellular infiltration within and around the glomeruli, accompanied by degenerative changes. The combination of Fis and CBZ showed moderate degenerative changes with swollen cells and cellular infiltration within the glomeruli.

The group treated with Fis-NLCs exhibited severe architectural changes with cellular infiltration in the glomeruli and increased cellular density. On the other hand, in the CBZ-NLCs group, a mild increase in cellular density with severe cellular infiltration within the glomeruli, mild degenerative changes in tissue, vacuolations, and cellular infiltration were observed. The combination of Fis-NLCs and CBZ-NLCs revealed moderate degenerative changes with swollen cells, hemorrhages, cellular infiltration, and architectural changes (Figure 22).

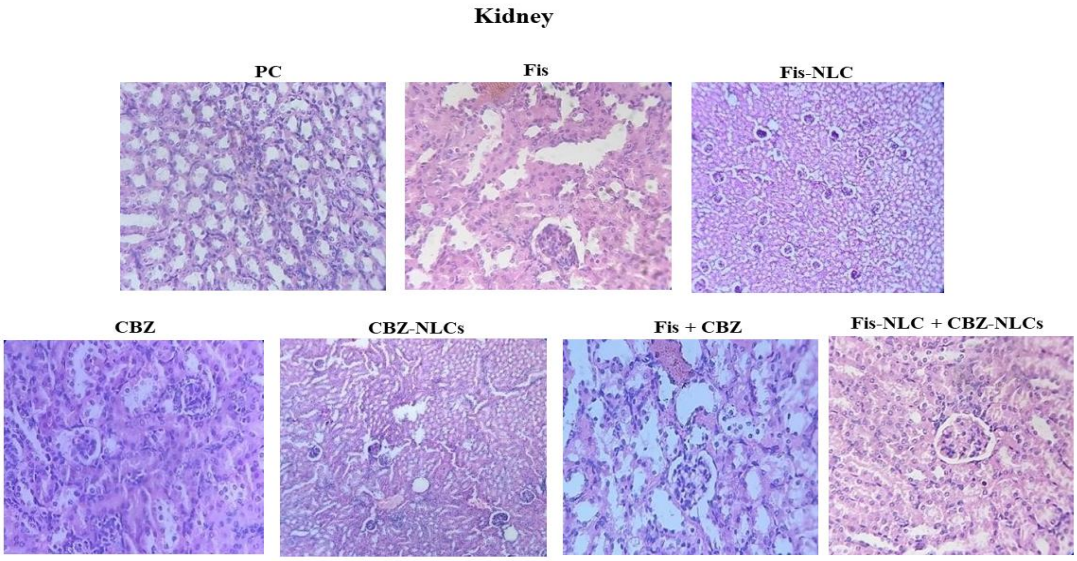

Fig. 22. Histological slides of kidney tissues after implantation of various formulations.

Histopathological parameters for the liver in different groups of animals

Severe changes in tissue architecture and density, with vacuolations of hepatocytes, diffusion of sinusoidal spaces, severe degenerative changes, and a congested central vein, were observed in the liver tissues of the positive control (PC) group. Mild changes in tissue architecture and density, cellular infiltration around the central vein, and moderate diffuse degenerative changes with loss of cellular pattern and diffuse vacuolation were observed in the animals treated with Fisetin (Fis).

In the CBZ group, a loss of cellular density and architecture, moderate multifocal degenerative changes, and vacuolations of hepatocytes were observed. The combination of Fisetin (Fis) and Cabazitaxel (CBZ) resulted in congestion in the central vein, swollen cells, and severe loss of architecture.

The group treated with Fis-NLCs exhibited severe cellular infiltration, swollen cells, and congestion in the central vein, while the architecture was maintained with normal cellular density. On the other hand, in the CBZ-NLCs group, a complete loss of architecture was observed, with multifocal swollen cells, vacuolations, and no sinusoidal spaces. The combination of Fis-NLCs and CBZ-NLCs resulted in a complete loss of architecture, with a mild increase in cellular density and swollen cells (Figure 23).

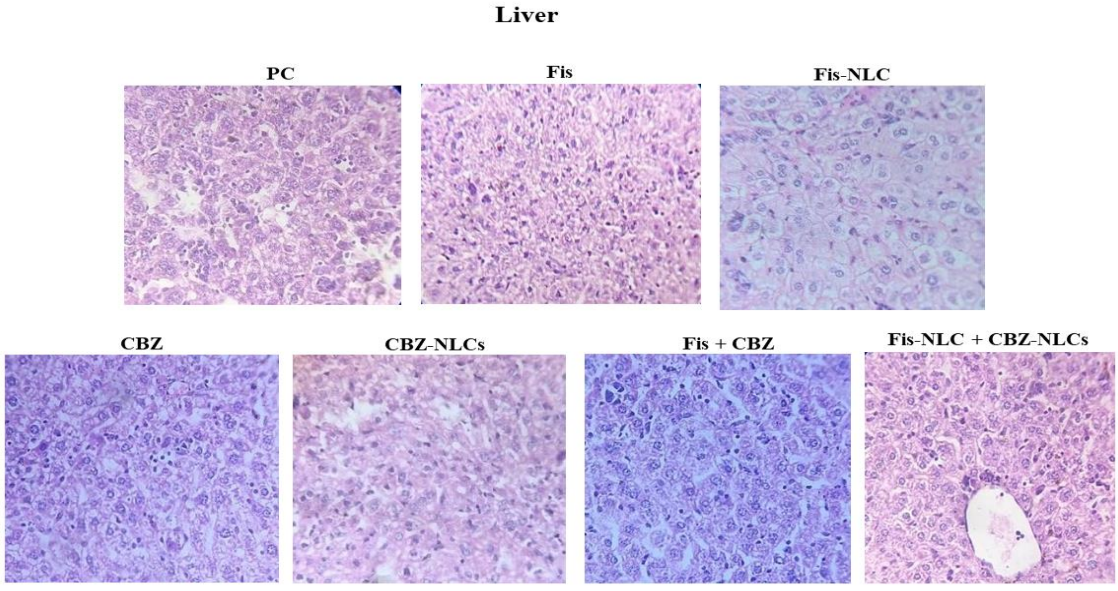

Fig. 23. Histological slides of liver tissues after implantation of various formulations

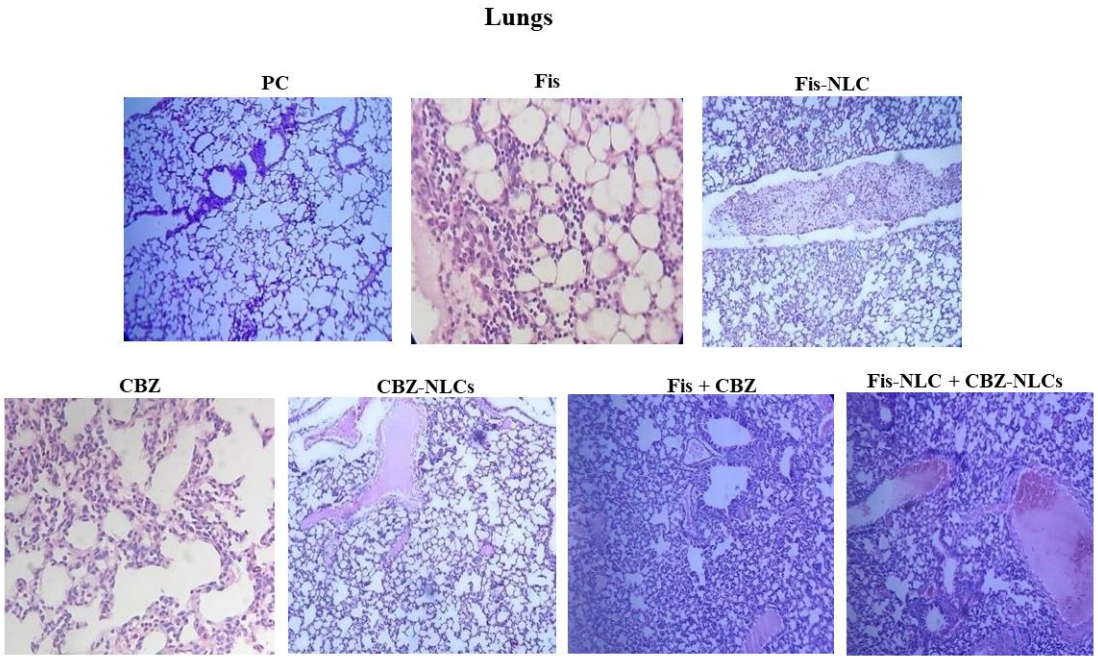

Fig. 24. Histological slides of lung tissues after implantation of various formulations.

Histopathological parameters for lungs in different groups of animals

The lung tissues of the positive control (PC) group showed severe degenerative changes, thickened interstitium, severe edematous lung parenchyma, and significant perivascular cellular infiltration. The animals in the groups treated with Fisetin (Fis) exhibited a normal tissue architecture, mild perivascular congestion, and diffuse cellular infiltration with an edematous alveolar cavity.

In the Cabazitaxel (CBZ) group, normal cellular density and architecture with normal-sized air spaces were observed. The combination of Fis and CBZ resulted in severe architectural distortion, uneven air spaces, increased cellular density, and significant degenerative changes. The group treated with Fis-NLCs showed a loss of architectural integrity, with a severe increase in cell density, hemorrhages, and degenerated cells.

On the other hand, in the CBZ-NLCs group, increased cellular density, uneven air spaces, thickened interstitium, and mild changes in architecture and cells were observed. The combination of Fis-NLCs and CBZ-NLCs revealed hemorrhages, with a severe increase in cell density, complete distortion of architecture, and significant degenerative changes in the alveolar epithelium (Figure 24).

Histopathological parameters of the spleen in different groups of animals

In the spleen tissues of the positive control (PC) group, there was no clear demarcation between the red and white pulp, and diffuse necrotic changes with severe cellular infiltration were observed. Slight architectural changes, loss of splenic cord arrangement, and cellular infiltration with moderate hydropic degeneration were observed in the animals treated with Fisetin (Fis).

In the Cabazitaxel (CBZ) group, clearly defined red and white pulp with an active marginal zone, splenic cords with increased cell density, and mild degenerative changes were observed. The combination of Fis and CBZ resulted in hemorrhage, degenerative changes, severe loss of architecture, and densified cellular architecture.

The group treated with Fis-NLCs exhibited active red and white pulp with splenic cords, degenerative cells, highly densified cell architecture, and germinal centers. On the other hand, severe degenerative changes and a complete loss of architecture were observed in the CBZ-NLCs group. The combination of Fis-NLCs and CBZ-NLCs showed active germinal centers with highly densified cell architecture and a complete loss of architecture (Figure 25).

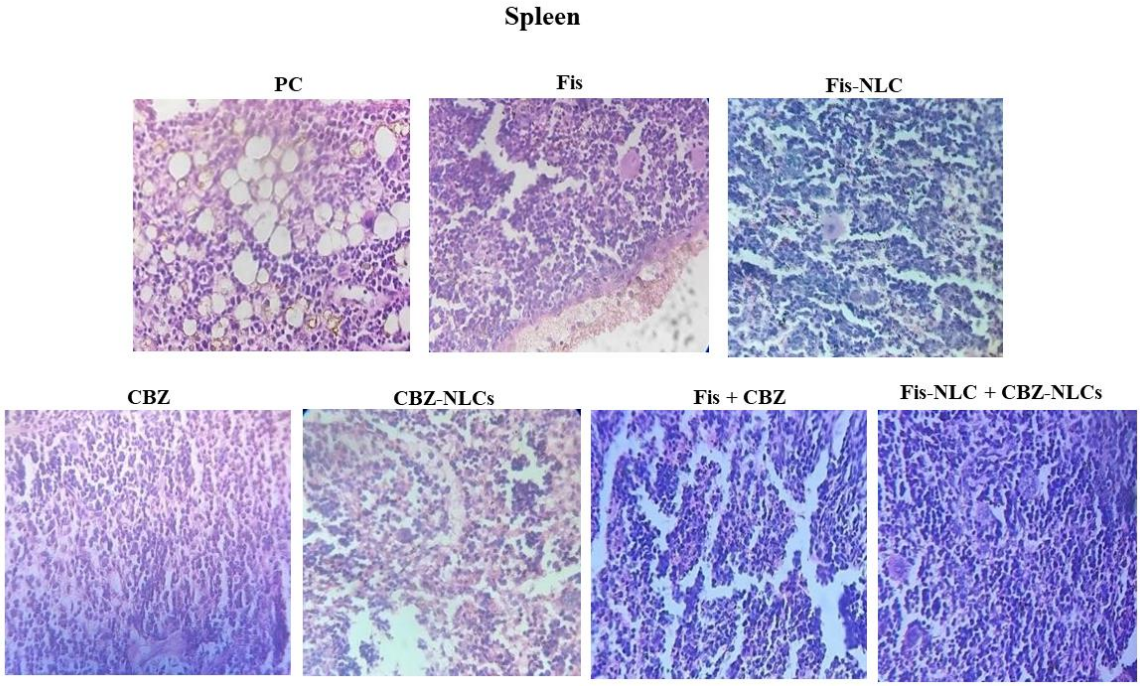

Fig. 25. Histological slides of spleen tissues after implantation of various formulations.

3.2.3.viii. Hematological Parameters

Hematological parameters

Hematological studies were conducted to investigate the effects of the drug and its NLCs on blood factors, such as white blood cells (WBCs), red blood cells (RBCs), and platelets, in both single and combination therapies. Table 3 shows that treatment with Cabazitaxel (CBZ) alone drastically decreased WBCs (5.0±1.1×10³/µL) and neutrophils (2.9±0.7%) in monotherapy and combination with pure Fisetin (Fis) (WBCs: 2.67±0.4×10³/µL and neutrophils: 1.8±0.5%). The hematological parameters of the other groups remained normal. Furthermore, the NLCs mitigated the toxicities of the pure drugs and increased the total blood counts compared to the pure medicines.

Table 3. Hematological results of mice blood after different treatments.

Sample	White blood cell (WBC) (×10³/µL)	Neutrophil (%)	Red blood cell (RBC) (×10⁶/µL)	Hemoglobin (HgB) (g/dL)	Hematocrit (HCT) (%)	Mean corpuscular volume (MCV) (fL)	Mean corpuscular hemoglobin (MCH) (pg)	Mean corpuscular hemoglobin concentration (MCHC) (g/dL)	Platelet count (PLT) (×10³/µL)
Negative Control	13.02±3.9	36.16±1.3	8.7±0.5	14.07±0.6	45.0±4.8	51.7±2.6	16.2±0.5	31.5±2.6	781.3±87.8
Positive Control	15.6± 1.4	28.8±1.4	8.1± 0.3	13.8± 1.0	41.7± 0.8	53.0± 1.8	15.6± 1.0	27.3± 1.2	1080.7± 119.2
Fis	16.7± 1.2	37.1±7.1	7.8± 0.4	12.6± 0.8	38.6± 3.1	49.3± 1.7	16.2± 0.5	32.8± 2.0	812± 117.5
Fis-NLCs	23.8± 1.3	35.8±0.7	7.5± 0.7	11.7± 1.0	39.4± 4.9	52.4± 1.6	15.7± 0.3	29.9± 1.3	676.3± 144.4
CBZ	5.0± 1.1	2.9±0.7	6.7± 0.9	12.0± 0.4	26.5± 1.5	50.1± 2.6	15.4± 0.9	29.4± 1.6	610.3± 38.2
CBZ-NLCs	9.2± 1.4	19.1±4.3	6.9± 1.1	11.5± 1.1	34.3± 7.1	49.4± 2.7	17.0± 2.8	34.6± 6.2	949.7± 29.9
Fis + CBZ	2.67± 0.4	1.8±0.5	5.7± 0.8	15.6± 0.3	34.7± 2.9	51.2± 1.3	19.0± 2.5	45.0± 3.6	705.7± 61.4
Fis-NLCs + CBZ-NLCs	10.6± 0.2	12.3±2.5	8.3± 0.3	13.0± 0.5	43.9± 1.5	51.5± 1.6	15.6± 0.1	29.6± 0.2	934.3± 78.1

In this research, we uncovered a significant potential for nanostructured lipid carriers (NLCs) to enhance the efficacy of Fisetin (Fis) in combating cancer cells. Our findings demonstrated that Fis-loaded NLCs increased cytotoxicity in specific breast cancer (BC) cell lines. The fold increase in cell growth inhibition was measured at two different time points: at 24 hours, the inhibition rates for the NLCs were 2.2 times higher for the MDA-MB-468 cell line, 2.0 times higher for MDA-MB-231, and 1.7 times higher for MCF-7. At the 48-hour mark, the results were even more striking, with increases of 3.1 times for MDA-MB-468, 3.0 times for MDA-MB-231, and 2.7 times for MCF-7. These compelling results suggest that incorporating NLCs in delivering Fisetin improves its effectiveness and presents new avenues for research and development in treating BC. The increased cytotoxicity observed with NLCs may lead to more effective therapeutic strategies, reducing the potential for resistance and enhancing patient outcomes. Overall, this study highlights the promising role of NLCs as a delivery system for cancer therapies, particularly for compounds like Fisetin, which may offer significant therapeutic benefits [51].

The cytotoxicity of CBZ-NLCs was assessed in vitro using human adenocarcinoma cell lines, specifically MDA-MB-468 and MCF-7 (which lack Her2/neu protein). These cell lines were selected to evaluate the effectiveness of CBZ-NLCs in inducing cell death across different breast cancer (BC) subtypes, providing insights into their potential as targeted cancer therapies. The findings revealed that CBZ exhibited dose-dependent cytotoxicity, likely associated with its role in modulating tubulin polymerization. To further evaluate the safety profile of the NLCs, experiments were also conducted on non-cancerous lung epithelial cells [52]. A concentration range of 0-400 µM was employed, which exceeds the 0-100 µM range used for the cancerous cell lines. At a concentration of 100 µM, after a 72-hour incubation period, the results indicated that Beas-2B (non-cancerous cells) exhibited approximately 58% cellular viability, in stark contrast to the 3.6% viability observed in MDA-MB-468 BC cells. This suggests that NLCs are safe and 16 times less toxic to non-cancerous cells. This could be because NLCs have a specific attraction to malignant cells, which may facilitate their internalization. Taxanes delivered via carrier-mediated transport have improved results and decreased toxicity at sub-therapeutic dosages [53–55].

In addition to monotherapy, combination therapy was evaluated on breast cancer (BC) cell lines through similar in vitro experiments. Initially, cell cytotoxicity was determined by combining Fisetin (Fis)-loaded NLCs and Cabazitaxel (CBZ)-loaded NLCs to identify the best combination index. Several patterns were used to identify more effective inhibition, including sequential and simultaneous treatments of both drug-loaded NLCs at different concentrations. The best therapeutic effect of combination therapy was achieved through simultaneous treatment, using various doses of Fis/Fis-loaded NLCs and an optimal concentration of CBZ/CBZ-loaded NLCs at 5 µM. Subsequently, the combination index was calculated using CompuSyn software, which showed that concentrations above 3.25 µM of Fis/Fis-loaded NLCs combined with 5 µM of CBZ/CBZ-loaded NLCs exhibited a synergistic effect. However, for further studies, we selected 5 µM and 10 µM concentrations of Fis/Fis-loaded NLCs, as they inhibited cell growth by approximately 50% of the population in combination with 5 µM and 10 µM concentrations of CBZ/CBZ-loaded NLCs. Combination studies on cell migration, cell apoptosis, and mRNA expression levels for Bax, Bcl2, Nrf2, and NQO1 suggested that the combination therapy of Fis-loaded NLCs and CBZ-loaded NLCs had a high potential to inhibit BC in vitro, compared to drug-loaded NLCs or monotherapy [56].

The study on the cellular uptake of pure Fisetin (Fis) and Fis-loaded NLCs revealed that cell growth inhibition was both dose- and time-dependent, with increased drug uptake observed over time [53–55].

The cell migration and apoptosis assay results showed that Fis-loaded NLCs were more than two times more effective than pure Fisetin (Fis) at 5 and 10 µM concentrations. Several studies have demonstrated that Fis exhibits dose-dependent activity against cancerous cell lines.

The migration of breast cancer (BC) cell lines MDA-MB-468 and MCF-7 was notably reduced after treatment with various concentrations of Cabazitaxel (CBZ) and CBZ-loaded nanostructured lipid carriers (NLCs). At lower concentrations of CBZ (1 µM and 5 µM), cell migration decreased by over 39% and 27%, respectively. Additionally, both cell lines showed a more than 29% reduction when treated with 1 µM of CBZ-loaded NLCs. At higher concentrations (10 µM and 15 µM for CBZ, and 5 µM, 10 µM, and 15 µM for NLCs), significant inhibition of cell migration was observed. These results suggest that CBZ, combined with NLCs, may

effectively reduce cell migration in BC, highlighting its potential as a therapeutic agent.

The apoptosis assay demonstrated results consistent with those obtained from the CBZ-loaded NLCs. Consequently, the observed decrease in cell migration, combined with the increased apoptosis rate in the CBZ-treated cells, supports the conclusion that these drug-loaded NLCs facilitate higher cellular uptake and exhibit enhanced cytotoxicity toward cancerous cell lines. We further evaluated the expression of regulatory genes with expert apoptosis and antioxidant properties after treating with Fisetin (Fis) and Fis-loaded NLCs. RT-PCR analysis showed that Fis-loaded NLCs effectively induced apoptosis by significantly downregulating the pro-apoptotic gene Bax and upregulating the anti-apoptotic gene Bcl2 compared to Fis alone. Moreover, Fis-loaded NLCs exhibited strong antioxidant properties by upregulating the NQO1 gene compared to Fis [57–59].

The in vivo biodistribution studies indicated that pure Fisetin (Fis) reached higher concentrations in the spleen and tumor than in other organs. However, the Cabazitaxel (CBZ) concentration was higher in the liver than in CBZ-loaded NLCs. The presence of the drug and NLCs was also higher in the blood serum, suggesting that NLCs enhanced blood circulation and reduced the elimination of drugs from nanometric systems.

Body weight and tumor volume were regularly monitored during tumor growth and treatment. The body weight of the CBZ group decreased, indicating drug-related toxicities in mice, whereas the CBZ-NLCs group did not show any toxicities. When both targeted NLCs were combined, the tumor volume was lower than that of monotherapy's, suggesting that combinatorial therapy exhibited a synergistic effect.

Histopathological studies were conducted to investigate the drug's toxic effects and to prepare NLCs for the tumor and other organs. It was observed that the pure drugs Fisetin (Fis), Cabazitaxel (CBZ), and their combination produced a mildly toxic impact on the liver, heart, and spleen. In contrast, the drug-loaded NLCs and their combinations were safe for administration [60].

The primary observation during drug dosing in mice was that CBZ and the combination of CBZ with Fis drastically decreased the white blood cell (WBC) and neutrophil counts to life-threatening levels. In contrast, the drug-loaded NLCs and their combinations for both drugs showed values more or less similar to those of the control and positive control groups. These experimental results confirmed that the efficacy of the combination therapy of Fis-NLCs and CBZ-NLCs was higher than that of the drug alone and monotherapy [48].

CONCLUSION

Formulation scientists have consistently focused on developing effective delivery systems, particularly for plant-derived products and lipophilic components, with potential bioapplications in biomedicine. However, the lipid carrier system should be biodegradable, highly potent, and capable of enabling a high drug payload. Nanostructured lipid carriers (NLCs) are lipid-based delivery systems that can deliver drugs that are either highly lipophilic or hydrophilic, with a high payload, and can target the therapeutic site of action inside the cell. Fisetin (Fis) is a natural bioactive compound used as an anticancer agent against several cancers, including breast cancer (BC). Cabazitaxel (CBZ) is a synthetic derivative of taxane moieties that is highly effective and sensitive against drug-resistant BC. In this work, in vitro cell line studies were conducted on NLCs, which demonstrated that the drug-loaded NLCs were significantly more effective, as the lipophilic characteristics of NLCs promoted drug targeting compared to pure drugs. Furthermore, the combination treatment results from cell line studies indicated that combining Fis-loaded NLCs and CBZ-loaded NLCs potentially inhibited BC cell growth more effectively than monotherapy. Animal experimental results confirmed that the efficacy of combination therapy with Fis-NLCs and CBZ-NLCs was higher than that of either drug alone or monotherapy.

Based on the observed outcome, the NLCs (for both mono- and combinations) were practical and potential nanocarrier systems for delivering bioactives such as Fis and CBZ.

ETHICAL CONSIDERATIONS

All animal experiments were conducted in accordance with the guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, under the Prevention of Cruelty to Animals Act, 1960. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of JSS Academy of Higher Education & Research, with approval number 261/PO/ReBi/S/2000/CPCSEA.

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No conflict of interest.

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