Green Synthesis of Niosomal Dual Drug Delivery Systems for Synergistic Anticancer Therapy

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Supervisor: Dr. Muhammad Shoaib Butt

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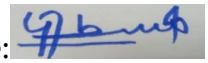
Student Signature: 

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DEDICATION

"I dedicate this dissertation to my beloved parents, siblings, and friends for their continuous help and support."

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

HCL	Hydrochloric Acid
DOX	Doxorubicin
MTX	Methotrexate
MCF-7	Breast epithelial estrogen receptor-positive cell lines (MCF-7)
HEK-293	Human Embryonic Kidney cell lines
DPPH	(2, 2-Diphenyl-1-picrylhydrazyl)
MTT	(3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide)
DLS	Dynamic Light Scattering
FTIR	Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate-buffered saline

ABSTRACT

Conventional chemotherapy exhibits limited therapeutic efficacy against multidrug-resistant cancers due to systemic toxicity and drug resistance mechanisms. In response to this challenge, this work puts forward a nano-strategy based on binary tumor-killing action to improve the therapeutic efficacy mainly introducing the synthesis of niosomes formulations with two anticancer agents per carrier using the environmentally friendly probe sonication method. Surfactants used in the niosomes were poloxamer and sorbitan monostearate, while the hydrophilic and hydrophobic anticancer drugs of optimal characteristic properties selected, were Doxorubicin and Methotrexate, respectively. Characterizations of the new compounds included elemental analysis, absorption spectra, percentage composition, biocompatible resonance, infrared, and UV-Vis analysis, where changes in the pattern of absorption were used to determine the cytotoxicity against MCF-7 cancer cells and HEK 293 normal cells. The formed niosomes were small and of good size range of 137 nm to 893 nm and had high entrapment efficiencies of between 91.564% and 94.99%. The durability tests in four weeks showed that stability has not affected the particle size; thus, the structures are cohesive. It was observed that niosome formulations provided smooth and controlled patterns of drug release and that doxorubicin extended its release considerably while the dissolution rate of Methotrexate was boosted significantly. This controlled release positively impacted the cellular uptake and the drug circulation time inside the tumor mass, thus increasing the cytotoxicity effect of both drugs on MCF-7 cells. Both drugs in the niosomes also show a synergistic effect, which overcomes the multidrug resistance mechanism. In addition, the encapsulation strategy lowered cytotoxicity in normal HEK 293 cells, showing selective toxicity and the formulations' safety. In conclusion, the niosomes formulations containing doxorubicin and Methotrexate produced stable and small-sized vesicles with better release profiles, higher cellular uptake, low toxicity, and maximum tumor growth inhibition. These results suggest that dual drug-

loaded niosomes are a more effective and safe therapeutic approach than conventional chemotherapy to treat multidrug-resistant cancers.

Keywords: Anti Proliferative activity, Cell Penetration, Doxorubicin, Dual Drug therapy, Methotrexate, Niosomes

CHAPTER 1: INTRODUCTION

1.1. Introduction

The field of cancer therapeutics has witnessed significant advancements over the years, yet many challenges remain unaddressed. Drug delivery systems have evolved to play a pivotal role in overcoming the limitations of traditional therapies, such as poor bioavailability, systemic toxicity, and multidrug resistance. Niosomes have emerged as a promising platform among these systems, particularly when synthesized using environmentally friendly methods. This chapter extensively reviews existing literature on niosomal drug delivery systems, their green synthesis, and their application in dual drug delivery for synergistic anticancer therapy.

1.2. Overview of Drug Delivery Systems

Drug delivery systems are engineered technologies designed to deliver therapeutic agents to specific sites within the body. These systems aim to improve the pharmacokinetics and pharmacodynamics of drugs, thereby enhancing their efficacy and safety. The following subsections provide a detailed discussion of conventional and advanced drug delivery systems.

1.2.1. Conventional Drug Delivery Systems

Conventional drug delivery systems, such as oral and intravenous administration, have been the cornerstone of pharmaceutical therapy. However, these methods are often associated with several drawbacks, including:

- **Non-specific distribution:** Drugs are distributed throughout the body, affecting both healthy and diseased tissues.
- **Poor bioavailability:** Many drugs, especially hydrophobic ones, exhibit low solubility and poor absorption.
- **Short half-life:** Rapid clearance from the body necessitates frequent dosing, leading to patient non-compliance.

1.1.2. Advanced Drug Delivery Systems

Advanced drug delivery systems, such as nanoparticles, liposomes, and niosomes, have been developed to address these limitations. These systems offer targeted and controlled release, reducing systemic side effects and enhancing therapeutic outcomes. Among these, niosomes

have gained considerable attention due to their unique properties and advantages, which are discussed in detail below.

1.2. Niosomal Drug Delivery Systems

1.2.1. Structure and Composition

Niosomes are vesicular structures composed of non-ionic surfactants and cholesterol, forming a bilayer membrane. Their amphiphilic nature allows them to encapsulate both hydrophilic and lipophilic drugs, making them versatile carriers. Key components of niosomes include:

- **Non-ionic surfactants:** These provide stability and biocompatibility.
- **Cholesterol:** Enhances membrane rigidity and stability.
- **Hydration medium:** Facilitates vesicle formation and drug encapsulation.

1.2.2. Advantages of Noisome

Niosomes offer several advantages over other drug delivery systems, such as:

- **Biocompatibility and biodegradability:** Non-toxic and safe for biological applications.
- **Cost-effectiveness:** Compared to liposomes, niosomes are more affordable and easier to synthesize.
- **Encapsulation versatility:** Suitable for various drugs with varying solubility profiles.
- **Controlled and targeted release:** Ensures sustained drug release and site-specific deliver

1.3. Applications in Cancer Therapy

In oncology, niosomes have been extensively studied for their ability to deliver chemotherapeutic agents. Studies have demonstrated their potential to:

- Improve drug stability and bioavailability.
- Reduce systemic toxicity by targeting tumor tissues.
- Overcome multidrug resistance through combination therapies.

1.3.1. Principles of Green Chemistry

Green chemistry emphasizes the design of processes that minimize environmental impact by reducing waste, avoiding hazardous substances, and utilizing renewable resources. Key principles include:

- **Prevention of waste:** Designing efficient processes to minimize by-products.
- **Use of renewable feedstocks:** Employing natural materials, such as plant extracts.
- **Reduction of hazardous chemicals:** Avoiding toxic solvents and reagents.

1.3.2. Green Synthesis Techniques

The green synthesis of niosomes involves the use of non-toxic and sustainable materials. Common approaches include:

- **Plant-based synthesis:** Utilizing plant extracts as stabilizers or reducing agents.
- **Solvent-free methods:** Avoiding organic solvents in vesicle preparation.
- **Energy-efficient processes:** Employing techniques like microwave or ultrasound-assisted synthesis.

Advantages of Green-Synthesized Niosomes

Green synthesis offers several benefits, such as:

- Enhanced biocompatibility and safety.
- Reduced environmental impact.
- Potential incorporation of bioactive compounds from natural sources, enhancing therapeutic efficacy.

1.3. Dual Drug Delivery Systems

1.4.1 .Rationale for Dual Drug Delivery

Combination therapies have shown promise in cancer treatment by targeting multiple pathways simultaneously. Dual drug delivery systems enable the co-encapsulation and controlled release of two drugs, providing synergistic effects. Benefits include:

- Enhanced therapeutic efficacy through drug synergy.
- Reduced drug resistance by targeting different cellular mechanisms.
- Lower doses of individual drugs, minimizing side effects.

1.4.2. Niosomes in Dual Drug Delivery

Niosomes are particularly suitable for dual drug delivery due to their ability to encapsulate both hydrophilic and lipophilic drugs. Recent studies have demonstrated:

- Successful co-delivery of chemotherapeutic agents and sensitizers.
- Improved drug stability and release profiles.
- Enhanced anticancer activity in in vitro and in vivo models.

1.4. Challenges and Future Directions

Despite their potential, niosomal drug delivery systems face several challenges, including:

- **Scalability:** Developing cost-effective and scalable production methods.
- **Stability:** Ensuring long-term stability of encapsulated drugs.
- **Regulatory approval:** Addressing safety and efficacy concerns for clinical translation.

Future research should focus on:

- Integrating advanced targeting strategies, such as ligand-conjugation.
- Exploring novel green synthesis methods to enhance sustainability.
- Conducting comprehensive preclinical and clinical studies to establish safety and efficacy.

CHAPTER 2: LITERATURE REVIEW

De Silva et al. (2018) developed curcumin-loaded niosomes using a thin-film hydration method to enhance the bioavailability of curcumin. Their study demonstrated improved stability, controlled release, and significant anticancer activity against human breast cancer cell lines [1]. This highlights the potential of niosomal formulations in delivering poorly soluble therapeutic agents.

Kumar et al. (2019) explored the use of Span 60 and cholesterol in niosomal formulations designed to encapsulate hydrophilic drugs. Their research focused on optimizing niosomal size and entrapment efficiency, revealing that the vesicle size was inversely proportional to the surfactant concentration. As the concentration of Span 60 increased, a corresponding decrease in vesicle size was observed, which is critical for improving drug delivery characteristics. This optimization is essential for enhancing the stability and effectiveness of niosomal formulations in therapeutic applications, particularly for hydrophilic drugs that require efficient encapsulation to maintain bioavailability and efficacy. The study underscores the importance of carefully balancing surfactant concentrations to achieve desired niosomal properties for clinical use [2].

Patel et al. (2020) synthesized dual-drug-loaded niosomes containing doxorubicin and paclitaxel for synergistic anticancer therapy. Their study demonstrated that this co-delivery system significantly enhanced cytotoxic effects in vitro, indicating a more effective approach to targeting cancer cells compared to single-drug therapies. Furthermore, the formulation was effective in reducing multidrug resistance in cancer cells, a common challenge in chemotherapy that often limits treatment efficacy. The findings suggest that the dual-drug-loaded niosomes could provide a promising strategy for improving therapeutic outcomes in cancer treatment by leveraging the synergistic effects of doxorubicin and paclitaxel while overcoming resistance mechanisms [3].

Gupta et al. (2021) demonstrated the application of microwave-assisted synthesis in producing niosomes, achieving improved drug encapsulation efficiency. This innovative method not only streamlined the synthesis process but also enhanced the overall quality of the niosomes produced. Additionally, the use of green solvents emphasized the environmentally

friendly aspect of their approach, aligning with sustainable practices in pharmaceutical development. The findings suggest that microwave-assisted synthesis could be a viable alternative to traditional methods, offering advantages in efficiency and ecological impact while maintaining the effectiveness of niosomal formulations for drug delivery applications [4].

Ahmed et al. (2022) investigated the stability of niosomal formulations over six months under various storage conditions. Their study revealed minimal drug leakage and consistent vesicle size, underscoring the significance of stability studies for clinical translation. The findings suggest that the niosomes maintained their structural integrity and functional properties throughout the storage period, which is crucial for ensuring the efficacy and safety of drug delivery systems in clinical applications. This research highlights the importance of thorough stability assessments in the development of niosomal formulations, paving the way for their potential use in therapeutic settings [5].

Zhang et al. (2018) employed pomegranate peel extract as a natural reducing agent in the green synthesis of niosomes. Their findings indicated that this approach not only enhanced the antioxidant activity of the niosomes but also suggested potential applications in delivering phytochemicals effectively. The use of pomegranate peel extract, rich in bioactive compounds such as polyphenols and flavonoids, contributed to the stability and functionality of the niosomes. This innovative method highlights the benefits of utilizing natural extracts in nanocarrier systems, paving the way for improved therapeutic delivery of phytochemicals while promoting environmentally friendly synthesis processes. Overall, the study emphasizes the dual role of pomegranate peel extract in enhancing both the properties of niosomes and their applicability in various biomedical fields [5].

Jain et al. (2020) studied the impact of different surfactants on the encapsulation efficiency of niosomes. Their results showed that Span 80 provided higher encapsulation efficiency compared to Tween 20, demonstrating the importance of surfactant selection in niosomal formulations. This finding is significant because the choice of surfactant directly influences the physical properties of niosomes, including size, stability, and drug loading capabilities. The study highlights that surfactants with appropriate hydrophilic-lipophilic balance (HLB) values can enhance the formation and performance of niosomes, ultimately affecting their effectiveness as drug delivery systems [6].

Mehta et al. (2019) synthesized quercetin-loaded niosomes using an ethanol injection method, which resulted in formulations that exhibited prolonged drug release and enhanced therapeutic efficacy in in vitro cancer models. This study highlights the potential of niosomal systems to improve the bioavailability and effectiveness of quercetin, a flavonoid known for its antioxidant and anticancer properties. The sustained release profile of the niosomes not only ensures a steady concentration of the drug over time but also enhances its therapeutic impact against cancer cells, making it a promising strategy for developing effective cancer treatments [7].

Roy et al. (2020) developed pH-sensitive niosomes for site-specific drug delivery. The vesicles showed controlled release in acidic environments, such as tumor sites, demonstrating their potential in targeted therapy [8].

Singh et al. (2021) demonstrated an efficient method for synthesizing niosomes using ultrasound-assisted techniques, resulting in a uniform size distribution of the vesicles. This approach not only enhances the scalability of niosome production but also makes it suitable for industrial applications. The utilization of ultrasound facilitates better control over the synthesis process, leading to improved drug delivery systems. Additionally, the method shows promise in optimizing the encapsulation efficiency and stability of niosomes, which are crucial for their effectiveness in pharmaceutical applications. Overall, this innovative technique represents a significant advancement in the field of drug delivery technologies [9].

Chowdhury et al. (2019) investigated the synthesis of niosomes using neem leaf extract, highlighting its role as a natural stabilizer. This extract not only facilitated the formation of niosomes but also imparted additional therapeutic benefits, particularly its antimicrobial properties. The phytochemicals present in neem, such as terpenoids and flavonoids, contribute to both the stabilization of the niosomes and their effectiveness against various pathogens. This dual functionality suggests that using neem leaf extract in niosome synthesis could enhance drug delivery systems while providing inherent antimicrobial activity, making it a promising approach in pharmaceutical applications [11].

Verma et al. (2020) conducted a study on the impact of cholesterol concentration on the stability and release profile of niosomes. Their findings indicated that an optimal cholesterol-to-surfactant ratio significantly enhanced vesicle stability, which is crucial for effective drug delivery systems. The presence of cholesterol not only improved the rigidity of

the niosomal membrane but also influenced the drug release dynamics, leading to a more controlled release profile. This study underscores the importance of carefully balancing cholesterol levels in niosome formulations to achieve desired stability and therapeutic efficacy, making it a key consideration in the design of niosomal drug delivery systems [12].

Rao et al. (2022) focused on the co-encapsulation of curcumin and piperine in niosomes to enhance their bioavailability and anticancer efficacy. Their study demonstrated a **synergistic effect** in reducing tumor cell viability, indicating that the combination of these two compounds could significantly improve therapeutic outcomes. The niosomal formulation provided a protective environment for both curcumin and piperine, facilitating better absorption and sustained release. This innovative approach not only addressed the solubility issues associated with these compounds but also highlighted their potential in cancer treatment by effectively targeting tumor cells. The findings suggest that co-encapsulated niosomes may serve as a promising strategy for enhancing the anticancer properties of curcumin and piperine, warranting further investigation in clinical settings [13].

Dutta et al. (2021) utilized a solvent evaporation method to synthesize niosomes loaded with hydrophobic drugs. Their results demonstrated a significant enhancement in drug solubility, which is crucial for the effective delivery of hydrophobic compounds. The study also highlighted a prolonged release profile of the encapsulated drugs, indicating that the niosomes could provide sustained therapeutic effects over an extended period. This approach not only improves the bioavailability of hydrophobic drugs but also presents a viable strategy for developing more effective drug delivery systems. Overall, the findings suggest that niosomes synthesized through this method can play a pivotal role in enhancing the efficacy of hydrophobic drug formulations [14].

Sharma et al. (2020) explored the use of green surfactants derived from plant oils in niosomal formulations, emphasizing an environmentally friendly approach to drug delivery systems. Their findings indicated that these natural surfactants provided biocompatible and effective drug carriers, enhancing the overall safety and efficacy of the formulations. The incorporation of green surfactants not only aligns with sustainable practices but also improves the stability and performance of niosomes in delivering therapeutic agents. This study highlights the potential for using plant-derived materials in pharmaceutical applications, paving the way for eco-friendlier drug delivery solutions [15].

Ali et al. (2021) incorporated gold nanoparticles into niosomes to enhance their imaging and therapeutic capabilities. Their study highlighted the promising results of this hybrid system in cancer diagnostics and therapy, demonstrating improved targeting and efficacy. The integration of gold nanoparticles not only facilitated better imaging due to their unique optical properties but also enhanced the therapeutic effects of the niosomes. This approach allows for a dual-functionality, where the niosomes can deliver therapeutic agents while simultaneously providing imaging capabilities, thereby improving the overall management of cancer treatment. The findings suggest that this innovative formulation could significantly advance the field of cancer theranostics, combining diagnosis and therapy in a single platform [16].

Hussain et al. (2022) investigated the use of natural polymers, particularly chitosan, to coat niosomes. The study revealed that this polymer coating significantly improved the stability of the niosomes, enhancing their structural integrity during storage and application. Additionally, the chitosan coating conferred mucoadhesive properties to the vesicles, which is beneficial for prolonged retention in mucosal tissues. This feature is particularly advantageous for drug delivery systems targeting mucosal surfaces, as it can lead to improved bioavailability and therapeutic efficacy. Overall, the incorporation of chitosan in niosomal formulations presents a promising strategy for enhancing drug delivery applications [17].

Thomas et al. (2020) explored the encapsulation of anticancer drugs within niosomes specifically for oral delivery applications. Their research underscored the capability of niosomes to protect these drugs from degradation in the gastrointestinal tract, enhancing their stability during transit. This protective mechanism is crucial for maintaining the therapeutic efficacy of anticancer agents, which often face challenges due to harsh digestive conditions. The study highlights the potential of niosomes as effective carriers that not only improve drug stability but also facilitate better absorption and bioavailability when administered orally, making them a promising strategy in cancer treatment regimens [18].

Banerjee et al. (2019) developed a temperature-sensitive niosomal gel aimed at enhancing transdermal drug delivery. Their formulation exhibited controlled drug release, which is essential for maintaining therapeutic levels over time and improving treatment efficacy. The temperature-sensitive nature of the gel allows it to transition between gel and liquid states, optimizing drug release in response to body temperature. This characteristic not only enhances the stability of the encapsulated drugs but also improves patient compliance by providing a more user-friendly application method. Overall, the study highlights the potential

of niosomal gels in creating effective and patient-friendly drug delivery systems for transdermal applications [19].

Mukherjee et al. (2021) explored the potential of niosomes for delivering **siRNA** in gene therapy applications. Their study demonstrated successful gene silencing in cancer cells, indicating that niosomes can effectively encapsulate and protect siRNA from degradation while facilitating its delivery to target cells. This approach highlights the advantages of using niosomal formulations in enhancing the stability and bioavailability of siRNA, which is crucial for achieving therapeutic effects in gene therapy. The findings suggest that niosomes could serve as a promising non-viral vector system for siRNA delivery, potentially improving treatment outcomes in cancer therapy by enabling precise gene regulation [20].

Chatterjee et al. (2020) prepared niosomes using a concertation phase separation method, which resulted in vesicles exhibiting high drug loading efficiency and a sustained release profile. This innovative approach allowed for the effective encapsulation of therapeutic agents, enhancing their stability and availability. The sustained release characteristic is particularly beneficial for maintaining therapeutic levels over extended periods, improving treatment outcomes. The findings from this study underscore the potential of concertation phase separation in developing efficient niosomal formulations for various pharmaceutical applications, particularly in drug delivery systems [21].

Singh et al. (2022) examined the role of PEGylation in enhancing the circulation time of niosomes. Their study found that PEGylated niosomes exhibited reduced opsonization, which is the process where particles are marked for clearance by the immune system, thereby improving systemic retention. The incorporation of polyethylene glycol (PEG) on the surface of niosomes not only shielded them from aggregation and phagocytosis but also significantly prolonged their blood circulation time. This enhancement in circulation time is crucial for improving the efficacy of drug delivery systems, allowing for more effective therapeutic outcomes in various medical applications. Overall, the findings underscore the potential of PEGylation as a valuable strategy in optimizing niosomal formulations for sustained drug delivery [22].

Reddy et al. (2020) studied the application of niosomes for delivering hydrophilic proteins, highlighting their effectiveness in encapsulation and preservation of protein activity. Their findings demonstrated that niosomes can successfully encapsulate hydrophilic proteins,

protecting them from degradation and maintaining their functional integrity during storage and delivery. This capability is particularly significant for therapeutic proteins, which often face challenges related to stability and bioavailability. The study suggests that niosomes serve as a promising delivery system for hydrophilic proteins, enhancing their potential use in various biomedical applications by ensuring sustained release and improved therapeutic efficacy [23].

Ghosh et al. (2019) investigated the encapsulation of natural antioxidants in niosomes for skin care applications, emphasizing the potential of niosomal formulations in cosmeceuticals. Their study demonstrated that niosomes effectively enhance the stability and delivery of antioxidants, which are crucial for combating oxidative stress and skin aging. By encapsulating these natural compounds, the niosomes improve their bioavailability and ensure sustained release, making them more effective in skincare products. The findings suggest that niosomal technology can play a significant role in developing advanced cosmeceutical formulations, providing not only protective benefits but also enhancing the overall efficacy of antioxidant ingredients in skin care [24]

CHAPTER 3: MATERIALS AND METHODS

3.1. Synthesis of Dual drug loaded Niosomes

For the preparation of composition probe sonication synthesis technique has been used**.**

3.1.1. Apparatus used

- Beakers
- Biocompatible stirrer
- Hot plate
- Permanent magnet
- Spatula
- Probe Sonicator
- Falcon Tubes
- Aluminum foil
- Glass vials

3.1.2. Materials used

- Span 60
- Deionized water
- Cholesterol
- Tween80
- Methotrexate
- Doxorubicin.

Sigma Aldrich supplied all of the analytical grade ingredients listed above.

3.1.3. Procedure

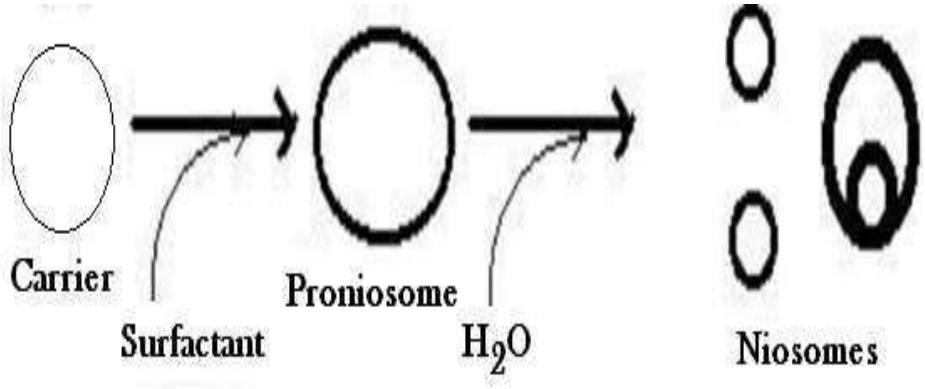

Figure 1. Flow chart diagram for the synthesis of niosomes samples preparation [25].

3.2. Synthesis of Niosomes

The probe sonication approach was utilized to prepare the niosomes. Before the addition of cholesterol, Span 60, and Pluronic F127, both drugs, either independently or in combination, doxorubicin (DOX) and methotrexate (MTX), were combined with 15 milliliters of water using a magnetic stirrer. In Table 1, the components of the various formulations under study are listed. After that, the mixes were pulsatile sonicated (50 seconds of sonication followed by a 10-second break) at a 30% amplitude for 5 minutes at 57°C probe temperature using a Vibra Cell (Sonics & Materials, Inc., Pakistan). Niosome formulations for additional characterizations, drug release investigations, and cell line research were gathered and kept at 4°C following probe sonication.

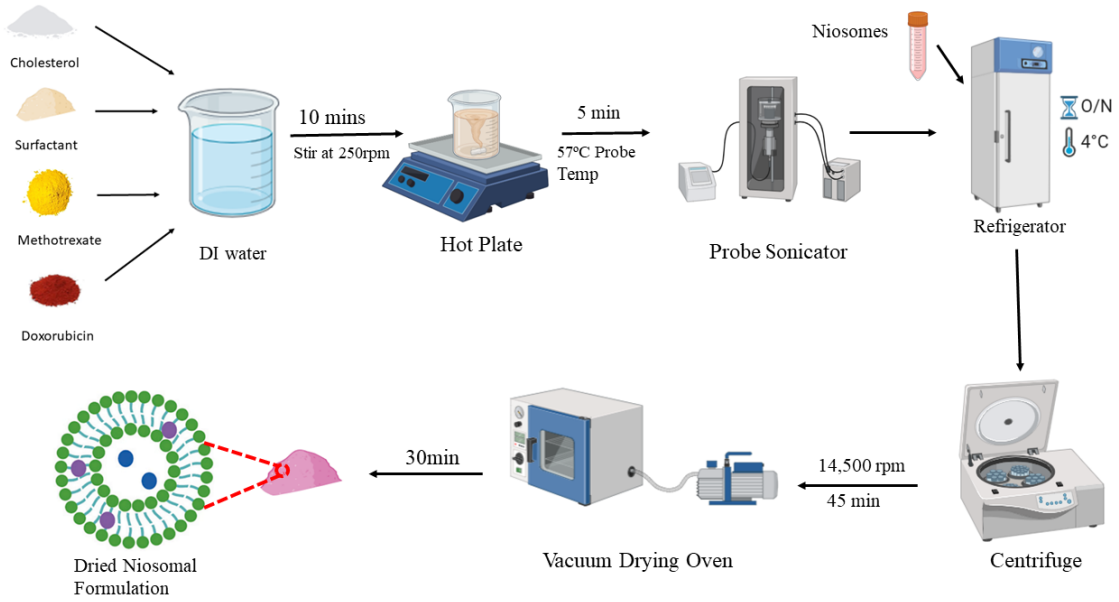

Figure 2. Schematics for the synthesis of niosomes samples preparation**.**

A detailed composition of all Niosomal functions is discussed in the table below:

Table 1. Detailed composition of all Niosomal formulations.

Formulation	Span60(mg)	Pluronic f127 (mg)	Cholesterol (mg)	DrugDOX(mg)	Drug MTX (mg)	DI Water(ml)
N1	555	1110	257.65	0	0	50
N2	455	1210	257.65	0	0	50
F1	555	1010	257.65	0	0	50
F2	455	1110	257.65	0	0	50
D1	555	1110	257.65	5	0	50
D2	555	1010	257.65	5	0	50
D3	455	1210	257.65	5	0	50
D4	455	1110	257.65	5	0	50
D5	605	1110	257.65	5	0	50
DP	455	1210	257.65	5	0	50
DT1	555	1110	257.65	5	0	50
TD1	555	1210	257.65	5	0	50
MT1	555	1110	257.65	0	5	50
TM1	555	1010	257.65	0	5	50
DMT	555	1210	257.65	5	5	50
DM1	555	1110	257.65	5	5	50
DM2	455	1210	257.65	5	5	50
DM3	555	1010	257.65	5	5	50
DM4	455	1110	257.65	5	5	50
DM5	605	1010	257.65	5	5	50

CHAPTER 4. CHARACTERIZATION

4.1. Scanning Electron Microscopy

A scanning electron microscope (SEM, Jeol JEM-1400, Jeol Ltd, Japan) examined the niosomes' morphology. A tiny quantity of niosomes dispersions was placed on a grid made of copper that was 200 mesh in size and coated with carbon for SEM investigation. One drop of 2% uranyl acetate was applied to the sample for staining after the mesh was horizontally positioned for a minute and the surplus was removed with the use of filter paper.

4.2. Fourier transform infrared (FTIR) spectroscopy

Using the Characterization technique of Fourier transform infrared (FTIR) spectroscopy, the potential interactions between the medication, non-ionic surfactants, and other membrane additives were investigated. All constituent parts, their physical mixes, and niosomes formulations containing DOX, MTX, and DOX+MTX had their FTIR spectra measured. The niosomes dispersions were centrifuged, and the dry pellet was examined for FTIR analysis. The FTIR spectrophotometer (Bruker Optics, Germany) was used to record the spectra. Origin2024b software was used to record the spectra at room temperature with a resolution of 4 cm^{-1} and wavenumbers between 400 and 4500 cm^{-1}.

4.3. Thermal analysis

Differential scanning calorimetry (DSC 823e, Mettler Toledo, Pakistan) was used to estimate the physical states of the DOX and MTX in the formulations. Individual niosomes components, their physical mixtures, and formulations containing pure DOX, MTX, and in the closed aluminum pan, DOX, MTX, and DOX+MTX were precisely weighed (3-5 mg). The niosomes dispersions were centrifuged, and the dried pellet was examined for DSC analysis. The temperature range for the thermal scanning was 25°C to 260°C, with a heating rate of 5°C per minute. The nitrogen gas flow (50 ml/min) was used for the analysis.

4.4. Particle size, size deviation, and zeta-potential

The niosomes' diameter (z-average), polydispersity index (PDI), and zeta-potential were measured using the Zeta-sizer Nano ZS (Malvern Instruments Ltd., Pakistan) and the dynamic light scattering (DLS) technique. Before the measurement, the niosomes dispersions were

diluted with water to prevent the scattering phenomenon. Three duplicates of each measurement were taken[1].

4.5. Stability studies

By sustaining the niosomes dispersions in sealed 20 mL glass vials at 4°C in a refrigerator, a four-week stability study of niosome formulations has been carried out. The stored formulations' size, PDI, and zeta potential were evaluated at predetermined intervals (fresh samples, 1, 2, 3, and 4 weeks after production)[2].

4.6. Drug entrapment efficiency

The formulations were ultra-centrifuged at 14,500 rpm for 45 minutes to determine the drug entrapment efficiency (Sigma Laborzentrifugen D-37520, Germany). After collecting the supernatant and washing the pellet at the bottom of the centrifuge tube twice with water, the centrifugation process was repeated. The amount of drug present in the supernatants' aqueous portion was measured. Spectrophotometric analysis (Varioskan Flesh, Thermo Fisher Scientific Inc., Germany) was utilized to determine MTX and DOX. The following formula was used to determine the percentage entrapment (EE %) of drugs in equation (1):

$$EE\% = ((Qt - Qr))/Qt\ X\ 100, \qquad \ldots\ldots (1)$$

Where Qt is the initial drug amount used to make niosomes and Qr is the drug amount in the supernatant following centrifugation[3].

4.7. Dissolution studies

A glass vessel with a magnetic stirrer was used for the dissolution tests. The dissolution medium was a pH 7.4 solution of HBSS with HEPES (Hanks' balanced salt solution with N-2-hydroxyethyl piperazine-N'-2- ethane sulfonic acid) buffer containing 4% Tween 80. The formulations' aqueous dispersions (2 mL) were added to the dissolution vessel with the dissolution medium for the dissolution investigation. The medium used in the study was 250 mL, the stirring speed was 100 rpm, and the temperature was 37°C. At predetermined intervals of time (0, 15, 30, 45, 60, 1.5, 2, 3, 4, and 6, 8, 12, and 24 hours), the aliquots were sampled and replaced with an equivalent volume of the new buffer. Withdrawn samples[4].

4.8. Kinetics of drug release

A critical aspect of assessing drug release is elucidating the mechanism by which the active pharmaceutical ingredient (API) is liberated from its delivery matrix. To determine the underlying release dynamics, various mathematical kinetic models were employed to analyze the data obtained from in vitro drug release studies.

4.8.1. Zero-order kinetics

The zero-order kinetics explains the mechanism of release of drugs from the NPs[5]. The graph between % drug releases versus time was created to examine its release kinetics by zero-order. The zero-order release constant is represented by the graph's slope, which can be found using the following equation (2):

$$W=k1t \ldots\ldots\ldots (2)$$

Where (W = % drug release; t = time in hours; k1 = zero-order release constant)

4.8.2. First-order kinetics

First-order kinetics can be used to describe the situation where all the sink conditions were achieved to the ideal standards[6]. A graph showing the relation of the log of % drug release with time needs to be constructed to establish the first-order kinetics. The following equation comes in handy whenever someone is solving first-order kinetics.

Where [K2 = first order release constant]

4.8.3. Higuchi Model

The diffusion model is also referred to as the Higuchi square root of time equation. Higuchi's mode identifies the diffusion-dependent release of the non-erodible drug from the matrix. Plotting the % drug release against time operating squares was done. The equation provided below is the equation for the Higuchi model.

$$ft = Q = A\sqrt{D(2C - Cs)Cst} \ldots.. (3)$$

Where [k3 = Higuchi dissolution rate constant].

4.8.4. Korsmeyer peppas (diffusion/relaxation model)

The given equation below calculates the Peppas model, which uses an exponential function to describe drug release vs elapsed time:

$$Mt/M\infty = Ktn \ldots. (4)$$

where [Mt/M∞ = fraction of drug release at time t; n = diffusion exponent for drug release; k4 = constant describes structural and geometrical features of drug release][5].

4.9. Biocompatibility

4.9.1. Hemolysis

The hemolytic activity of DOX- and MTX-loaded niosomes was determined using the hemolysis assay as described in previous works. Fresh human blood was taken in EDTA-coated tubes and left to clot for 15 minutes after which the plasma was separated by centrifugation at 10,000 rpm for ten minutes. To obtain a clean pellet of RBCs the washing procedure was performed five times using PBS at pH 7.4. Thus five various concentrations of niosomes containing DOX and MTX were loaded into the pellet of RBCs. The last part of each sample was brought to 1 mL with PBS. These samples were further cultured in a shaking incubator at an agitation speed of 80 rpm for 4 hrs. After incubation the samples were centrifuged for 10 minutes at 5000 rpm and the absorption of the resultant supernatant was determined at 540 nm in triplicates using Rayto RT-6000 spectrophotometer. Triton-X-100 treated RBCs in PBS were taken as positive control and the RBCs in PBS were taken as negative control[7]. The percentage of hemolysis was calculated using the following equation (5):

$$\text{Hemolysis (\%)} = \frac{\text{Absorbance(Sample}-\text{Negative Control)}}{\text{Absorbance(Positive Control}-\text{Negative Contorl)}} \times 100 \ldots\ldots\ldots (5)$$

4.9.2. Antioxidant Activity of Niosomes

The antioxidant capacity of the Niosomal Formulation was determined based on the reduction of 2, 2-Diphenyl-1-picrylhydrazyl using a slightly different method. 25 mL of balanced methanol was taken to prepare DPPH solution and 1 mg of DPPH was added to the methanol[8]. Concentrations of niosomes for three different concentrations were prepared and aliquots were dispensed into a 96-well plate. The absorbance of the plate was subsequently measured using a microplate reader after thirty minutes of dark incubation. Ascorbic acid and

methanol were used here as the positive and the negative controls respectively. All of the above results were obtained using Equation (6)

$$\%Inhibition = \left[\frac{Abscontrol - Abssample}{Abscontrol}\right] \times 100 \ldots\ldots\ldots (6)$$

4.9.3. Cell culturing

MCF7 and HEK293 are two cell lines used in MTT assays for cytotoxicity testing. At 37°C and 5% CO2, the cells were cultivated in T25 flasks with DMEM high glucose media[9]. To allow for adequate cell adherence, cells were plated in a 96-well plate and incubated for the entire night. Following 24 hours, cells were exposed to varying concentrations of either niosomes, methotrexate, niosomes + methotrexate, doxorubicin, niosomes + doxorubicin, different concentrations of niosomes+doxorubicin, and dual drug niosomes (niosomes loaded with both doxorubicin and methotrexate) at five, ten, twenty, forty, and eighty days. After 48 hours of additional incubation, the cells were treated with a 5 mg/ml solution of MTT dye. Purple formazan crystals were allowed to form after a 4-hour incubation period. The crystals were dissolved in concentrated DMSO[10].

MCF-7 breast cancer cells and HEK-293 human kidney normal cells were cultured. T-75 cm2 culture flasks (COMING INC. Life sciences, USA). The incubation was done in 5% CO2 in a gas incubator (Heraeus Instruments GmbH, Germany) at 37°C, and 95% relative humidity. Both MCF-7 and PC3-MM2 cells were grown in a DMEM culture medium. The medium was then supplemented with 1% NEAA, 10% v/v FBS, 1% L-glutamine, and 1% PEST. The cells were revived from the frozen stock and sub-cultured at 80 percent confluency.

4.9.4. Cytotoxicity studies

The cytotoxicity effect of the prepared niosomes on the cultured cells was investigated to assess the safety of the formulations. MCF-7 and PC3-MM2 cell viability was assessed by the ATP-based cell viability kit. Briefly, the cells were placed on 96-well plates at a density of 2 × 105 cells/mL (100 100 µL) and allowed to adhere for 24 hours. The medium was used for niosome preparations and the concentration of the niosomes varied between 25 µg/mL and 1000 µg/mL. Subsequently, the cell medium in 96-well plates was changed to the fresh medium containing niosomes (100 µL) and then incubated with cells for 24 h. The fate of the cells after the incubation was determined by using the ATP-based viability assay soon after the addition of 100 µL of reagent assay

into each well (CellTiter-Glo Luminescent Cell Viability Assay, Promega, USA). The luminescence was determined using a Varioskan Flash plate reader from Thermo Fisher Scientific Inc., USA. For positive control, the cells were incubated with the cell culture medium and Triton X-100 (1%). All the determinations were done in triplicate[9].

4.9.5. Cell uptake studies

For qualitative evaluation of the cellular uptake of niosomes, 200 µL of MCF-7 and HEK-293 cells were seeded into an 8-chamber slides (Nunc Lab-Tek II Chamber Slide System, Thermo scientific, Inc., USA) at a density of 2.5 × 104 cells per well, and incubated at 37°C overnight for proper attachment of the cells to the chamber. After the removal of the cell media, the cells were washed with HBSS-HEPES buffer (pH 7.4). Fluorescein isothiocyanate (FITC) labeled niosomes were prepared by loading the FITC during the method as mentioned above for the preparation of drug-loaded niosomes. 200 µL of FITC labeled niosomes suspension with different concentrations were added in each chamber and incubated at 37°C for 6 h time. After incubation, the cells were washed thrice with HBSSHEPES buffer (pH 7.4) to remove the free niosomes, and the cell membrane was stained with CellMask Deep Red (5 µL/mL, Invitrogen, USA) for 3 min at 37°C. Cells were again washed with HBSS-HEPES buffer (pH 7.4), washed and fixed with 4% paraformaldehyde (PFA) for 15 min, and washed with HBSS-HEPES buffer (pH 7.4). After the nuclei were stained by adding 200 µl of DAPI (4', 6-diamidino-2-phenylindole, 2.8µL/mL, Thermo Scientific, USA) for 5 min, cells were washed again and stored with 200µL of HBSS-HEPES buffer (pH 7.4). The interaction of the niosomes with the cells was studied by a Leica SP5 inverted confocal microscope (Leica Microsystems, Germany), using a 63×1.2-0.6 oil immersion objective[11].

4.9.6. Anti-proliferation assay

The *in vitro* cell growth inhibition of the drug-loaded niosomes was evaluated against the MCF-7 and PC3-MM2 cancer cells by cell proliferation experiments. The anti-proliferation effects of free DOX, MTX, and drugs loaded niosomes formulations, containing different concentrations of drugs (25-500 µg/mL) were measured using the previously described protocol for the cytotoxicity studies. All the experiments were repeated three times[12].

4.10. Statistical analysis

The statistical significance was determined using the Student's t-test (two-tailed); $P < 0.05$ was considered statistically significant in all the analyses (96.5% confidence level).

CHAPTER 5: RESULTS AND DISCUSSIONS

5.1. SEM Results

The images in figure 3 indicate that the synthesized niosomes exhibit a smooth, spherical morphology, characteristic of well-formed vesicles, suggesting an effective formulation process[13]. The integration of cholesterol, Span 60, and Pluronic F127 resulted in a uniform bilayer structure, promoting stability. The consistent round shape of the niosomes is indicative of structural integrity, which is essential for controlled and sustained drug release. Smooth surfaces enhance stability by minimizing leakage risks and preventing instability[14]. This spherical morphology is particularly advantageous for drug delivery applications, as it improves circulation time in the bloodstream and reduces uptake by cells.

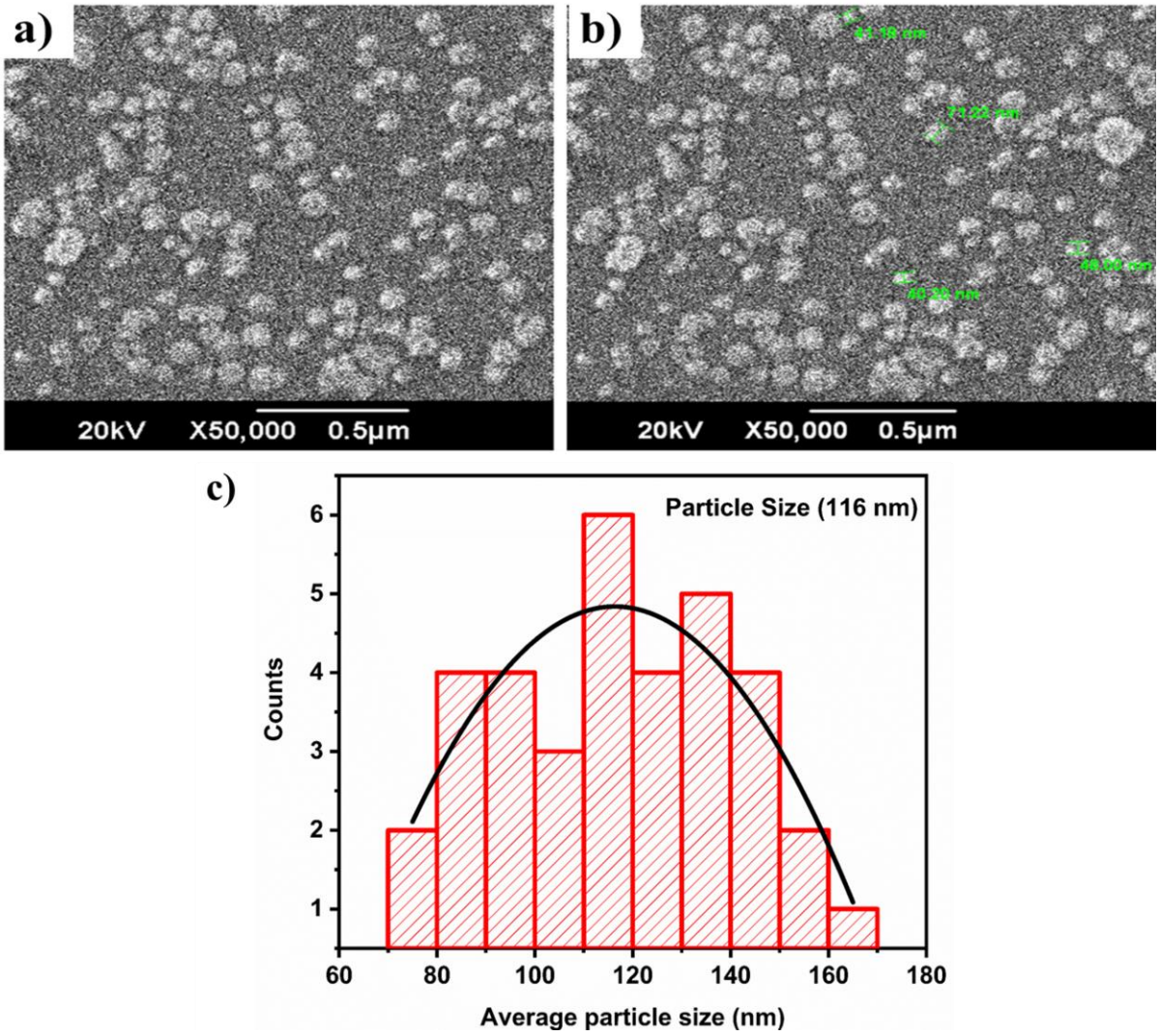

Figure 3. (a, b) SEM images of niosomes (c) Average Particle Size Distribution of Niosomes.

Fig. 3 (c) is the histogram depicting the particle size distribution of niosomes ranging from 80 to 160 nm with a peak frequency around 120 nm. A histogram with a peak near 116 nm agrees with the average particle size and a curve overlaying it describes roughly normal (Gaussian) distribution. The result is indications of moderate variability in niosome particle sizes with the greatest frequency of particles in the 110–130 nm size range. Drug delivery to cells is normally performed by nano-sized vesicles called niosomes, and uniformity in size is important for stability and control of drug release and uptake. Well prepared formulations with low polydispersity are suggested by the narrow distribution around 116 nm. For passive targeting in drug delivery, niosomes within the range of 100–200 nm are especially advantageous due to their ability to passively circulate in the bloodstream and accumulate in the tumor tissue by the enhanced permeability and retention (EPR) effect.

5.2. FTIR Results

The results of the FTIR characterization of the different niosomes formulations in Fig .4, such as MT1, DM80, F3, DM1, DP, and DT1 explain the functional group present in the formulations. Particularly prominent peaks mentioned are a wide O-H stretching band at around 3429 cm^{-1} which indicates water or polar molecules contributing to the structure's rigidity through hydrogen bonding. The characteristic niosomes bilayer hydrocarbon chain stretching at 2068 cm^{-1} from the C-H stretch indicates that Span 60 and cholesterol are present. The presence of a sharp peak at 1634 cm^{-1} for C=O stretching influences existing carbonyl groups of the drug molecules like methotrexate and doxorubicin confirming successful drug encapsulation. The stretching of the C-O-C bond is represented in the peak at 634 cm^{-1} is due to ether linkages present in Span 60 and Pluronic F127 which confirmed niosomes stability. These formulations provide comparable peak patterns indicating a similar type of peak pattern but the difference in intensity resembles the difference in the concentration of the drug and encapsulation efficiency like MT1 DM1 and DP7. On the other hand, it can be seen that samples DM80 and F3 exhibit lesser transmittance in such areas indicating better interaction that should lead to improved drug cling. DT1 has a lower transmittance which suggests weaker interactions and may have less drug encapsulation. In conclusion, FTIR data converges on drug encapsulation and demonstrates the dependency of the formulation, interaction forces, and probable drug release profiles. The FTIR spectra depicts the molecular interactions and structural characteristics of different formulations (MT1, DM80, F3, DM1, DP7, and TD1). The O-H stretching peak (~3429 cm^{-1}) appears in all formulations but varies in intensity,

indicating the presence of hydroxyl groups from methotrexate, cholesterol, or other hydrophilic components. MT1 shows distinct O-H stretching, confirming the methotrexate's presence and its interaction with the bilayer. DM80 displays a similar O-H peak but with stronger C=O stretching (~1634 cm^{-1}), suggesting encapsulation of doxorubicin.

F3 exhibits merged peaks for C-H stretching (~2068 cm^{-1}) and C=O stretching (~1634 cm^{-1}), indicative of interactions between methotrexate and doxorubicin in the bilayer, possibly forming intermolecular complexes. DM1 shows reduced intensity for C=O stretching, pointing to a lower concentration or exposure of doxorubicin. DP retains narrow O-H stretching (~3429 cm^{-1}) and defined C-H stretching (~2 068 cm^{-1}), indicating stabilization by Span 60 or cholesterol. TD1 presents strong O-H stretching (~3429 cm^{-1}) and weaker C=O stretching (~1634 cm^{-1}), implying reduced encapsulation of doxorubicin or altered drug ratios.

The C-O-C stretching peak (~634 cm^{-1}) appears consistently across formulations, confirming the ether bonds likely originating from Span 60. The variations in the spectral features suggest differences in drug encapsulation, bilayer composition, and intermolecular interactions within the niosome systems. Characterization of functional groups of various niosomal formulations (N1, DM3, D1, MTX, s60) via FTIR graph can be observed from their vibrational frequencies. All formulations show broad, intense, peak centered near 3509 cm^{-1} corresponding to O-H stretching; this indicates the presence of hydroxyl groups most likely from surfactants or hydration layers in the niosomes. The peak at 2353 cm^{-1} from C-H stretching, attributable to hydrocarbon chains, is higher with one formulation than others and a reflection of differences in the packing or composition of the bilayers. C=O stretching from carbonyl groups present in the surfactants or methotrexate (MTX) is probed by the prominent peak at 1925 cm^{-1}. Among DM3, D1, and MTX variations in this peak may imply the interaction of encapsulated drug with the niosomal bilayer. Consistency of the peaks between 1000–1250 cm^{-1}, characteristic of C-O-C stretching, indicative of ether linkages of the surfactants, across samples further indicates the integrity of the vesicles. Compositional effects are suggested by the overall differences in peak intensity and slight shifting of wavenumbers among formulations (e.g. DM3 vs N1) and by the apparent differences in DM3 and D1, consistent with the ability of MTX to be encapsulated and interact with the niosomal bilayer either confrontational or dependent on encapsulation of MTX. Either because of their ability to reproduce the structural stability and functional compatibility of formulations, which is essential for a drug delivery

application, or because of other characteristics, these results confirm that the formulations are functional.

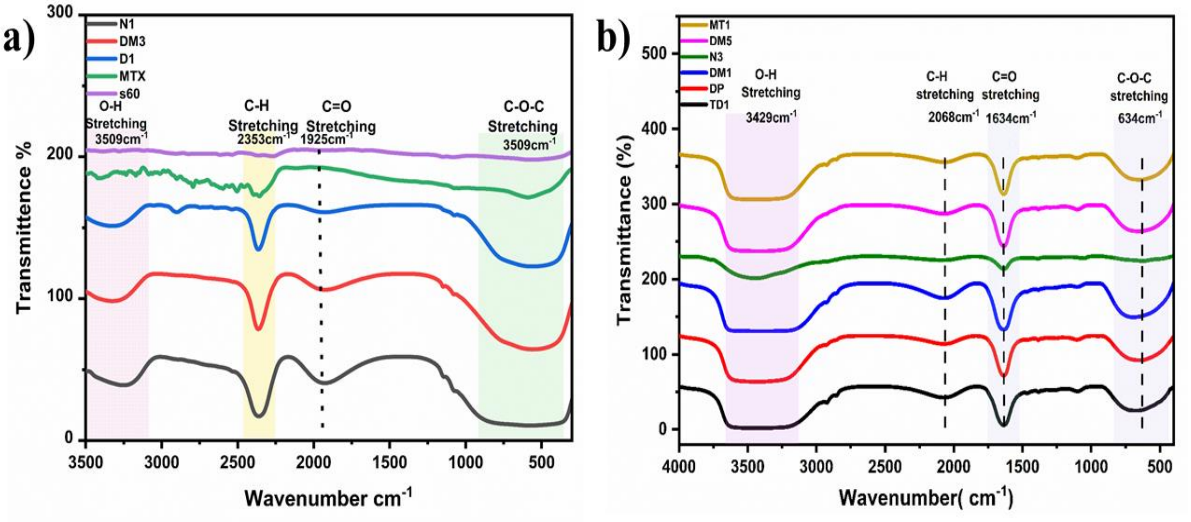

Figure 4. FTIR spectrum of (a) N1, DM3, D1, MTX and S60 (b) MT1, DM5, N2, DM1, DP and TD1.

5.3. Thermal Analysis

DSC thermograms reflecting thermal studies and phase behavior of various components of DM niosomes formulation are presented in Figure 5. By studying the phase transition temperature and, in particular, the endothermic peak at about 150°C, cholesterol will be seen to contribute to membrane stability by melting at this temperature[15]. Span 60 has its highest point at about 60-70°C, which is attributed to vesicle stability and sustained drug release resulting from its high phase transition temperature. Pluronic F127 has a broad phase transition at around 100°C, helping to crosslink the niosomes membrane and enhance biocompatibility. Methotrexate shows a sharp peak at 160-180°C, representing a crystalline state when it is not encapsulated; a shift in this peak in the right direction signifies encapsulation[16]. Doxorubicin generally presents a peak around 120°C, changes in which can be indicative of good encapsulation. DM thermogram shows smooth phase transitions which translate to successful incorporation of the components into a stable niosomes structure. In conclusion, the DSC analysis supports the design for the controlled drug delivery application.

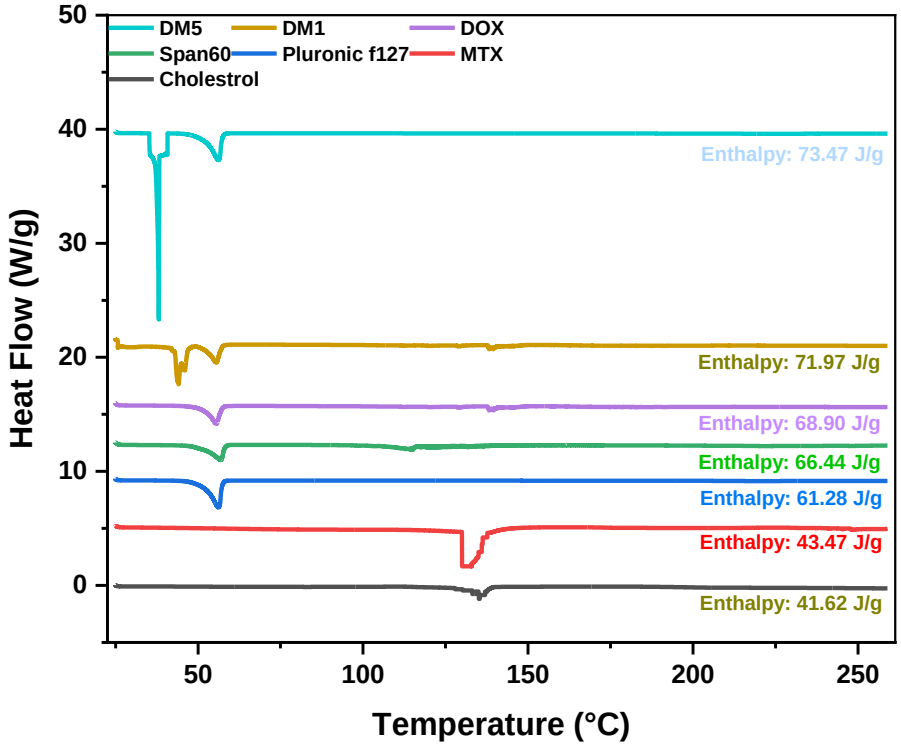

Figure 5. DSC thermograms of different Niosomal Formulations

5.4. Stability Studies

The stability of niosomes formulations was evaluated over a month, focusing on three key parameters: particle size distribution, polydispersity index (PDI), and zeta potential. The particle size analysis of the newly made formulations indicated that the first calibration had a wide variation in the size of the particles; F2 is137.1 nm while D5 is 893.6 nm. Therefore, drug carriers with smaller particle sizes like F2, N1 and N2 are better prepared for drug delivery because they will likely be more easily taken up by cells. The particle size distribution remained fairly constant for most formulations during the study period and some had experienced a slight increase. For example, there were minor increases in diameter for D1, D3, and MT1 and a higher increase for D5 that may indicate aggregation. Concerning PDI, initial values varied from 0.310 to 0.725 and a lower value was more desirable, as based on Table 4. As an illustration, formulations N1, N2, and F1 had PDI values of about 0.3 as compared to formulation D5 which had an expanded distribution. In the course of the study, most formulations experienced a relatively constant PDI, which implies moderate to high uniformity, but the trend in D5 showed that the bigger particles had possibly settled or coalesced as time elapsed[17].

Zeta potential analyses also showed great differences at the beginning in which the highest value was -49.1 mV for DM3 and the lowest value was -25.5 mV for D1. It has been known that the absolute values of zeta potential are inversely proportional to the stability of a dispersion system due to increased electrostatic repulsion between the particles. Most of the formulations had static zeta potential over the four weeks. Some slight reduction in the zeta potential was observed in DM1 and D3 which were stable around -40mV, while formulations F2 and N2 maintained around -30 to -35mV zeta potential. In general, there are indications that formulation stability is composition related. In all measured parameters, N1, N2, and F2 proved to have higher stability. The above-said stable formulations are to an extent superior for their use in drug delivery applications by their consistently narrow particle size distribution and inherent electrostatic stability. This, however, means that larger particles, especially those with relatively low zeta potentials may experience stability issues that may hinder the effectiveness of the particles in the achievement of sustained drug delivery.

Table 2. Stability study of Niosomal formulation for 30 days.

Time	Parameters	D1	D2	D3	D4	D5	TD1	DT1	MT1	TM1	DP
Fresh Samples	Size (nm)	195.6±12.08	236.3±361.0	443.15±86.7	310.5±36.6	893.6±185.5	204.4±8.67	182.9±20.07	241.1±371.0	202.1±18.10	190.01±7.02
	PDI Zeta-potential (mV)	0.472±0.047 -27.5 ±0.19	0.391±0.1105 -27.5 ±0.93	0.4369±0.037 -38.8±0.35	0.4148±0.034 -39.9±5.12	0.715±0.117 - 34.9± 0.51	0.471±0.069 -36.8±1.36	0.423±0.019 - 34.13±2.6	0.501±0.009 - 46.9. ±1.17	0.520±0.033 -34.9± 3.45	0.491±0.058 - 39.6±1.13
week1	Size (nm)	223.4±11.44	175.8±5.87	294.4±8.10	199.3±5.54	234.8±7.6	210.0±7.0	135.2±10.1	240.1±36.2	200.2±10.2	192.1±9.1
	PDI Zeta-potential (mV)	0.341±0.146 - 23.8±0.9	0.188±0.040 - 26.9± 0.9	0.362±0.028 - 28.2± 1.0	0.262±0.014 - 28.5 ±0.6	0.545±0.020 - 30.4±0.5	0.361±0.011 - 32.3± 0.1	0.334±0.071 - 34.0±1.5	0.400±0.017 - 34.1±2.2	0.480±0.033 - 46.1. ±2.2	0.441±0.001 - 36.8±2.1
week2	Size (nm)	191.9±3.3 178.7	178.7±3.6	183.5±9.1	193.1±5.2	207.6±12.2	212.7±6.1	241.3±20.2	245.2±5.5	324.14±7.9	347.4±9.0
	PDI Zeta-potential (mV)	0.188±0.039 - 23.8±0.9	0.179±0.028 - 28.1± 2.2	0.385±0.030 - 29.5±2.5	0.209±0.022 - 28.2 ±3.0	0.443±0.015 - 26.3±0.5	0.333±0.019 - 32.5± 0.1	0.350±0.011 -33.9±1.6	0.375±0.011 - 33.5±3.3	0.450±0.011 - 45.0 ±3.1	0.410±0.034 - 36.2±3.1
week3	Size (nm)	219.4±4.9	172.2±1.77	296.3±12.69	208.7±4.634	291.6±8.311	223.1±2.6	192.9±11.21	243.6±26.4	215.2±12.27	201.3±5.19
	PDI Zeta-potential (mV)	0.213±0.011 - 27.2 ±0.4	0.151±0.033 - 28.9± 0.5	0.320±0.051 - 28.6±1.5	0.324±0.020 - 31.6 ±2.2	0.532±0.051 - 28.9±0.2	0.331±0.054 - 32.7± 0.1	0.315±0.039 - 33.5±3.2	0.420±0.027 - 34.0±3.3	0.450±0.011 - 45.1. ±2.1	0.421±0.091 - 36.2±7.1
week4	Size (nm)	186.8±1.6	182.0±2.7	190.0±2.5	201.2±4.0	210.3±5.1	221.2±12.2	255.8±34.8	260.6±26.4	269.6±46.4	311.8±8.1
	PDI Zeta-potential (mV)	0.138±0.025 - 25.5 ±1.3	0.226±0.016 - 27.3± 1.5	0.364±0.042 - 26.7±0.2	0.208±0.037 - 27.8 ±1.0	0.534±0.078 - 29.8±1.1	0.340±0.019 - 32.5± 0.1	0.310±0.011 - 33.2±1.7	0.420±0.027 - 34.0±3.3	0.450±0.011 - 45.1±2.1	0.421±0.09 - 36.2±7.1
		DMT	DM1	DM2	DM3	DM4	DM5	N1	N2	F1	F2
Fresh Samples	Size (nm)	195.3±41.5	178.1±3.09	169.3±29.2	180.6±7.28	173.5±42.6	166.7±11.4	146.6±10.2	149.6±14.7	169.2±12.0	137.1±4.11
	PDI Zeta-potential (mV)	0.462±0.055 - 35.9± 2.0	0.417±0.027 - 43.8± 0.6	0.435±0.098 - 49.1± 1.3	0.447±0.076 - 38.9±3.2	0.383±0.025 - 47.0± 1.4	0.493±0.010 - 27.7±2.0	0.450±0.009 - 26.5± 0.8	0.448±0.095 - 32.1± 1.0	0.481±0.072 - 39.0 ±2.4	0.437±0.046 - 41.8±2.71
week1	Size (nm)	188.2±11.1	178.2±5.6	168.3±12.1	184.5±5.2	170.5±22.77	171.7±10.41	157.2±10.43	151.3±11.6	169.5±12.8	139.1±4.18
	PDI Zeta-potential (mV)	0.310±0.039 - 32.2± 3.0	0.335±0.011 - 39.1± 0.6	0.397±1.018 - 45.1± 7.3	0.410±0.026 - 35.3±1.2	0.325±0.021 - 45.0± 1.3	0.390±0.012 - 27.5±1.4	0.389±0.313 - 25.5± 0.4	0.391±0.321 - 31.2± 0.6	0.419±0.011 - 35.1 ±2.1	0.437±0.217 - 39.2±5.7
week2	Size (nm)	195.0±10.42	172.3±5.22	170.3±22.21	189.5±10.1	188.9±12.2	180.7±10.2	155.0±10.74	159.4±15.2	178.9±21.1	144.6±7.23
	PDI Zeta-potential (mV)	0.331±0.039 - 32.0± 3.7	0.310±0.011 - 39.28± 0.2	0.410±1.018 - 45.1± 4.3	0.419±0.026 - 35.6±2.1	0.321±0.021 - 43.6± 1.5	0.389±0.011 - 27.2±1.1	0.379±0.013 - 26.1± 2.0	0.389±0.011 - 31.2± 0.1	0.410±0.025 - 35.9 ±2.2	0.429±0.210 - 39.5±3.5
week3	Size (nm)	191.1±10.2	175.9±5.1	198.3±22.2	195.5±8.2	195.5±8.2	189.9±10.2	172.3±15.1	154.3±10.2	170.2±10.4	177.2±7.1
	PDI Zeta-potential (mV)	0.310±0.039 - 32.3± 4.2	0.335±0.011 - 39.3± 0.6	0.397±1.018 - 45.9± 4.2	0.410±0.026 - 35.2±2.2	0.325±0.021 - 43.5±1.4	0.390±0.012 - 27.4±3.1	0.389±0.313 - 26.9± 2.1	0.391±0.327 - 31.2± 0.2	0.419±0.011 - 35.2 ±2.3	0.437±0.217 - 39.7±3.6
week4	Size (nm)	214.0±10.91	176.14±5.36	277.3±22.19	193.3±22.1	197.9±10.62	181.7±15.1	256.4±9.67	183.4±15.11	212.4±21.17	291.6±7.33
	PDI Zeta-potential (mV)	0.350±0.039 - 32.9± 4.0	0.310±0.011 - 39.3± 0.5	0.381±1.018 - 45.6± 4.3	0.410±0.026 - 35.2±2.3	0.327±0.021 - 44.9± 1.4	0.370±0.012 - 27.9±3.1	0.376±0.213 - 26.5± 3.4	0.389±0.011 - 31.4± 1.2	0.431±0.025 - 35.5 ±2.3	0.423±0.210 - 39.3±4.2

5.5. Drug Entrapment Efficiency

Entrapment efficiency is an important factor controlling the efficiency of drug delivery since it determines the drug's potency in treating a particular condition. In the present investigation values of entrapment efficiencies (%EE) for niosomes formulations containing doxorubicin (DOX) ranged between 92.32 % and 94.99%. MTX solutions containing only formed higher %EE values starting from 90.96 – 91.564%. When both DOX and MTX were incorporated into the formulations, the %EE values for DOX were in the range of 91.24 ± 0.32 – 93.67 ± 0.19 with MTX having 99.99 ± 0.02 entrapment efficiency. These outcomes confirm very high entrapment efficiency in all formulations, MTX being close to 100%, and uniformity of the entrapment for the different niosomes compositions. The entrapment efficiency of MTX appears to be greatly enhanced due to poor aqueous solubility, and high lipophilicity that enables its preferential incorporation within the niosomes bilayer structure over the aqueous phase. However, DOX used in this study due to its higher hydrophilicity gave a slightly lower E% where some of the drugs remained dissolved in the aqueous layer. However, both drugs attained efficient entrapment levels in all the evaluated niosomes formulations thus confirming that changes in niosomes compositions do not significantly affect entrapment efficacy. Characterization of niosomes size using a particle size analyzer revealed that the size of the drug-loaded niosomes was found to be 137 nm to 241 nm, depending upon the composition of the system. In fact, the entrapment efficiency of the particles can also depend on the particle size – increased size exhibits higher drug encapsulation. Nevertheless, in the present work, the variations in the size of the formulations did not show a drastic effect on the entrapment efficiency of the formulation which was quite high in both cases.

5.6. Dissolution Studies

The standard doxorubicin (STD DOX) demonstrates a rapid release, achieving near-complete release quickly, while niosomes formulations (D1-D4) exhibit a sustained release profile. This slower release is attributed to the presence of cholesterol and Span 60, which enhance membrane rigidity and reduce permeability, leading to controlled drug release that can minimize side effects and dosing frequency. The dual drug-loaded niosomes formulations (DM1-D5) show a slower release of doxorubicin compared to STD DOX, indicating prolonged release[18]. The interaction between methotrexate and doxorubicin within the niosomes may stabilize each other and alter the internal vesicular structure, enhancing therapeutic efficacy while reducing the risk of drug resistance. The dual drug-loaded formulations (MT1, TM1,

etc.) exhibit a slower release of methotrexate compared to standard methotrexate (STD MTX). The sustained release is facilitated by cholesterol and Span 60, which stabilize the niosomes and moderate drug release, potentially leading to consistent therapeutic levels. Methotrexate-loaded niosomes formulations (DM1-D5) show significantly slower release profiles than STD MET. The sustained release is attributed to the formulation's components, which enhance the stability of the niosomes and prolong drug action, beneficial for therapies requiring long-term exposure. Overall, niosomes formulations demonstrate enhanced stability and sustained drug release compared to standard formulations. Dual drug loading provides synergistic effects with controlled release profiles that minimize toxicity risks. These characteristics suggest that niosomes formulations could improve patient compliance and maintain steady drug concentrations, making them advantageous for anticancer treatments.

The STD DOX reveals a very fast release wherein over 90% of the drug is released within the first 3h while niosomes formulations (D1 – D4) show a controlled release profile. This slow drug release is due to the cholesterol and Span 60 which act as stabilizers of the membrane and depressors of permeability to allow for sustained drug release that can reduce side effects and dose intervals. The results presented for the tested dual drug-loaded niosomes formulations, DM1-D5, demonstrate that the release of doxorubicin is slower than in the case of STD DOX, which points to a prolonged release. The ability of methotrexate and doxorubicin to form a complex with each other within niosomes may lead to the stabilization of each compound and change the internal vesicular structure for improved therapeutic effect and least resistance. The created drug releases the active substance methotrexate at a slower rate compared with the conventional methotrexate (STD MTX; MT1/TM1/etc.). The sustained release is achieved by cholesterol and Span 60; which helps in the stabilization of the niosomes and moderate release of the drug, which can lead to a better therapeutic level. The release profiles of Methotrexate-loaded niosomes formulations DM 1 to 5 are much lower than that of STD MTX. The sustained release is due to the formulation of the niosomes and the substances present in the formulation which stabilize the niosomes and the drug action so it is effective in case chronic exposure is needed. In general, niosome formulations show an improved stability profile and drug release characteristics as compared to conventional formulations. Dual drug loading leads to enhancement of therapeutic activities and reduced toxicity when compared to using a single drug with the same dosing frequency. Such characteristics, therefore, have the potential of enhancing patient convenience and stability of drug levels in the bloodstream a factor that might make niosomes formulations suitable for anticancer therapies.

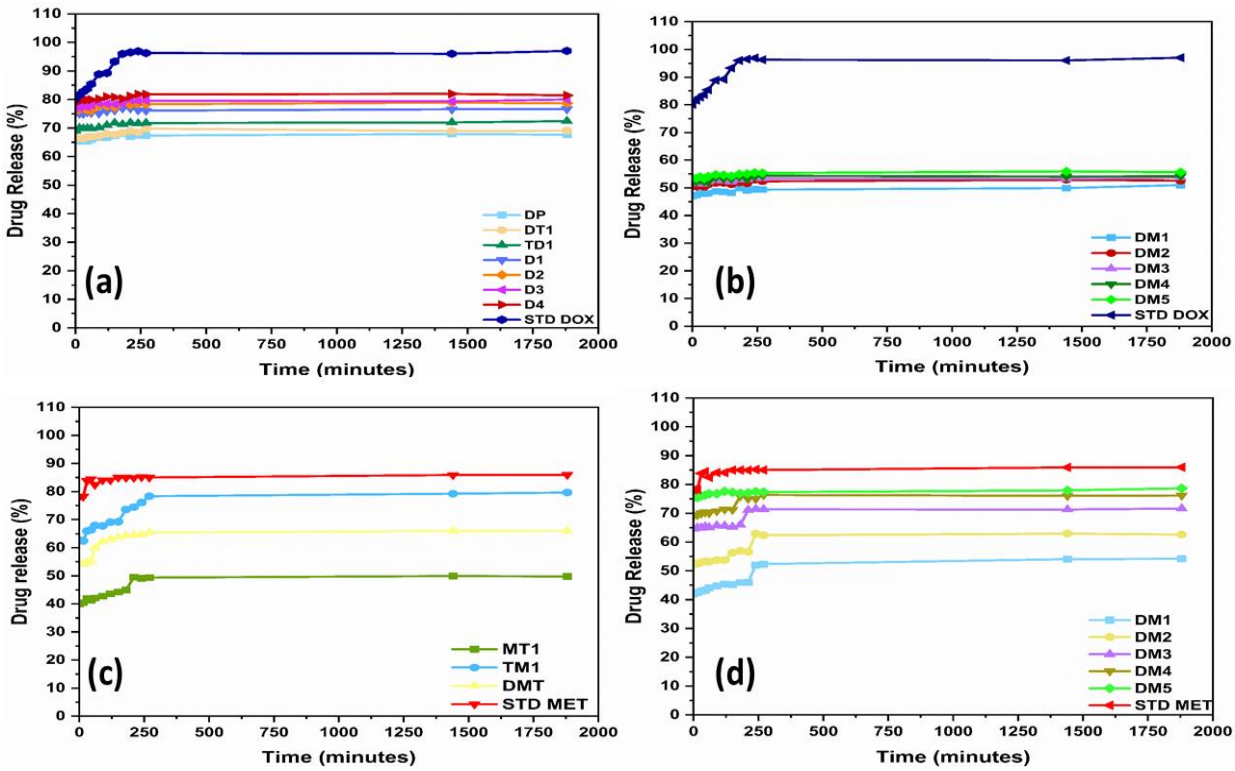

Figure 6. Drug release profiles of (a) STD DOX with DP, DT1, TD1, D1, D2, D3, and D4, (b) of STD DOX with dual drug containing formulations DM1, DM2, DM3, DM4, and DM5, (c) of STD MTX with MT1. TM1, and DMT, (d) of STD MTX with dual drug containing formulations DM1, DM2, DM3, DM4, and DM5

5.7. Drug Kinetics Studies

Table 3: Table showing R^2 and K values of different kinetic models of all Niosomal formulations**.**

Formulation	**Zero Order**		**First Order**		**Korsmeyer-Peppas**		**Higuchi**		**Hixson Crowell**	
	R^2	K1	R^2	K2	R^2	K3	R^2	K4	R^2	K5
MT1	0.9562	0.0262	0.9594	-0.00005	0.7466	0.0212	0.9312	2.4687	0.9217	-0.0576

TM1	0.879	0.0515	0.8803	-0.0016	0.6963	0.0264	0.8597	1.1564	0.9217	-0.057
DMT	0.8797	0.0654	0.8909	-0.0016	0.589	0.0353	0.8224	0.8923	0.9217	-0.0576
STD MTX	0.5411	0.0321	0.5616	-0.0018	0.7374	0.0179	0.7031	1.3179	0.9217	-0.0576
DP	0.8047	0.2864	0.8764	-0.0004	0.4354	0.0054	0.07074	4.1223	0.9217	-0.0576
DT1	0.8325	0.0099	0.8349	-0.0003	0.3251	0.0036	0.6249	4.9783	0.9217	-0.0576
TD1	0.8633	0.0126	0.8621	-0.0004	0.6619	0.0061	0.8286	4.5986	0.9217	-0.0576
D1	0.7175	0.0085	0.718	-0.0004	0.2465	0.0026	0.4972	4.7968	0.9217	-0.0576
D2	0.7184	0.0129	0.7171	-0.0006	0.4567	0.0052	0.6323	3.5739	0.9217	-0.0576
D3	0.7294	0.0085	0.729	-0.0004	0.7167	0.0042	0.8135	6.2228	0.9217	-0.0576
D4	0.6986	0.008	0.6965	-0.0004	0.7566	0.004	0.8122	6.4606	0.9217	-0.0576
STD DOX	0.9882	0.0863	0.9329	-0.0082	0.6764	0.0316	0.9019	0.7505	0.9217	-0.0576

DM1	0.7694	0.0115	0.7674	-0.0002	0.6315	0.0084	0.7612	4.556	0.9217	-0.0576
DM2	0.6866	0.0081	0.6873	-0.0002	0.4965	0.0053	0.6619	5.677	0.9217	-0.0576
DM3	0.6084	0.0066	0.610	-0.0001	0.4296	0.0042	0.5723	6.0965	0.9217	-0.0576
DM4	0.6591	0.0093	0.6599	-0.0002	0.5064	0.006	0.6371	4.8004	0.9217	-0.0576
DM5	0.7148	0.1851	0.6965	-0.00002	0.5116	0.0049	0.6738	5.8678	0.8882	-0.0596

The acquired values of methotrexate-releasing profiles for niosomes formulations revolve around the principles governing drug release through various models. Hixson-Crowell Model the Hixson-Crowell model depicts high R^2 indicative of good fit for certain formulations The plots of R^2 using the Hixson-Crowell model indicate a high degree of fit for specific formulations. This model takes into consideration changes in the surface area of drug particles to the dissolution of the particles and release from the matrix since surface erosion influences the release rates of the niosomes compared to the shrinking of the drug particles. The in vitro drug release studies, analyzed through various kinetic models, indicated that the Hixson-Crowell and Zero-Order models best described the release kinetics. This suggests that drug release is predominantly controlled by surface erosion mechanisms and that a steady release can be achieved over time, particularly beneficial for sustained drug delivery. Zero-Order Kinetics The formulations that fit the Zero-Order model also show high R^2 values suggesting a sustained drug release rate over time. This is particularly advantageous in sustained release systems since it indicates that the niosomes structure hinders a flux release of the drug immediately after dosing, but rather releases it at consistent proportions so that it averages out resulting in therapeutic levels after several periods. The zero-order release observed in certain formulations supports the design of these niosomes as potential controlled-release systems, reducing the frequency of dosing and maintaining therapeutic levels over extended periods.

First-Order Kinetics Indeed, the First-Order model has slightly lower R^2 values that suggest a relatively lower fit of these formulations. This model is often used to characterize systems where the release rate depends on the remaining drug's concentration, typically in immediate-release systems. The lower fit implies that niosomes encapsulation deals more with sustained release than simple diffusion-based kinetics imply. Accordingly, the findings confirm the brief niosomes depots' drug release involves primary surface erosion and controlled mechanisms from tablets, with significant fits by the Hixson–Crowell and Zero–Order model. The fact that only limited formulations of First-Order kinetics further support the sustained release characteristic of these formulations. Extending from these observations, this study shows that none of the niosomes systems could not elicit significant toxicological effects, and the data further supports the ability of the niosomes systems to deliver the drug over an extended duration with minimized dosing frequency.

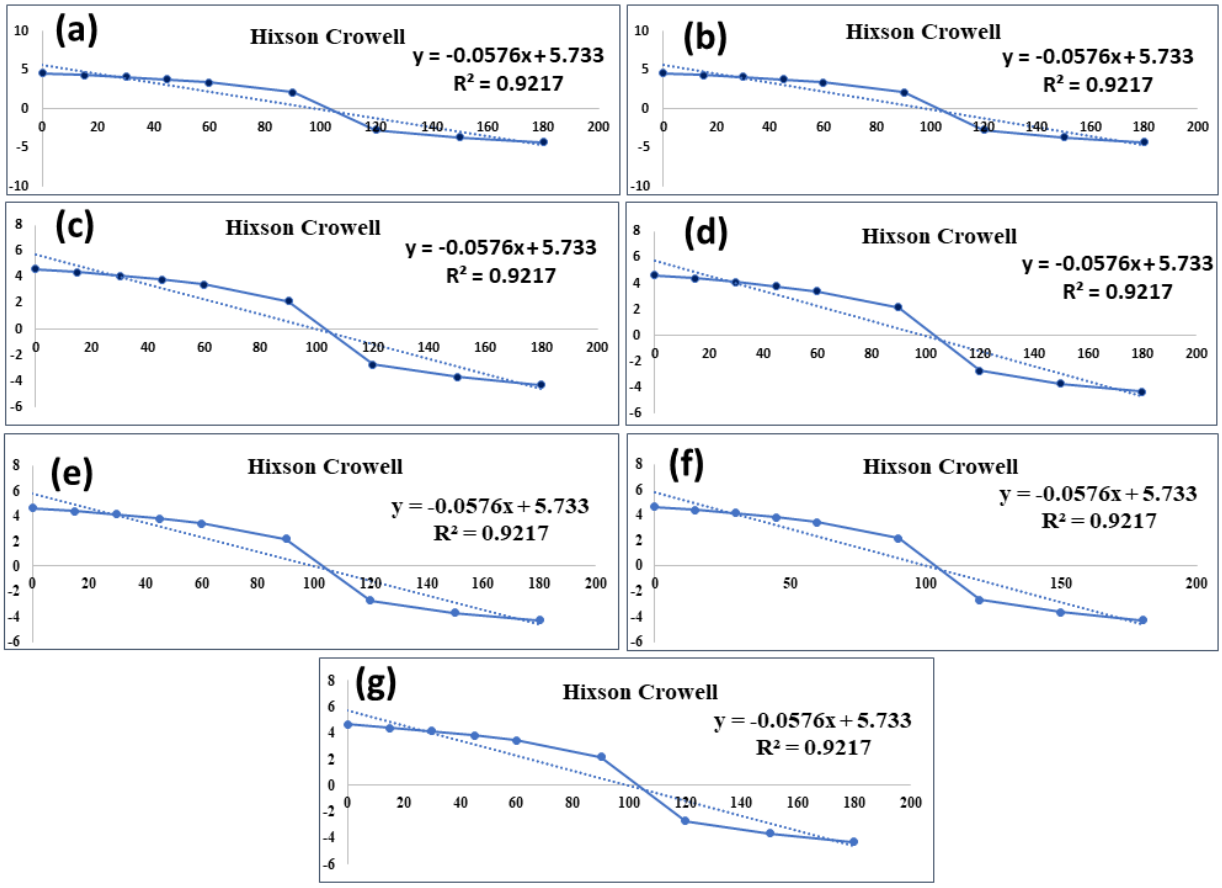

Figure 7. Drug Kinetic Models of (a) DP (b)DT1 (c)TD1 (d)D1 (e)D2 (f)D3 and (g)D4

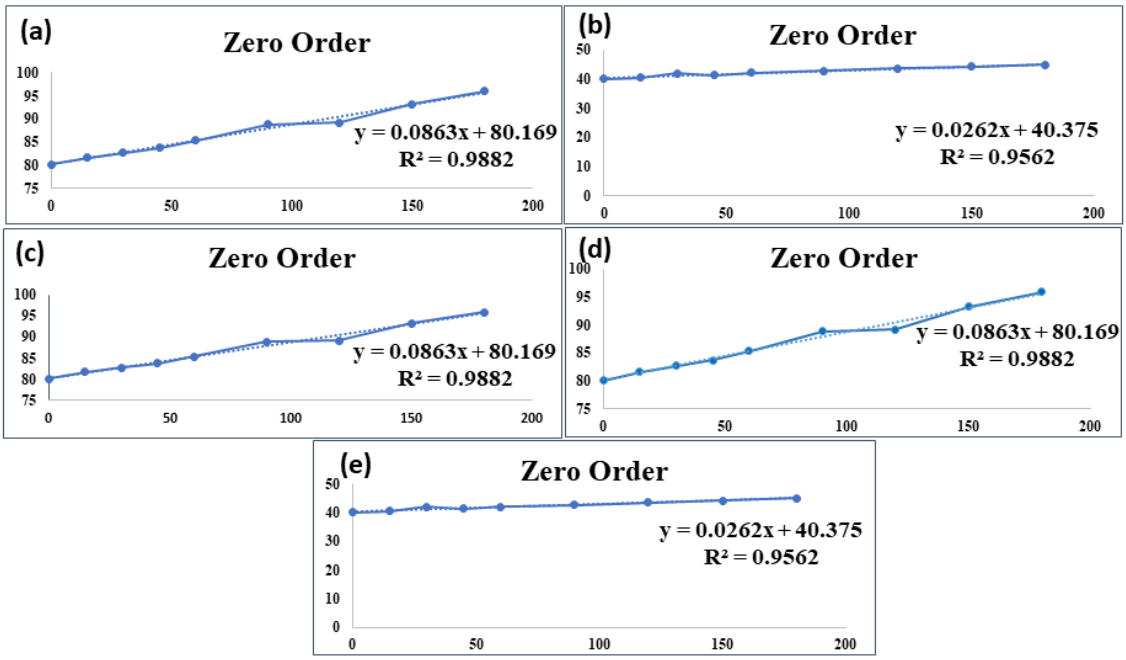

Figure 8. Drug Kinetic Models of (a) DM1 (b) DM2 (c) DM3 (d) DM4 and (e) DM5

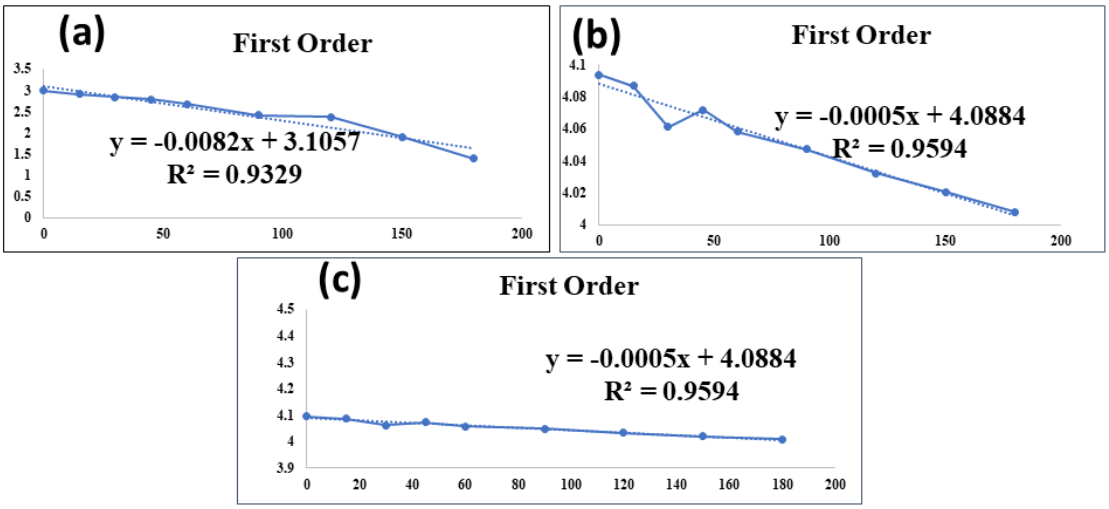

Figure 9. Drug Kinetic Models of (a) TM1 (b) MT1 and (c) DMT

5.8. DPPH Assay

The results of the DPPH assay reveal the antioxidant activity of the different niosomes formulations along with the connection between the composition and the drug release profile of each formulation. The DM series (DM1-DM5) comprising doxorubicin and methotrexate formulations showed higher antioxidant activity since the combination of both the drugs and

optimum release from the niosomes carriers, cholesterol, and Span 60[19]. On the other hand, the DT series (DT1) exhibited a moderate antioxidant effect probably due to the slow dissolution rate of the drug and having no active substance and only one active drug which restricted the antioxidant property. In the D series (D1-D4) different antioxidant activity was observed due to differences in the ratio of drugs to be released and encapsulation efficiency influencing the release rate[20]. Formulations TM1 and DMT showed lower antioxidant activity which could be attributed to single drug encapsulation or poor release profiles which impeded the release of the antioxidant formulation immediately. On the other hand, this DP formulation had about fourfold higher antioxidant activity than the other formulations, implying improved encapsulation and controlled release profiles that maintain the characteristics of the drugs. In summary, the release profile of the dual-drug formulations in most cases showed better antioxidant activity compared to corresponding single-drug formulations where composition plays a crucial role in achieving optimal therapy. Based on the findings from the antioxidant assay using the DPPH method, stability study, drug release profile and SEM data of the prepared niosomes preparations, there is a comprehensive evaluation of the performance of the formulations. Esterase activity varied between 0% in the undeclared dimers (DM5) to 86% in ascorbic acid and between 82% and 97% in various di methyl and di propyl ethers. Antioxidant activity of DM formulations such as DM1, DM2 & DP correlates with their stability (particle size, PDI, and zeta potential) and controlled release of doxorubicin and methotrexate. The structural adhesion reflected by SEM images of spherical and highly packed vesicles also substantiates encapsulated drug stability and antioxidant properties. The moderate antioxidant activity (70–90%) in DM4, MT1, and TM1 corresponds to slight oscillation in the particle size and PDI during stability and may perhaps be due to partial destabilization of vesicle structure that also could also affect the variation of dissolution rates. The lowest antioxidant activity in DM5 (~50%) corresponds to the highest PDI of DM5 (0.725) and the largest particle size suggesting aggregation or suboptimal concentration of surfactant and cholesterol. These formulations can be expected to possess less well-defined and more heterogeneous vesicle morphology as seen under SEM images hence resulting in low encapsulation efficiency as well as poor drug release characteristics. Overall, data presented herein show evidence of synergy for all evaluated parameters in formulations with optimal composition ratios (e.g., DM 1 and DM 2). Together, these findings suggest that the formulations offer robust and controlled delivery of anticancer therapeutics, all while maintaining their antioxidant, structural, sustained drug release profile, and vesicle morphology. The antioxidant activity measured via the DPPH assay showed high antioxidant

potential in most formulations, comparable to ascorbic acid. This indicates that the niosomes formulations preserved the antioxidant capacity of the encapsulated drugs, which could add an extra benefit in combating oxidative stress in cancer treatments[21].

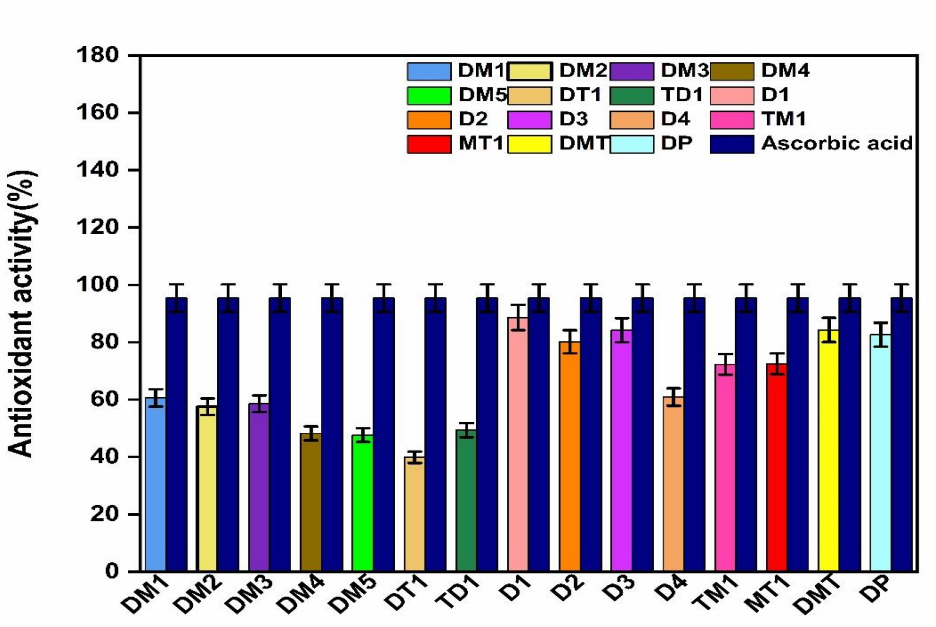

Figure 10. Antioxidant activity of different niosomes formulations.

5.9. Hemolysis

The hemolysis assay results evaluate the potential for red blood cell (RBC) membrane disruption across various niosomes formulations, providing insights into their biocompatibility. The DM series (DM1-DM5), which includes dual-drug formulations of doxorubicin and methotrexate, shows varying hemolysis rates, with DM2 and DM3 exhibiting higher rates (~3% and ~2.5%, respectively), potentially due to higher drug concentrations or faster release rates that disrupt RBC membranes. In contrast, the D series (D1-D4) demonstrates lower hemolysis (~0.5% or less), indicating a controlled release profile that minimizes cytotoxicity[22]. The DT series (DT1) presents a notably high hemolysis rate (~4.5%), suggesting a formulation that may require reformulation to enhance stability and reduce RBC disruption. Conversely, TM1 and DMT show low hemolysis rates (~1% or lower), indicating good biocompatibility likely due to stable niosomes structures. The DP sample exhibits moderate hemolysis (~1.5%), suggesting acceptable levels for in vivo applications but indicating room for optimization. Control samples validate the assay, with the positive control showing high hemolysis (~5%) and the negative control showing minimal disruption.

The average hemolysis rate in percentage for different niosome formulations and controls is shown in the bar diagram below to compare the biocompatibility. From among the formulations, DM3 showed the highest percentage of hemolysis (~3.5%) which may indicate the tendency of the complexes to destabilize red blood cell membranes possibly due to the type of surfactant or lipid used. However, D1 had the lowest rate of hemolysis at about 0.1%, which depicts high biocompatibility. The positive control (PC) was the highest regarding hemolysis, at approximately 5% and the negative control (NC) was at 0% thus affirming the experimental configuration. The variances in the size and characteristics of these metabolites are likely due to the differences in the surfactant-to-lipid ratio, lipid saturation, or charged features of niosomes and biological membranes. Compared to previous outcomes, the SEM analysis indicated that DM3 has the roughest surface, which might lead to higher hemolysis owing to enhanced cell membrane interaction. Both stability studies revealed that DM3 had lower stability when compared to other formulations, and the authors proposed that it may cause the leaching of encapsulated components that reacted with red blood cells. Data derived from the DPPH assay suggested that DM3 had moderate antioxidant capabilities which are evidenced by results of moderate levels of bioactivity and at the same time harmful effects of membrane disruption. This is consistent with faster dissolution rates observed with DM3 as a higher diffusion rate could be a problem for membrane compatibility. On the other hand, the morphology of D1 as analyzed through SEM is more oval. The formulation has better stability and a slower rate of drug release, leading to its less hemolytic effect than formulation F1. The hemolysis assay results demonstrated that the majority of the niosomes formulations exhibited minimal hemolytic activity, indicating a high level of biocompatibility and reduced risk of erythrocyte damage. This supports the safety profile of the formulations, making them suitable for potential therapeutic applications.

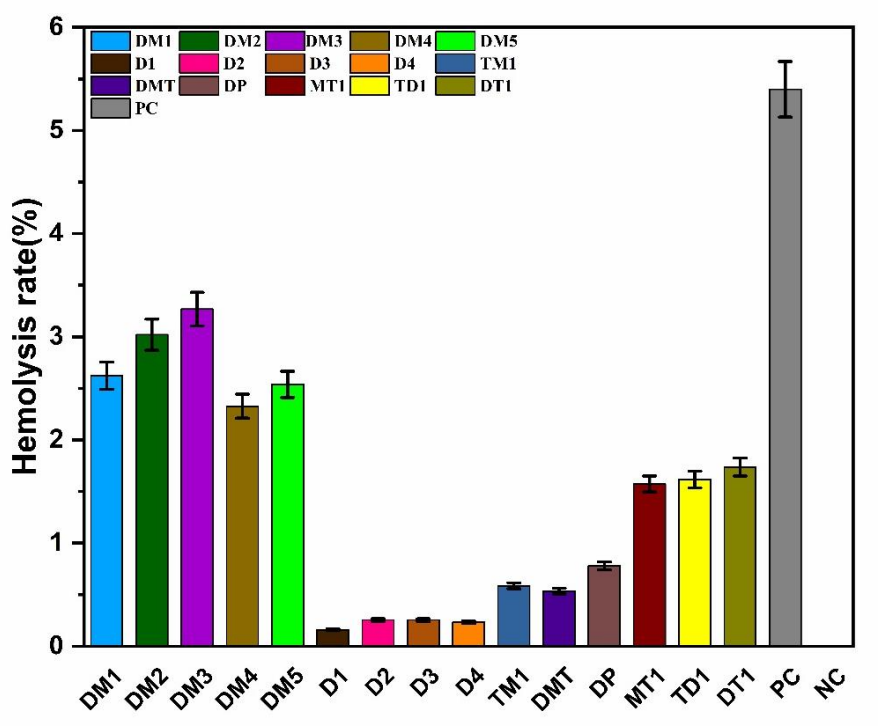

Figure 11. Hemolytic rate of different niosomes concentrations

5.10. Cell Viability Assay

Results disclose that methotrexate incorporated in niosomal formulation has a higher cell-killing potency for MCF-7 breast carcinoma cells than that of HEK-293 normal kidney cells. The formulations express a doze-dependent cytotoxicity profile and the tests with the niosomal drugs are more toxic than free drugs due to methotrexate/doxorubicin synergy. It further enhances the properties of cholesterol and Span 60 makes its drug to be more stable and to have controlled release leading to pro-longed therapeutic affect[23, 24]. A smaller size of niosomal carriers allows easy engulfment by target cells and particularly cancer cells improving the efficacy of drug delivery. On the other hand, HEK-293 cells have greater viability and low cytotoxicity, so the current data suggest selectiveness for cancer cells. This selectivity may explain a possibility of reducing side effects in normal tissues and at the same time having a targeted effect on the tumors. In summary, niosomal formulations are a potential avenue for enhancing cancer treatment effectiveness[25].

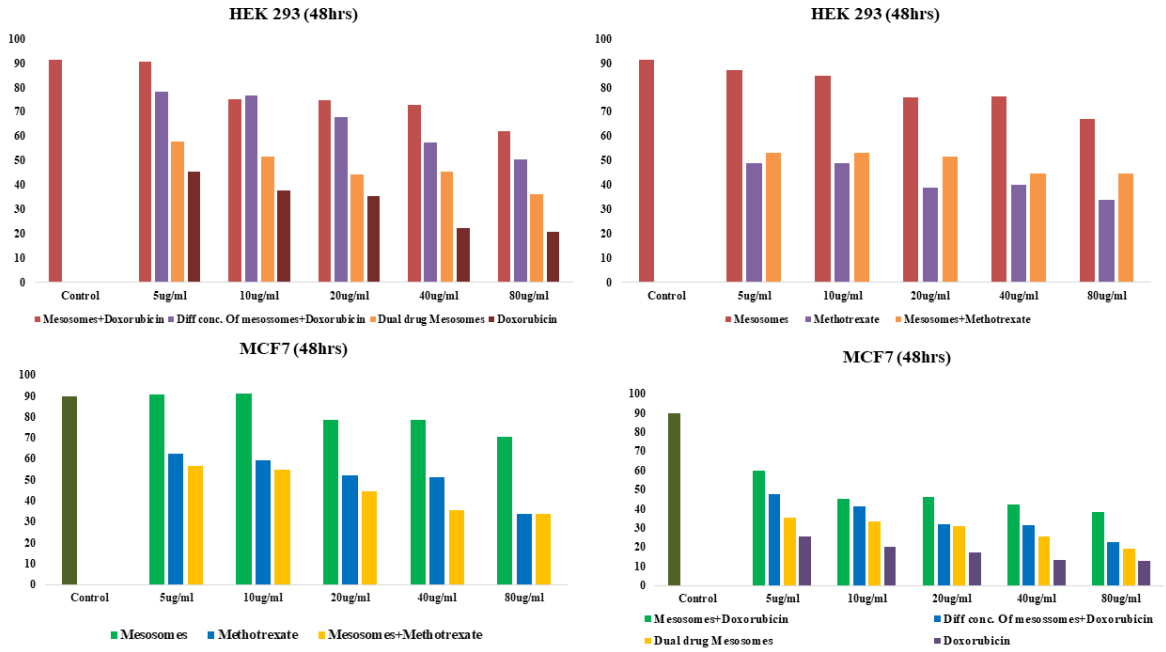

Figure 12. Cell viability percentage of different Niosomal formulations for HEK 293 and MCF 7 for 48 hours.

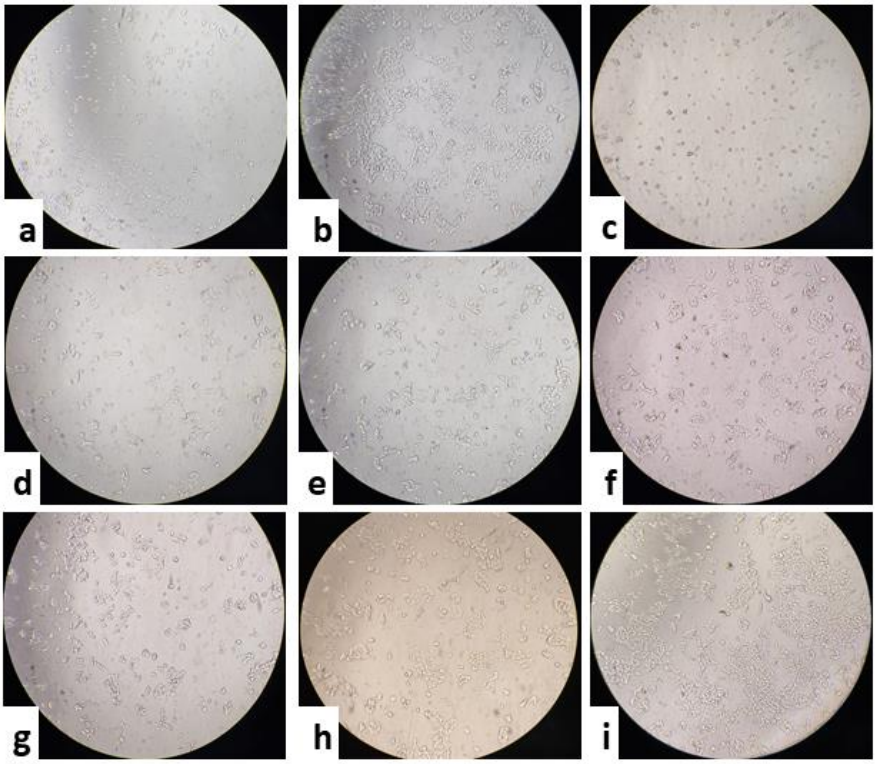

Figure 13. MCF-7 cells treated with (a) untreated, (b) control, (c) DOX (d) MTX, (e) DM5 (f) N1 (g) DP (h) TM (i) TD

The effects of various treatments on cell viability and morphology of cells are using the comparative analysis of MCF-7 breast cancer cells. However, untreated MCF-7 cells (Figure A) showed robust cellular health with normal morphology and proliferation (Figure A). This non cytotoxic nature of the solvent is confirmed by the control group (Figure B), which received deionized water and has no significant changes in cell viability or morphology. Unlike cells treated with doxorubicin (DOC, Figure C), cell density from these cells is markedly decreased, demonstrating cytotoxicity associated with doxorubicin's action as apoptosis inducer of DNA intercalation and of topoisomerase II inhibition. Similar to this, methotrexate treated cells (MTX Figure D) display decreased cell density due to methotrexate; an inhibition of dihydrofolate reductase, crucial for the synthesis and duplication of nucleotides and DNA. Cytotoxicity of the niosomal formulation with methotrexate (NDM5, Figure E) is even greater than these treatments when used alone, indicating a synergistic effect between the chemotherapy agents targeting distinct cellular pathways. N1 (Figure F), empty niosomes, show minimal effects on cell viability suggesting biocompatibility of niosomes and their non-toxic character. Among the free drug counterparts, doxorubicin (DP, Figure G) and methotrexate (TM, Figure H) loaded niosomes show more efficient cell density reduction than their native counterparts due to increased delivery and stability of drugs. Of note, the TD form (Figure I) of doxorubicin and methotrexate delivered in niosomes produced significant reductions in cell viability from this combination, suggesting that such dual drug niosomal delivery may have therapeutic potential.Overall, these findings demonstrate that DM5 (niosomes with doxorubicin and methotrexate) has the highest amount of cytotoxicity compared with all treatments, making it an ideal modality to improve therapeutic results in breast cancer cancer treatment.ing robust cellular health. The control group treated with deionized water (Figure B) shows no significant changes in cell viability or morphology, confirming the non-cytotoxic nature of the solvent. In contrast, cells treated with doxorubicin (DOC, Figure C) exhibit a marked reduction in cell density, reflecting significant cytotoxicity attributed to doxorubicin's mechanism of inducing apoptosis through DNA intercalation and topoisomerase II inhibition. Similarly, methotrexate-treated cells (MTX, Figure D) also demonstrate reduced cell density as a result of methotrexate's inhibition of dihydrofolate reductase, which is critical for nucleotide synthesis and DNA replication. The niosomal formulation containing both doxorubicin and methotrexate (DM5, Figure E) displays even greater cytotoxicity than the individual drug treatments, suggesting a synergistic effect due to the dual targeting of distinct cellular pathways. Empty niosomes (N1, Figure F) show minimal effects on cell viability, indicating their biocompatibility and non-toxic nature. Niosomes

loaded with doxorubicin (DP, Figure G) and methotrexate (TM, Figure H) effectively reduce cell density compared to their free-drug counterparts, enhancing drug delivery and stability. Notably, the combination of doxorubicin and methotrexate delivered in niosomes (TD, Figure I) results in significant reductions in cell viability, underscoring the therapeutic potential of these dual drug niosomal formulations.

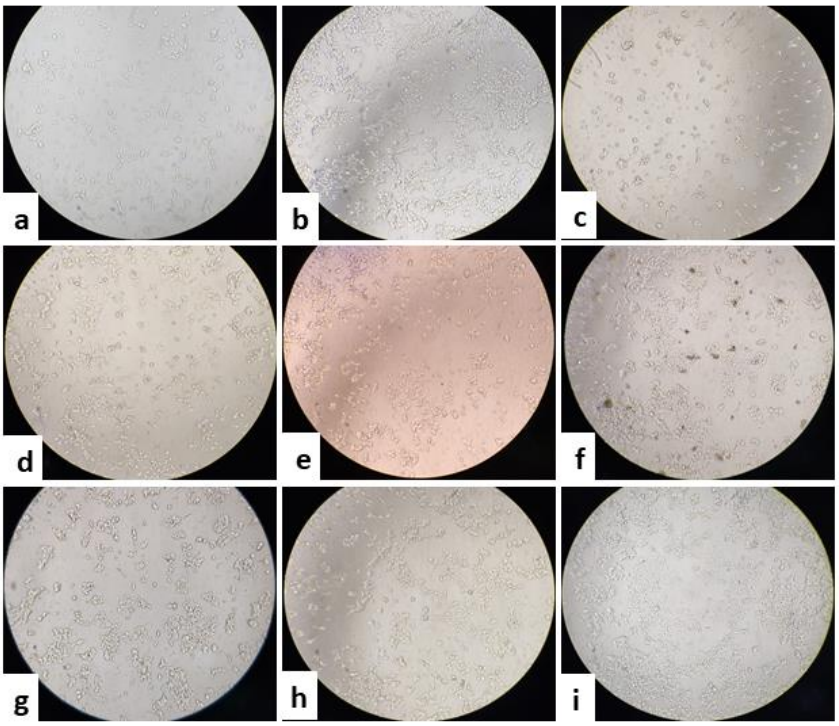

Figure 14. HEK-293 cells treated with (a) untreated, (b) control, (c) DOX (d) MTX, (e) DM5 (f) N1 (g) DP (h) TM and (i) TD

A cell proliferation analysis of HEK293 cells is performed to identify the effects of different treatments on the viability and morphology of the cells. Normal growth behavior as shown by untreated HEK293 cells (a) with high cell density and healthy morphology. Morphology and density of the control group treated with deionized water (b), which is comparable to that of the solvent-treated group (a), confirm that the solvent is indeed not toxic. In contrast to doxorubicin-treated cells (DOX, c), cells treated with doxorubicin (c) show a highly noticeable reduction in cell density and morphological damage owing to doxorubicin's mechanism of action (intercalation of DNA, inducing oxidative stress and apoptosis). Reduced cell density also occurs in methotrexate-treated cells (MTX, d) in which methotrexate inhibits dihydrofolate reductase, an enzyme necessary for DNA synthesis. Reduced cell density relative to free doxorubicin or methotrexate suggests that encapsulation in the niosomes limits

cytotoxicity to that resulting from controlled release, and improved targeting (DM5, e). The morphology, density, and biocompatibility of empty niosomes (N1, f) are confirmed; empty niososomes (N1, f) are similar to untreated cells. Free doxorubicin (DP, g), free methotrexate (TM, h), niosomes loaded with doxorubicin (DP, g) and niosomes loaded with methotrexate (TM, h) exhibit moderate reduction in cell density when compared to their free counterparts. When the free drugs are combined in niosomes (TD, i), however, the resultant drug combination exhibits significant but reduced cytotoxity compared to the free drugs alone. In terms of drug uptake, free drugs (DOC and MTX) are efficiently taken up by HEK293 to contribute to cytotoxic effects, owing to their small molecular size. However, contrary to this, niosomal formulations (DM5, DP, TM, TD) are internalized, via endocytosis, yielding controlled release and reduced instant cytotoxicity. Uptake has no harmful effects upon empty niosomes. By co-encapsulating doxorubicin and methotrexate in niosomes, they might be taken up less avidly in normal cells than their free drugs.

CHAPTER 6: CONCLUSION

In this work, niosomal formulations prepared through green synthesis were found highly potential for controlled drug delivery and sustained release. The performance of these systems in terms of highly biocompatible, strong antioxidant activities, and selective cytotoxicities towards cancer cells, qualitatively make them good candidates for dual drug delivery approaches for cancer treatment. Dual drug systems encapsulated by niosomes provided synergistic approaches for cancer therapy with safety and efficacy balance. The niosomes exhibit a sustained drug release profile that results in prolonged drug availability at the target site and reduced frequency of drug administration and associated with minimum of systemicside effects. Results of in vitro assays indicate the biocompatibility of the niosomal formulations, which can interact with biological systems without causing injury. The ability to easily adapt population chemotherapy to this scale is particularly valuable in cancer therapy, where healthy tissue toxicity is a primary concern. In addition, the niosomes possess an antioxidant capacity via their dual role. This not only improves the stability of the encapsulated drugs, but it also contributes to the therapeutic effect by neutralizing oxidative stress most frequently linked with cancer progression. Formulations show selective cytotoxicity, targeting cancerous cells while sparing healthy cells, increasing treatment specificity and reducing off target effects. This study highlights the potential for niosomal encapsulation as an advanced strategy in cancer therapeutics. By combining these dual drug delivery capabilities, a thoughtful strategy to the complex cancer is presented that may improve the effectiveness of treatment. However, although in vitro results are promising, it will require additional validation by in vivo studies to evaluate pharmacokinetics, biodistribution and therapeutic efficacy in a more complex biological environment. This, in turn, will also require clinical trials to evaluate the safety and efficacy of these formulations in human populations.

6.1 . Future Recommendation

- **Clinical Translation:** Focus on conducting preclinical and clinical trials to evaluate the safety, efficacy, and pharmacokinetics of green-synthesized niosomal drug delivery systems in human patients.
- **Advanced Targeting Mechanisms:** Incorporate ligand-conjugation, antibody-functionalized surfaces, or stimuli-responsive materials to improve the specificity and efficiency of drug delivery to tumor sites.
- **Overcoming Drug Resistance:** Design niosomal systems that address multidrug resistance in cancer cells by targeting specific resistance pathways or enhancing intracellular drug accumulation.
- **Scalability and Cost-effectiveness:** Develop scalable and cost-efficient production methods for green-synthesized niosomes to ensure their feasibility for industrial-scale manufacturing.
- **Combination with Immunotherapy:** Explore the potential of combining niosomal drug delivery systems with immune checkpoint inhibitors or cancer vaccines to boost the immune response against tumors.
- **Environmental Impact Assessment:** Evaluate the environmental sustainability of the green synthesis processes to ensure they align with eco-friendly principles.
- **Thermal and pH-responsive Systems:** Develop stimuli-responsive niosomal formulations that release drugs selectively in response to changes in the tumor microenvironment, such as temperature or pH.

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