

Formulation and Optimization of Xanthohumol loaded Nanostructured lipid carriers for the treatment of Lung cancer

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In

MOLECULAR BIOLOGY AND GENETIC ENGINEERING

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DECLARATION

I, **SHUBHAM SINGH**, bearing Registration No. **12224392**, hereby declare that the research work embodied in the Ph.D. thesis titled “**Formulation and Optimization of Xanthohumol loaded Nanostructured lipid carriers for the treatment of Lung cancer**” submitted to **Lovely Professional University** is the original and bonafide work carried out by me under the supervision of **Dr. Sachin Kumar Singh**, (HOD of Pharmaceutical Science), Department of Pharmaceutical Science, Lovely Professional University .

I further declare that:

- a)** The work presented in this thesis is a genuine contribution to the field of research and is a result of investigations carried out by me during the period of my doctoral study.
- b)** The research work has been conducted in accordance with the academic and ethical standards prescribed by the university.
- c)** Due acknowledgements and references have been duly given wherever the work of others has been cited or used.
- d)** The thesis has not been submitted either partly or fully to any other university or institution for the award of any degree or diploma.

I understand that any violation of the above declarations may lead to the cancellation of my degree by the university at any stage.

Shubham Singh

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled “**Formulation and Optimization of Xanthohumol loaded Nanostructured lipid carriers for the treatment of Lung cancer**” submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the **Department of Bioengineering and Biosciences**, is a research work carried out by **SHUBHAM SINGH, 12224392**, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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"The goal of education is the advancement of knowledge and the dissemination of truth." – John F. Kennedy.

"Honesty is the first chapter in the book of wisdom." – Thomas Jefferson.

"Success is the sum of small efforts, repeated day in and day out." –Robert Collier.

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It's warm memories of the past and big dreams for the future...

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Abstract

Lung cancer (LC) ranks among the most prevalent causes of cancer incidence and associated mortality among humans. As per the last report released by the “International Agency for Research on Cancer (IARC)” in February 2024, about 2.5 million new cases of LC were reported in 2022, with mortality of 1.8 million people, about 18.7% of the total cancer deaths. The main causes of lung cancer (LC) are smoking, exposure to secondhand smoke, radiation, radon gas exposure, asbestos exposure, and other carcinogens. With an exceptional targeting capability, novel drug delivery uses nanoscale material that is particularly effective against lung cancer such as nanostructured lipid carriers (NLCs). NLCs are second generation of solid lipid nanoparticles (SLNs), which are made up of a mixture of solid and liquid lipids, this mixture results in an imprecise matrix structure, which allows space to add pharmaceuticals to the matrix that has been widely used for drug formulation. Xanthohumol (Xn) is a prenylated chalconoid that is isolated from the female inflorescences of *Humulus lupulus*, more often referred to as hops. Xn has been reported for anti-cancer activity against breast, leukemia, and NSCLC with an increase in the amount of the protein p21. It also activates p53 to start the cell cycle arrest. Despite these challenges, Xn possesses poor aqueous solubility and low absorption from GIT. Hence, an alternative hypothesis has been proposed. Wherein, Xn will be loaded in NLCs and administered via intranasal route so that it can be targeted to lungs, particularly at tumor site. Upon reaching tumor site Xn can be easily penetrated in the tumors owing to their nanometer size and effective treatment of LC can be achieved by multifactual action of Xn on molecular pathways of LC.

The present research aims were to develop and optimize Xn loaded NLCs for treating LC. It has been discovered that Xn and its formulation are useful in the treatment of cancer. As this bioactive compound has medicinal importance, hence, a novel, precise, and sensitive HPLC method should be developed. In the present study, an RP-HPLC method has been developed and validated as per ICH Q2(R1) guidelines using column C18 having particle size 5 μ m and dimension 250 $\times$ 4.6mm. The detection wavelength (λ) used was 370 nm. The mobile phase consisted of a combination of HPLC grade Methanol and HPLC grade water buffer at a ratio of 95:5 v/v, with a flow rate of 1.0 mL/min. The total run time was 10 minutes with Xn retention at 4.5 minutes. The calibration plot was found linear in the range 2–10 μ g/ml. The recovery of 96.3% indicated good accuracy of method. The method's precision is within 2% of acceptable limits. LOD and LOQ values of 0.85

and 2.6 µg/ml indicated good sensitivity of method. The uniqueness of the research work is relying on achieving peak of Xn at lesser retention time as compared to existing methods. Further the results of specificity studies revealed absence of any interference of Xn peak with the excipients used in the NLCs. Overall, the study provided an accurate, precise, sensitive and specific method to quantify Xn in bulk and NLCs.

Xn-NLCs were successfully prepared by hot high-pressure homogenization in conjunction with the ultra-sonication method and optimized using a pseudo-ternary phase diagram followed by the Box–Behnken design (BBD). The NLCs were solidified using rotary evaporation followed by tray drying/vacuum drying. Mannitol was used for the solidification of L-Xn-NLCs. The BBD was operated using 3-factors and 3-levels for optimization of the formulation. The dependent variables were zeta potential (R1), particle size (R2), and drug entrapment efficiency (R3), while the independent variables were Beeswax (A), Lauroglycol-90 (B), and Tween-80 (C). The optimized liquid NLCs (L-Xn-NLCs) showed particle size (PS) of 101.60 ± 1.47 nm, polydispersity index (PDI) of 0.772 ± 0.029 , zeta potential (ZP) of -25.6 mV, and an entrapment efficiency (% EE) of $97.72 \pm 1.05\%$. As a result, NLCs have a higher drug encapsulation efficiency. After drying, followed by reconstitution, the solid NLCs (S-Xn-NLCs) showed PS of 278 nm, PDI of 0.22, ZP of -44.3 mV, and EE% of 95.5. The results of SEM and PXRD revealed the complete adsorption of S-Xn-NLCs on the surface of mannitol. The *in vitro* drug release and *in vitro* MTT assay were performed on A549 LC cells to evaluate the anticancer efficacy of S-Xn-NLCs. In 24 hours, the L-Xn-NLCs, S-Xn-NLCs, and pure drug suspension had a cumulative drug release rate of $80.09 \pm 0.8\%$, 79.8 % and $40 \pm 1.1\%$, respectively. It was found that both pure Xn and S-Xn-NLCs reduced the proliferation of A549 cell lines at 28.21 µM, 56.42 µM, 84.63 µM, 112.84 µM and 141 µM. However, S-Xn-NLCs exerted higher reduction in proliferation than pure Xn. The pure Xn and the S-Xn-NLCs showed anticancer potential against A549 cell lines and the IC₅₀ was found to be 140.186 µM for the pure Xn and 84.63 µM for the S-Xn-NLCs. The S-Xn-NLCs showed higher cytotoxicity potential to A549 cells as compared to the pure Xn. The cytotoxic efficacy of developed S-Xn-NLCs was further confirmed by Rhodamine 123 staining. The retention of rhodamine 123 was utilized to detect changes in mitochondrial membrane potential ($\Delta\Psi_m$) in A549 cells (after treatment). In this study, the results demonstrate a decrease in fluorescence intensity at IC₅₀ concentration of Pure-Xn and S-Xn-NLCs in comparison to the untreated control cells (100%), indicating loss of MMP. Further, cell migration assays, DCFH-DA Staining and q-

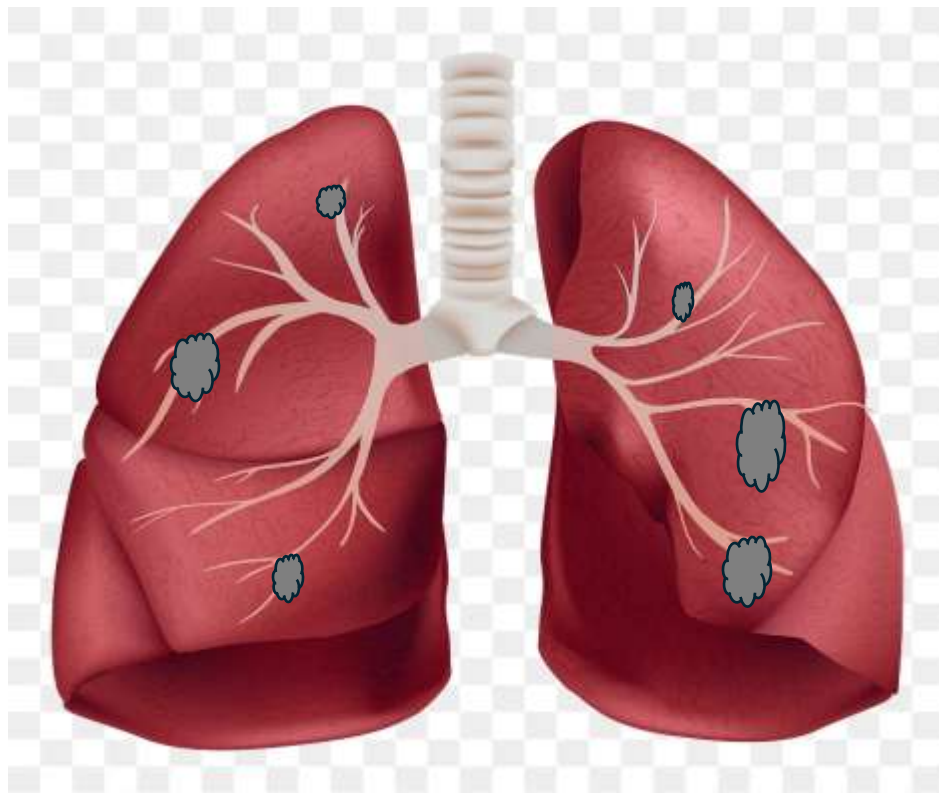
RT-PCR were performed. The percentage wound closure assay showed that S-Xn-NLCs reduced wound closure by more than 80% at lower IC_{50} values than the control group and pure Xn. This suggests that S-Xn-NLCs are more effective than pure Xn at preventing A549 cells from migrating and invading. DCF fluorescence intensity was used to determine ROS levels. It was found that the ROS level was higher in the treated groups of Pure Xn and S-Xn-NLCs than in the control group, which showed no green fluorescence.

Further, the cytotoxicity of S-Xn-NLCs and Pure Xn on A549 cells is related to cell cycle distribution. Flow cytometry was used to quantify cell populations in each phase of DNA content. The percentages of cells in G0-G1, S, and G2/M phases were determined. Cell cycle analysis showed that S-Xn-NLCs caused A549 cells to be arrested in the G2/M phase of the cell cycle. The results demonstrated that S-Xn-NLCs induced G2/M arrest significantly higher than pure Xn. The expression of important apoptotic genes was investigated by q-RT-PCR as a possible mechanism of action for the growth-inhibiting effects of pure-Xn and S-Xn-NLCs on the A549 cell line. The study found that pro-apoptosis genes p53 and caspase-3, were dramatically increased while anti-apoptosis genes BCL-2 were downregulated and NF- κ B were also downregulated by the action of S-Xn-NLCs. Thus, it was concluded that the optimized NLCs showed very good efficacy against LC.



Chapter 1

Lung Cancer



Introduction ...

CHAPTER 1

INTRODUCTION

1. Introduction

Lung cancer (LC) is the most leading cause of deaths across the globe. As per the report of GLOBOCAN published (Bray et al., 2024) in 2022, about 2.5 million people were diagnosed with LC and among them 1.8 million people died as shown in fig.1.1 (Sung et al., 2021a).

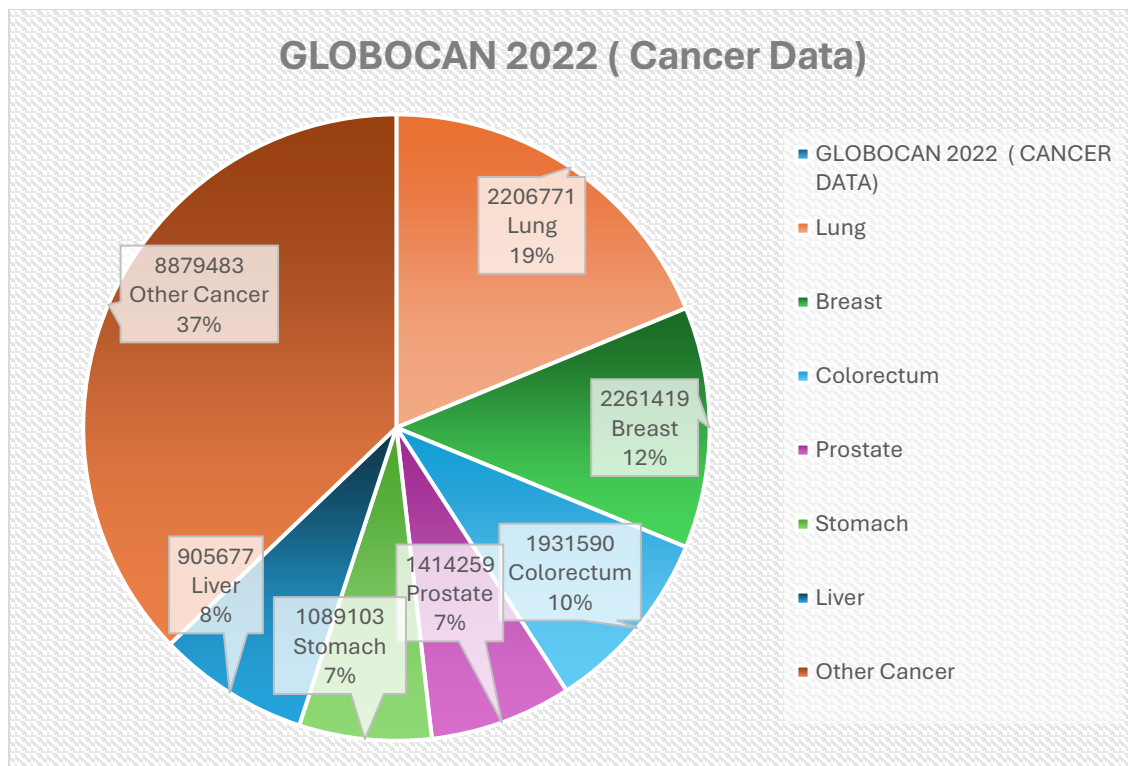


Fig. 1.1 : Global Observatory Data (2022) - Cancer

LC primarily arises due to smoking, inhalation of secondhand smoke, radiation exposure, and contact with radon gas, asbestos, or other carcinogenic substances. It is categorized into two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Small cell lung cancer, which constitutes 10-15% of total lung cancer cases, is particularly known for its rapid metastasis. SCLC is most common among heavy smokers. NSCLC accounts for about 85% of all LC cases (Rosell & Karachaliou, 2016a).

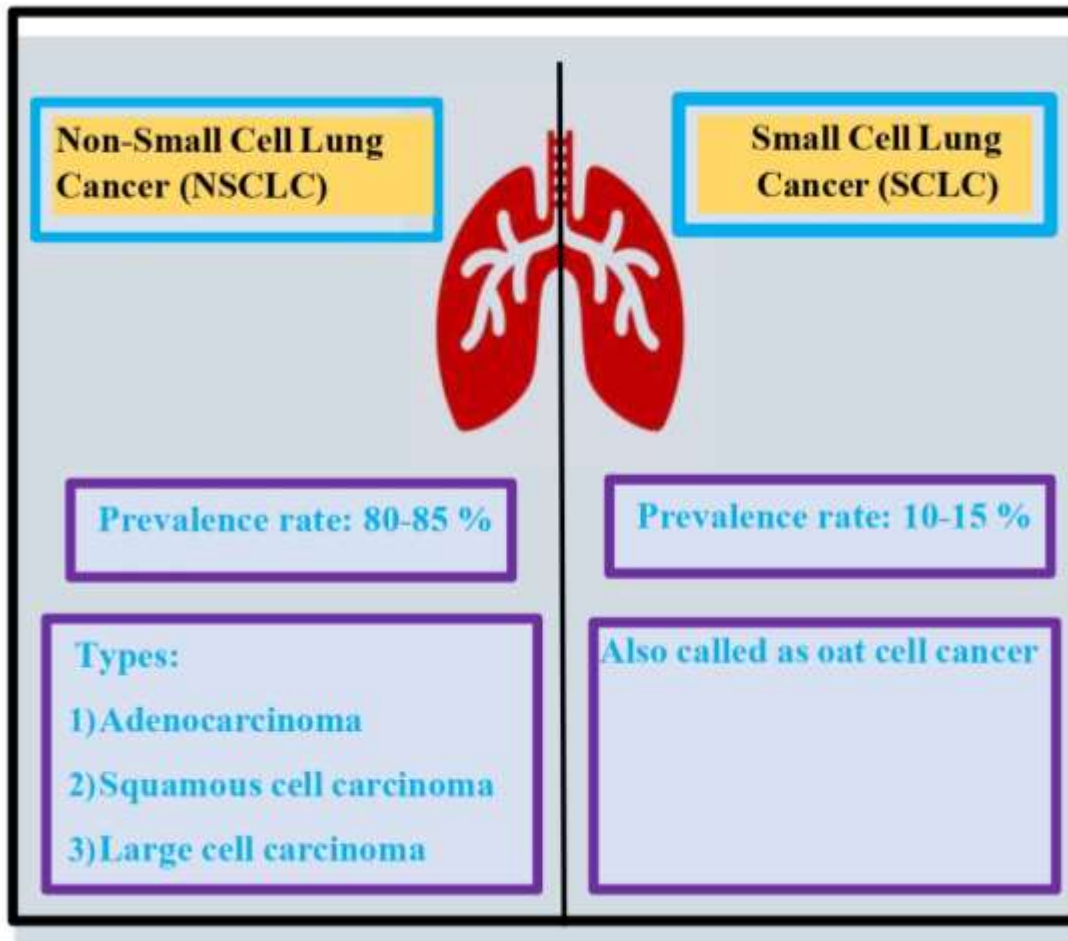


Fig. 1.2 : Figure demonstrating different types of LC along with prevalence rate

Despite the research done to manage NSCLC as well as advancement in LC targeted therapies, there exist only 16% of the total cases where about 5-years survival rate has been observed. NSCLC can develop both in smokers and non-smokers. This type of cancer primarily originates in the bronchi, which are the main air passages to the lungs, as well as in the peripheral lung tissues. About 90% of deaths related to cancer are caused by metastases (Bielenberg & Zetter, 2015a). Various types of LC are shown in Fig.1.2 &1.3.

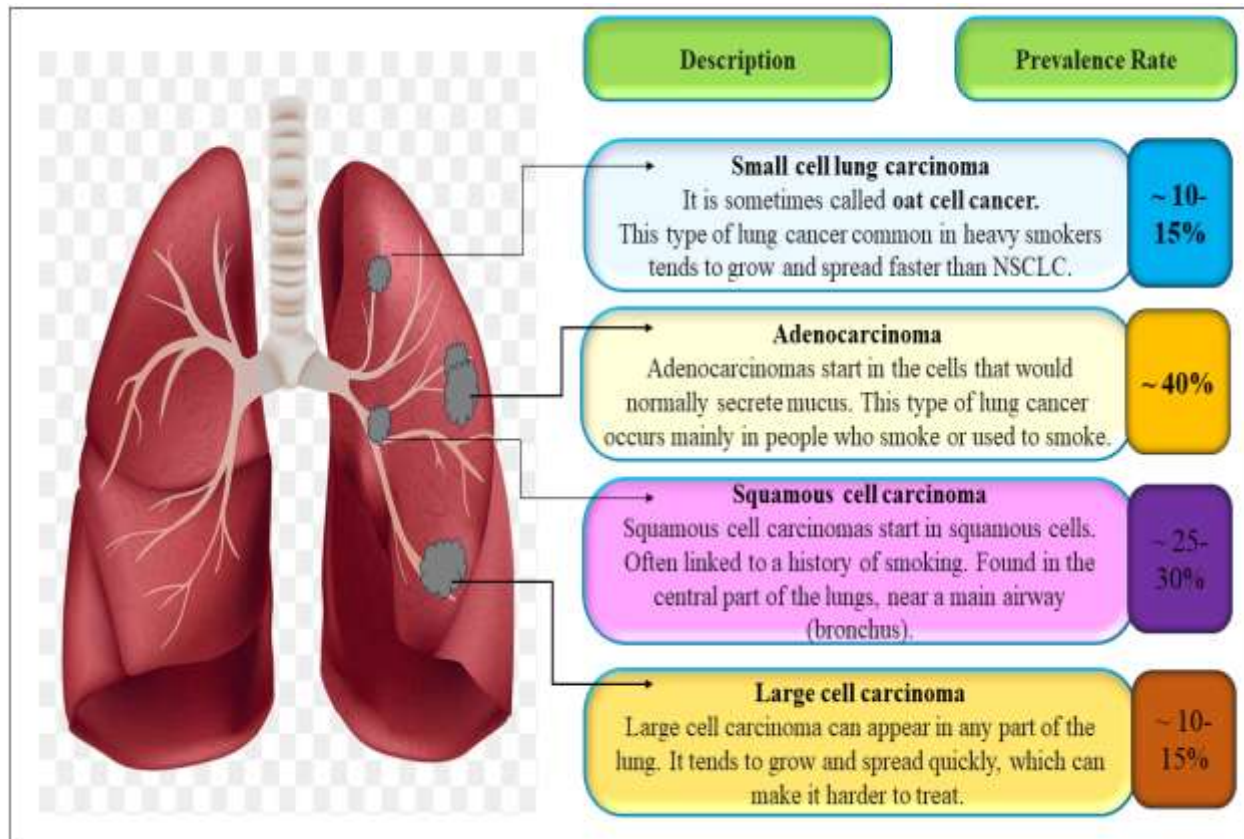


Fig. 1.3 : Types of lungs cancer along with their description

Non-small cell lung cancer (NSCLC) is primarily divided into three distinct types: adenocarcinoma, which originates in the mucus-secreting glands of the lungs; squamous cell carcinoma, which develops in the flat cells lining the airways; and large cell carcinoma, recognized by its large and abnormal cancer cells, and can occur in any part of the lung.

Among these, adenocarcinoma is the most prevalent form, comprising approximately 40% of all NSCLC cases. Adenocarcinomas develop in epithelial cells that line the outer surface of the lungs. The interior surface of the lungs is composed of flat cells known as squamous cells. The cancer that originates in squamous cell carcinoma. About 25-30% of all NSCLC cases belong to squamous cell carcinoma. Large cell carcinoma represents the least prevalent form of NSCLC, comprising approximately 10% of all NSCLC cases.

1.1 LC: Key Gene and Signaling Pathways

LC commonly involves changes or alterations in significant genes, including epidermal growth factor receptor (EGFR), Wnt/ β -catenin signaling pathway, anaplastic lymphoma kinase (ALK), mesenchymal epithelial transition factor receptor (MET), Kirsten rat sarcoma (K-Ras), and p53.

These genetic alterations play a crucial role in the signaling pathways that are responsible for tumor growth, differentiation, and the survival of cancer cells (*Herbst2008*, n.d.; Minna et al., 2002; Sekido et al., 2003; S. Sun et al., 2007).

The overexpression of the receptor tyrosine kinase EGFR occurs in 15% to 30% of patients with NSCLC, thereby activating critical signaling pathways, including RAS/RAF/MEK/ERK and PI3K/AKT. This activation plays a crucial role in the onset of tumors, their spread to other parts of the body, the formation of new blood vessels, and the survival of cancer cells. Consequently, it leads to lower survival rates among patients and diminishes the effectiveness of chemotherapy in treating tumors (*Herbst2008*, n.d.; Nicholson et al., n.d.; Sekido et al., 2003; Shigematsu et al., 2005). As a potential therapeutic target for treating LC, the EGFR protein is already being studied (Marchetti et al., 2005; Woodburn et al., 1999). The EGFR T790M mutation, along with c-MET gene amplification, are significant mechanisms that contribute to both primary and acquired resistance to EGFR-TKI therapy (Cross et al., 2014; S. Wang et al., 2015; Z. Wang et al., 2014) treatment in LC. KRAS and HER2 (ERBB2) co-mutations are being reported (Johnson et al., 2007; Morgillo et al., 2007).

In NSCLC patients, the tumor-suppressor gene known as p53 is often deregulated, occurring in 50% to 70% of cases. This deregulation is linked to a decrease in the process of programmed cell death, known as apoptosis, which in turn is associated with an increased survival rate of cancer cells (*Herbst2008*, n.d.; Minna et al., 2002; Sekido et al., 2003). On chromosome 17p13.1, the p53 gene is mutated in more than 50% of LC cases, however smokers are more likely to develop p53 mutations than non-smokers (Le Calvez et al., 2005). The downstream genes p21, BAX, and Phosphatase and tensin homolog (PTEN), which have an impact on cell division, cell death, and genomic integrity, are controlled by the protein p53, which also mediates a complex signaling network (*Herbst2008*, n.d.; Minna et al., 2002; Okada et al., 2014; Sekido et al., 2003). Targeting the p53 tumor-suppressor pathway presents an intriguing approach to treating LC, as this pathway plays a crucial role in controlling cell division and preventing tumor growth.

The current treatment of LC includes chemotherapy, radiotherapy, surgery and immunotherapy (Singh, Saxena, Sharma, Gupta, et al., 2024a). Cancer treatments, particularly chemotherapy and radiation therapy, commonly cause hair loss (alopecia) (Singh, Saxena, Sharma, Gupta, et al.,

2024a). For cancer patients, surgery is extremely stressful, and immunotherapy is too costly. Hence, safe, effective and economical treatment of LC has always been the prime objective of scientists working in the area of cancer. Owing to these objectives, the research has been tremendously shifted in the last ten years towards exploration of phytomedicines for treating LC. Among all plant-based medicines, more focus has been found towards the development of flavonoids-based formulations.

1.2 Xanthohumol

Xanthohumol (Xn) is a prenylated chalconoid isolated from the female inflorescences of *Humulus lupulus*. It has been proven to have good anti-cancer properties to treat LC, breast cancer and blood cancer ((Liu et al., 2015); Gieroba et al., 2020; Lee et al., 2007; Yong et al., 2015; Zhang et al., 2015). Xn is reported to have multiple biological targets in human cancer as it stimulates p53 and increases p21 protein levels causing cell cycle arrest. Treatment using Xn has been reported to lower Bcl-2 and survivin expression and promoted cancer cell death by altering various signaling pathways such as epidermal growth factor receptor (EGFR), Wnt/ β -catenin signaling, mitogen-activated protein kinase (MAPK), protein kinase B (PI3K/AKT), extracellular signal-related kinases (ERK), nuclear factor kappa B (NF- κ B), and signal transducer and activator of transcription 3 (STAT3) (Girisa et al., 2021). Additionally, it is reported to modulate the expression of proteins including Caspases, Bcl-2, CyclinD1, oxidative markers and tumor suppresser gene (Girisa et al., 2021a).

Even though Xn has amazing anticancer potential, there are several physiochemical and biopharmaceutical issues such as poor solubility and permeability that hinder its bioavailability, permeability to cancer cells and poor site specificity to lungs. With a Log P of 3.91, Xn has a poor solubility in water (1.3 mg/L at 23 °C). The hydrophobic characteristics and low bioavailability of Xn make it difficult to successfully deliver to its target location. As a result, Xn fails to achieve a therapeutic impact on the target site in cases of LC. Therefore, to overcome the challenges associated with nanotechnology-based approaches could be an alternative option to enhance bioavailability and provide an efficient therapeutic effect against lung carcinoma cells.

Nanotechnology has been extensively applied in health and medicine for various biomedical applications, particularly in the area of cancer. Among the various nanocarriers that have been utilized till date, the use of lipid based nanocarriers (LNCs) has been widely preferred by the formulation scientist for drug delivery. Among them, Solid lipid nanoparticles (SLNs) as well as

nanostructure lipid carriers (NLCs), have particular advantages towards delivering lipophilic drugs. SLNs and NLCs are gaining attention as potential nano-carriers for administering the drugs at local site since the last decade owing to their unique features such as it protect the drug inside the lipid matrix from degradation and oxidation, enhanced the drug loading and provide stability to reach to the target site. SLNs are composed of solid biocompatible lipid matrices, while NLCs contain both liquid and solid lipids (Viegas et al., 2023). Among both LNCs, some of the bottlenecks have been observed with SLNs such as poor loading of drug and possibility of leakage of drug from formulation during storage. While such challenges have not been observed in the case of NLCs. The NLCs are composed of a mixture of solid as well as liquid lipid which leads to a more complex matrix structure, reduced lipid density, and enhanced capacity for drug integration within the matrix (Chauhan et al., 2020a) As a result, better encapsulation of drugs and enhanced storage stability for long time has been observed with NLCs as compared to SLNs (Chauhan et al., 2020a; Elmowafy & Al-Sanea, 2021a; Viegas et al., 2023). When it comes to safety and biocompatibility, NLCs are generally preferred to polymeric nanoparticles (NPs) and are thought to be the less harmful (Chauhan et al., 2020a).

1.3 Nanostructured lipid carriers (NLCs)

Nanostructured lipid carriers (NLCs) are part of the second generation of SLNs and consist of a blend of solid lipids, liquid lipids, and surfactants, designed to enhance drug delivery systems through improved stability and bioavailability. NLCs has several advantages, including biocompatibility, non-immunogenicity, high drug loading capacity, greater stability, controlled drug release, and a simple fabrication process (Fig.1.4). As a result, better encapsulation of drugs and enhanced storage stability for long time has been observed with NLCs as compared to SLNs. The most common polymeric nanoparticles (NPs) in terms of safety and biocompatibility are NLCs, which are also thought to be the least hazardous when given in vivo. The present research aimed to prepare Xn-loaded nanostructured lipid carriers (NLCs) by the method of melting film ultrasonic and High-speed homogenization, and to assess the anti-tumor effect of the preparations on A549 cells line.

Novel drug delivery system has nanoscale material that is highly effective with excellent targeting abilities against LC (Kaur et al., 2022; Kazmi et al., 2023; Khursheed et al., 2022; Kumari et al., 2023). In the early 1990s, Professor R.H. Muller from Germany discovered solid lipid nanoparticles (SLNs) as an novel formulation. However, SLNs have some limitations like poor

drug loading capacity and drugs leakage during storage. To address these challenges, NLCs were developed .

NLCs are a blend of solid lipids, liquid lipids, and surfactants that create a unique matrix for drug delivery. It generates more space to incorporate drugs in the matrix (Almurshedi et al., 2021). As a result, when compared to SLNs, NLCs provide various advantages, including higher drug encapsulation efficiency (EE) and long-term storage stability. NLCs are made using emulsifiers and a biodegradable as well as suitable lipid (solid and liquid).

1.4 Composition of NLCs

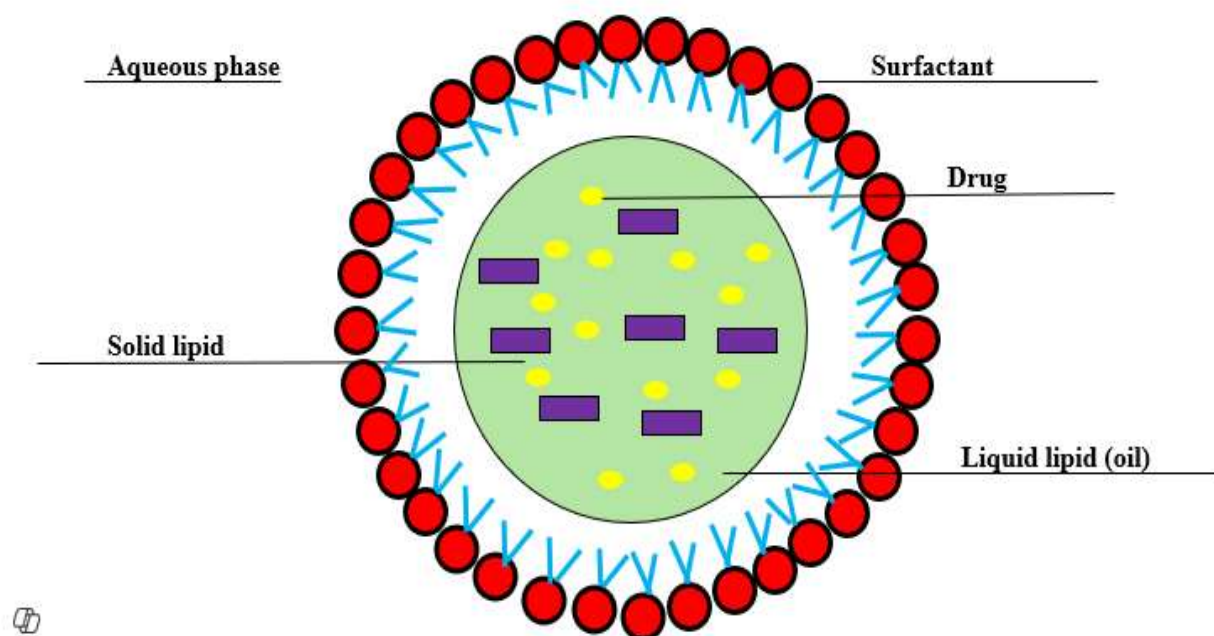
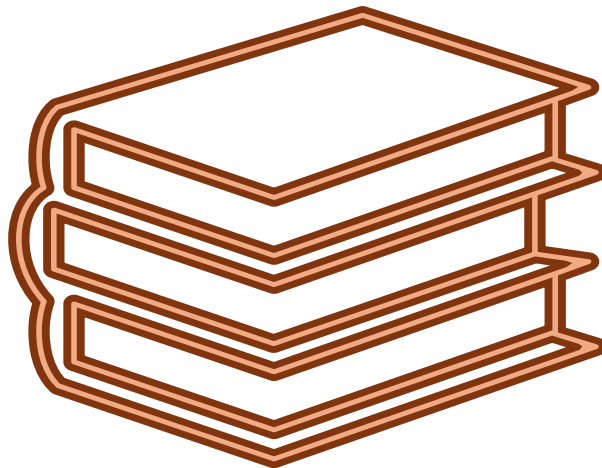


Fig. 1.4 : Composition of NLCs.

The current study investigates the possibility of Xn-loaded NLCs as a novel drug delivery system for treatment of LC (Singh, Saxena, Sharma, Gupta, et al., 2024a). Therefore, to overcome these challenges of Xn, the Xn loaded NLCs have been developed and characterized. A549, a cell line model of NSCLC, was used for study because it has been widely used to evaluate target identification and validation of anti-cancer activity (Solanki et al., 2025).



Chapter 2



Literature Review....

CHAPTER 2

Literature review

2.1 Lung Cancer

LC is one of leading causes of cancer. Worldwide, related deaths represent the highest mortality rates for both men and women. As per the report of GLOBOCAN published (Bray et al., 2024) in 2022, about 2.5 million people were diagnosed with LC and among them 1.8 million people died. LC is classified into two main categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which together comprise 96% of all lung cancer cases. (Lemjabbar-Alaoui et al., 2015; Singh, Saxena, Sharma, Paudel, et al., 2024).

2.1.1. Non-small-cell lung cancer (NSCLC)

NSCLC is the predominant type of lung cancer, characterized by its slow growth, and comprises approximately 80% of all LC cases (Singh, Saxena, Sharma, Paudel, et al., 2024). The main categories of non-small cell lung carcinoma (NSCLC) include adenocarcinoma, squamous cell carcinoma, Pancoast or pulmonary sulcus tumors, and large cell undifferentiated carcinoma.

Adenocarcinomas: Adenocarcinoma is a type of cancer that develops in glandular or secretory cells. It represents the most prevalent form of lung cancer, specifically categorized as a subtype of non-small cell lung cancer (NSCLC). These tumors typically present in the peripheral regions of the lungs and generally exhibit a slower growth rate compared to other forms of lung cancer. Adenocarcinoma is predominantly diagnosed in individuals who currently smoke or have a history of smoking.

Squamous cell carcinomas: Squamous cells, which are flat epithelial cells lining the air passages of the lungs, are frequently associated with a history of smoking. Typically located in the central areas of the lung near the primary airway, or bronchus, these cells exhibit a relatively slower growth rate compared to other cell types.

Pancoast tumor: A Pancoast tumor represents a rare subtype of LC occurring in the apex, or uppermost section, of the lung adjacent to the chest wall. It is characterized by its invasion into nearby structures such as the ribs and nerves. The tumor's location makes surgical intervention particularly complex. Notably, Pancoast tumors constitute less than 5% of all primary LC cases.

Large cell carcinomas: Large cell carcinoma, a subset of LC, is marked by the presence of large, undifferentiated cells. These cells have a rapid growth and spread pattern, which can complicate

treatment efforts, posing challenges in effectively managing this aggressive form of cancer.

2.1.2. Small-cell lung cancer (SCLC)

SCLC cancer is a fast-growing and aggressive form of LC that is almost always caused by smoking. Only about 15-25% of all LC are SCLC. SCLCs are also known as neuroendocrine cancer or oat cell cancer. SCLC usually spreads more rapidly than NSCLC.

Rarer types of LC include:

- ❖ **Mesothelioma:** Mesothelioma is a highly aggressive form of cancer affecting the mesothelium, the delicate protective lining of internal organs, most frequently the lungs and chest wall. This disease is commonly associated with exposure to asbestos.
- ❖ **Carcinoid:** Carcinoid cancer is a neuroendocrine tumor characterized by its slow growth, frequently originating in either the gastrointestinal tract or the lungs.
- ❖ **Sarcoma:** Sarcoma is an uncommon type of cancer that originates in the body's connective tissues, such as bones, cartilage, muscles, fat, or blood vessels.

2.2. Risk Factors and Screening Recommendations for LC

2.2.1. Risk Factors for LC

The risk factors for developing lung cancer include smoking and exposure to second-hand smoke, both of which significantly increase the likelihood of the disease. Additionally, contact with carcinogen substances known to promote cancer—such as radon, asbestos, coal smoke, and diesel fumes further elevates the risk. Individuals with a personal history of cancer or those who have undergone chest radiation also face higher chances of lung cancer. A family history of LC and personal medical conditions like Chronic Obstructive Pulmonary Disease (COPD) are additional factors that contribute to this risk. Recognizing these risk factors is essential for both prevention and early identification.

2.2.2. Symptoms of LC

In the early stages, LC typically presents without any symptoms, rendering it asymptomatic. As the condition progresses, individuals may experience a range of symptoms commonly associated with LC. These include a persistent cough, hemoptysis (coughing up blood), chest pain, and difficulty with breathing or swallowing. Other symptoms may consist of fatigue, dyspnea (shortness of breath), frequent pneumonia, wheezing, hoarseness, and notable changes like weight loss or reduced appetite.

2.2.3. Stages of LC

The TNM (Tumor, Node, Metastasis) staging system works by assessing three key factors for solid tumors (Chheang & Brown, 2013; Lemjabbar-Alaoui et al., 2015). The abbreviation TNM refers to the classification system that details the tumor size and the extent to which the cancer has metastasized to other regions :

- ❖ T (Primary tumor) : indicates the size of the tumors and local spread of the primary tumor. The 'T' category is numbered 1-4, with higher numbers indicating a larger size and more extensive invasion
- ❖ N (Node) : indicates if the cancer has spread to nearby lymph nodes. 'N0' means no spread to lymph nodes, while 'N1, N2, or N3' indicates that cancer has spread to one or more lymph nodes, with numbers often reflecting the number, size, and location of the affected nodes
- ❖ M (Metastasis) : indicates that cancer has spread to other parts of the body. 'M0' means no metastasis, while 'M1' means the cancer has spread to distant parts of the body.

Non-small cell lung cancer (NSCLC) is categorized into five distinct stages, which are represented using Roman numerals ranging from 0 to IV (4) (Chheang & Brown, 2013). The stages of NSCLC are as follows in Table 2.1.

Table 2.1 : Stages of NSCLC

Stage 0 (Carcinoma in situ)	Stage 0, known as carcinoma in situ, is characterized by the presence of atypical cells confined to the lining of the airways, indicating an early stage of abnormal cell development.
Stages IA & IB (1A & 1B)	In Stages IA and IB, the abnormal cells have developed into cancer, characterized by a small tumor size and the absence of lymph node involvement. Specifically, Stage IA is defined by a tumor measuring 3 centimeters or less, while Stage IB is characterized by a tumor size ranging from 3 to 4 centimeters.
Stages IIA & IIB (2A & 2B)	In Stage IIA, the lung tumor measures between 4 and 5 centimeters in size and has not metastasized to the lymph nodes. In Stage IIB, the lung cancer tumor is 5 centimeters or smaller and has spread to lymph nodes located within the same

	region of the chest as the original tumor, usually those within the lung itself or adjacent to the bronchus.
Stages IIIA, IIIB, & IIIC (3A, 3B, & 3C)	At this stage, the cancer has advanced by spreading to the lymph nodes, though it has not yet metastasized to other areas of the body. The tumor might have increased in size or the cancer may now reside in a more challenging location compared to its initial stages. Stages IIIB and IIIC frequently warrant treatment approaches similar to those for Stage IV cancers.
Stage IV (4)	In these cases, the LC tumor can vary in size and may have metastasized to the lymph nodes, extending beyond a single lung. It can affect the opposite lung, as well as the fluid surrounding the heart or lung, or other regions of the body. Common sites for LC metastasis include the brain, bones, liver, kidneys, or adrenal glands, although it is capable of spreading to any area.

2.2.4. Diagnostic tools for LC

Major types include:

- **Chest x-ray** : A chest X-ray is a basic radiographic examination of the lungs, commonly employed as the initial diagnostic tool for LC. Lung tumors typically present on X-rays as a white-grey mass. While this method offers the advantages of speed and cost-efficiency, it does not provide as detailed an image as computed tomography (CT) or magnetic resonance imaging (MRI) scans (Park et al., 2020; J. Shao et al., 2023).
- **CT and MRI scans** : CT (computed tomography) and MRI (magnetic resonance imaging) use computers to provide detailed 3-D images that are helpful in the diagnosis of LC and can determine a tumor's size, shape, and location. CT scans are more likely to detect lung cancers than normal chest x-rays. CT and MRI scans can be useful in identifying if LC has spread (Park et al., 2020; J. Shao et al., 2023).
- **PET scans** : A PET (positron emission tomography) scan is an imaging test for LC that detects cellular activity using a radioactive tracer, highlighting potential tumors as bright

spots. PET takes advantage of the fact that cancer absorbs more sugar than the normal tissues. A tagged sugar is injected for this scan, and a specialized camera captures pictures of its path (Park et al., 2020; J. Shao et al., 2023). PET scans provide a more accurate picture of the stage of LC. It helps to identify whether the cancer has spread outside the lungs. PET scans are more expensive, more time-consuming, and involve more radiation exposure than other types of scans.

In order to determine the type and stage of cancerous tumors detected by scans, a sample of tissue or fluid will be obtained (Park et al., 2020; J. Shao et al., 2023). There are numerous methods for obtaining a sample. Common ones consist of:

- ***Needle biopsy*** : A needle biopsy is a procedure to remove some cells or small pieces of tissue and looked at under a microscope that can be taken from the lung or a part of the body where the cancer has spread.
- ***Sputum cytology*** : A sputum cytology is a procedure to collect the sample of sputum (mucus produced by a cough) and looked at under a microscope.
- ***Thoracentesis*** : Thoracentesis is a technique to remove excess fluid or air from the pleural space, which is located between the lungs and the chest wall. A hollow needle is placed into the chest wall to drain the fluid. A sample of the fluid can be sent to a lab to be examined for the presence of cancer.
- ***Bronchoscopy*** : Bronchoscopy (a flexible tube-like instrument) is a medical procedure that uses a thin, lighted tube with a camera (a bronchoscope) to examine the airways in the lungs and to collect tissue samples. Local anesthesia and mild sedation are generally used.
- ***Navigational bronchoscopy*** : Navigational bronchoscopy is an image-guided procedure that uses a computer-generated, GPS-like system to help physicians navigate a bronchoscope to biopsy hard-to-reach parts of the lungs.
- ***Liquid biopsy*** : A liquid biopsy is a non-invasive medical test that analyzes a bodily fluid, most commonly blood, to detect signs of disease like cancer, such as circulating tumor cells (CTCs) or fragments of tumor DNA (ctDNA).

2.2.5. Biomarkers for Diagnosis and Treatment

The molecules known as biomarkers are indicators of a disease or condition. Sometimes they are referred to as cancer markers or molecular markers (V. Li & Vigneswaran, 2022). Biomarker testing, which can be performed using tumor tissue, blood, or sputum samples, is essential for

identifying various indicators related to diseases. Comprehensive testing is most effectively conducted through next-generation sequencing (NGS), allowing for the simultaneous analysis of multiple biomarkers, providing detailed insights into an individual's health condition (V. Li & Vigneswaran, 2022; Villalobos & Wistuba, 2017). Biomarker testing, which can also be referred to as tumor marker testing, genomic testing, mutation testing, or molecular testing, involves the analysis of specific biological markers associated with tumors or other relevant genetic components. These biomarkers can be used to kill cancer cells or halt their proliferation in a variety of novel cancer treatments. Several commonly detected LC biomarkers are as follows:

- ❖ *EGFR (epidermal growth factor receptor)*: A protein located on the cell surface that plays a crucial role in stimulating cell division and proliferation.
- ❖ *ALK (anaplastic lymphoma kinase) gene*: ALK gene, when mutated, has the potential to accelerate the growth of cancer cells.
- ❖ *ROS1 (c-ros oncogene 1)*: Mutations in this gene lead to the proliferation of cancerous cells.
- ❖ *KRAS (Kristen RNA Associated Rat Sarcoma 2 Virus Gene)*: This gene is responsible for the production of the KRAS protein, which plays a crucial role in regulating cell signaling pathways, cellular growth, and apoptosis.
- ❖ *BRAF (B-Raf proto-oncogene, serine/ threonine kinase)*: When mutations occur within this gene, the resulting alteration in the BRAF protein can trigger abnormal cell growth and division. This uncontrolled proliferation of cells can lead to the formation of tumors, contributing to the development of cancer.

2.2.6. LC: Key Gene and Signaling Pathways

LC is often characterized by the alterations of key genes such as epidermal growth factor receptor (EGFR), Wnt/ β -catenin signaling, Anaplastic lymphoma kinase (ALK), mesenchymal epithelial transition factor receptor (MET), Kirsten rat sarcoma (K-Ras) and p53 and downstream signaling pathways associated with tumor growth, differentiation, and survival (Herbst et al., 2008; Minna et al., 2002; Sekido et al., 2003; Sun et al., 2007). These pathways are intimately associated with miRNAs. A list of various pathways associated with tumor suppressing miRNAs and their mechanism is shown in Table 2.2 and in Fig.2.2.

2.2.7. *MicroRNAs*

MicroRNAs (miRNAs) are a class of small non-coding RNAs comprising 20 to 22 nucleotide-long single-stranded molecules. They regulate gene expression at the post-transcriptional stage and may significantly contribute to the suppression of tumorigenesis. Oncogenic miRNAs (Oncomirs) are miRNAs whose level gets increased in cancer cells. They promote carcinogenesis by blocking tumor suppressor genes (Esquela-Kerscher & Slack, 2006). Tumor suppressor microRNAs, conversely, are microRNAs that are downregulated and generally inhibit cancer development by suppressing the expression of proto-oncogenes (Dimopoulos et al., 2013).

2.2.8. *Gene silencing*

According to the evidence presented in various literature creation miRNA appears to be a well-defined and conserved mechanism. The transcription of the primary miRNA (pri-miRNA), which contains a local stem-loop structure is crucially regulated by RNA polymerase II in the nucleus. In the nucleus, RNaseIII, Drosha, and the Drosha auxiliary regulator DGCR8 work together to splice this stem-loop structure out of the pri-miRNA to create a precursor miRNA (pre-miRNA). Then the exportin-5/RAN-GTP complex subsequently shuttles the pre-miRNA into the cytoplasm, where it is further broken down by the enzyme Dicer to produce a duplex miRNA. The resultant duplex structure is unraveled, and the mature miRNA guide strand is subsequently loaded onto AGO proteins to create the RNA-induced silencing complex (RISC) (Davis-Dusenbery & Hata, 2010). The RNA-induced silencing complex (RISC complex) formation is to mediate transcriptional repression or mRNA degradation of the targeted gene. The mature miRNA controls how the RISC complex silences targeted that is shown in Fig.2.1.

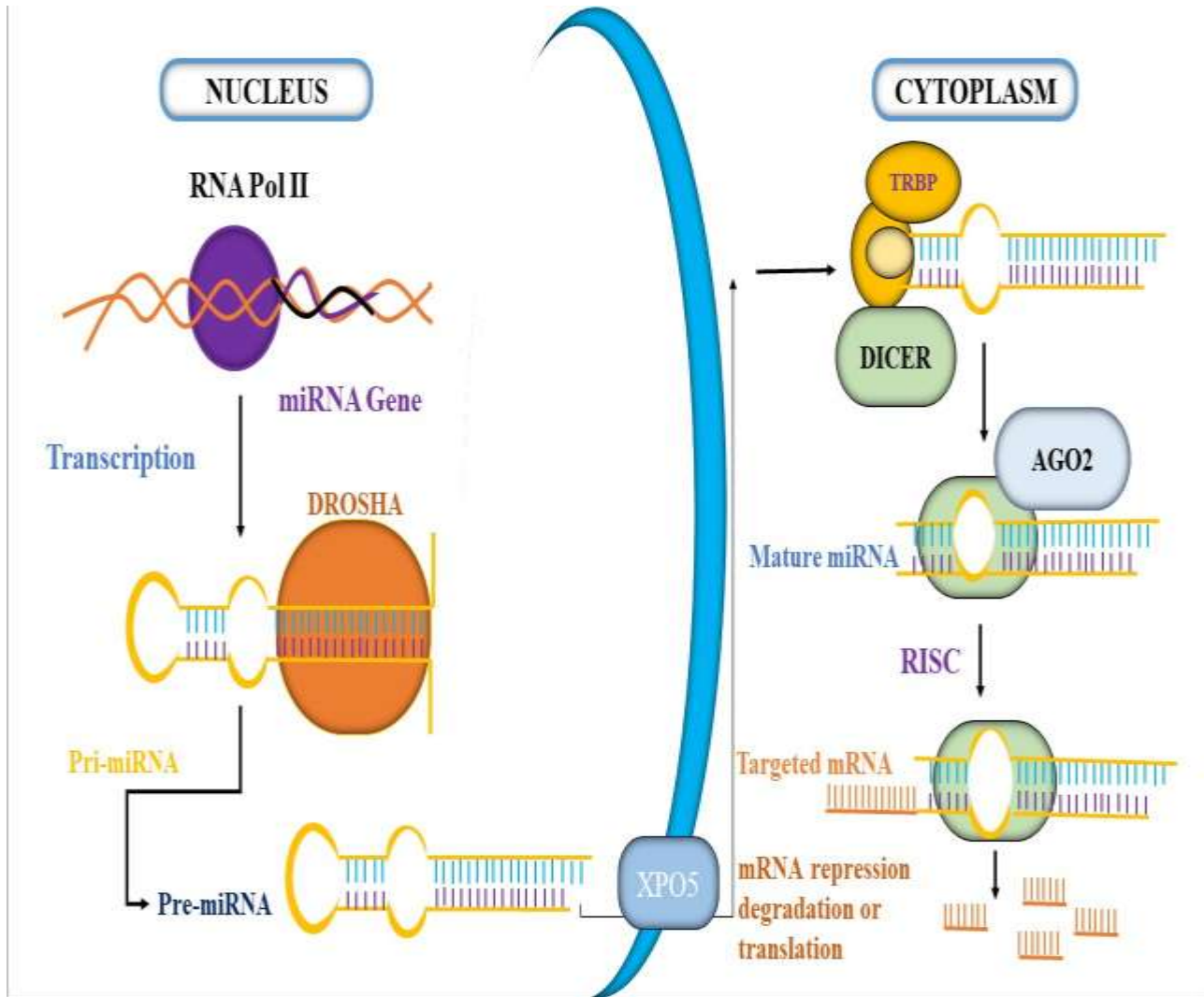


Fig. 2.1 : Gene silencing: By the process of RISC complex formation to mediate transcriptional repression or mRNA degradation of the targeted gene.

Literature review

Table 2.2 : Targets for oncogenic and tumor-suppressive by miRNAs in LC

miRNA	Lung cancer	Targets	Potential mechanisms	Ref.
mir-Let7 family	NSCLC	RAS, Myc, cyclin D, CDK6	- Let 7 is a tumor suppressor miRNA. - Let 7 directly influences several pivotal proto-oncogenes involved in the cell cycle, such as RAS, CDC25a, CDK6, and cyclin D, thereby modulating cellular proliferation by decreasing activity through the pathways that facilitate the transition from G1 to S phase.	(Johnson et al., 2007)
mir-34a	NSCLC	MAPK, c-MYC, E2F, CDK4, CDK6), anti-apoptotic proteins (Bcl2, SIRT1).	- MicroRNA (miRNA)-34a plays a crucial role in regulating tumor suppression. - The miR-34 family significantly influences the effects of p53 by promoting cell cycle arrest and apoptosis, achieved through the targeted inhibition of c-MYC, CDK6, and c-MET.	(Misso et al., 2014)
mir-134	NSCLC	EGFR	- mir-134 functions as a tumor suppressor in NSCLC. - By causing cell cycle arrest and/or apoptosis, miR-134 reduced EGFR-related signaling and decreased the proliferation of NSCLC cells.	(Qin et al., 2016)

Literature review

mir-16-1	NSCLC	p ²⁷ , Bax, procaspase 3, Bcl-2	• It is located at chromosome 13q14. The miR-16-1 has shown that it controls cell growth and apoptosis in vitro thereby acting as a tumor suppressor gene in cancer. • Triggers upregulation of p27 and p21 and causes G1 phase arrest in cells and halts cell division. • Overexpression of miR-16-1 led to an increase in the expression levels of Bax, procaspase 3, and cleaved caspase 3, while simultaneously reducing the expression of Bcl-2. This suggests that miR-16-1 overexpression may induce apoptosis in NSCLC cells through the regulation of these specific proteins.	(W. Wang et al., 2016)
mir-320 a	NSCLC	STAT3	• miR-320a directly modulates the signal transducer and activator of transcription 3 (STAT3) and influences its associated signaling pathways, including Bcl2, Bax, and Caspase 3. • miR-320a substantially inhibited the levels of p-STAT3 within the nucleus of cancer cells treated with miR-320a. This inhibition affects the translocation of p-STAT3 into the nucleus, where it typically regulates	(Lv et al., 2017)

Literature review

			gene expression by binding to promoter elements, ultimately leading to the expression of downstream proteins such as Bcl-2, Bcl-XL, and survivin.	
mir-148 a	NSCLC	Wnt1	• miRNA-148a suppressed LC cell migration and invasion by directly targeting Wnt1. • The overexpression of miRNA-148a effectively suppressed the expression of Wnt1 protein in cancer cells.	(Chen et al., 2017)
mir-125a-5p	NSCLC	STAT3	• miR-125a-5p has been found to limit the growth and invasion of LC cells while promoting their programmed cell death, or apoptosis, by inhibiting the activity of STAT3, a protein that can lead to cancer cell survival and proliferation when overactivated. • The findings indicate that the overexpression of miR-125a-5p effectively inhibited the expression of STAT3.	(Zhong et al., 2017)

Literature review

mir-497	NSCLC	FGFR1	• Overexpression of miR 497 slowed down cell division and migration in the NSCLC cell line A549. • Overexpression of miR497 resulted in decreased A549 cell growth and migration as well as decreased FGFR1 protein and mRNA levels. • The inactivation of the protein kinase B (AKT) and c-Jun N-terminal kinase (JNK) signaling pathways leads to a reduction in matrix metalloproteinase 26 expression, all of which are governed by the fibroblast growth factor receptor 1 (FGFR1).	(Huang et al., 2019)
mir-29	NSCLC	Wnt1, Mcl-1, Bcl-2	• miR-29a functions as a tumor suppressor by inhibiting the signaling pathway associated with Wnt inhibitory factor-1 (WIF-1). • In NSCLC, miR-29 achieves this suppression through the demethylation of WIF-1, thereby modifying its activity and contributing to the regulation of this cancer type.	(Alizadeh et al., 2019)
mir-133a	NSCLC	LASP1 and TGF- β /Smad3	• LASP1 has been identified as a direct target of miR-133a, a microRNA that plays a significant role in cellular processes.	(Shen et al., 2020)

Literature review

			• The overexpression of miR-133a effectively inhibits the pathways related to TGF-β/Smad3 and epithelial-mesenchymal transition (EMT) by regulating LASP1. • Additionally, when miR-133a levels are reduced, there is an increase in the vitality of A549 cells, whereas increasing miR-133a expression results in decreased cell viability.	
mir-29a-3p	NSCLC	Wnt/ β -catenin	• miR-29a-3p has been shown to reduce tumor growth, cell proliferation, migration, and invasion in NSCLC by inhibiting the Wnt/β-catenin signaling pathways. • The overexpression of miR-29a-3p significantly decreased the levels of Wnt3a and β-catenin proteins in NSCLC.	(K. Zhang et al., 2022)

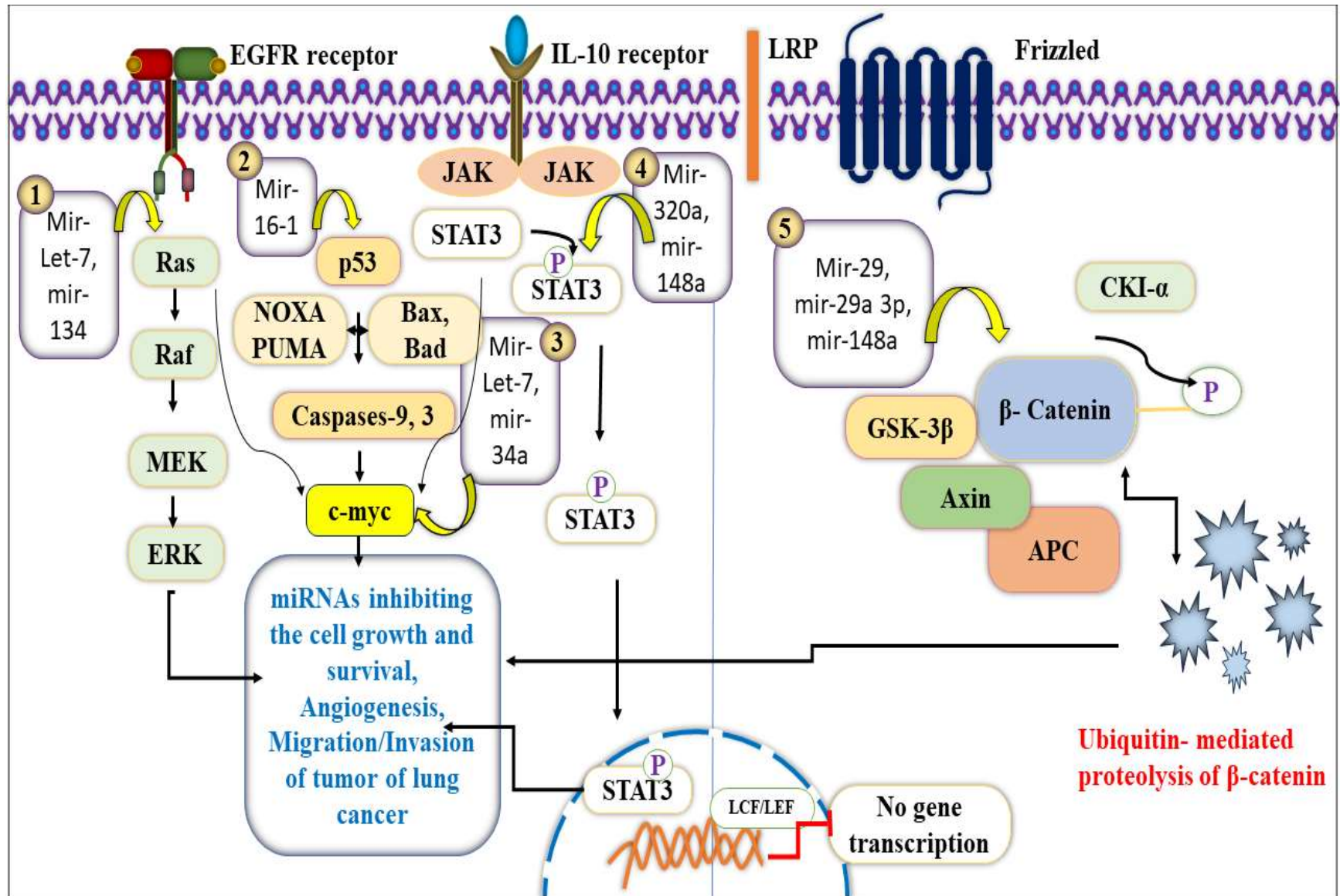


Fig. 2.2 : Tumor suppressing miRNAs targeting various signaling pathways in Lung Cancer.

The above illustration represents the role of various miRNAs targeting various signaling pathways and inhibiting cell growth and survival, angiogenesis, migration of tumor of LC. 1) Mir let-7, mir-134 target and act on the Ras, Raf, MER, ERK signaling pathway of EGFR of LC and inhibit the action c-myc protein for proliferation and migration/invasion of tumor cell. 2) Mir-16-1 acts on tumor suppressing p53 and initiates the caspases activity to inhibit the tumor cell. 3) Mir let-7, mir-34a act directly on c-myc protein and inhibit the action of tumor cells. 4) Mir 320a, mir-148a acts on JAK signaling pathways causes the phosphorylation of STAT-3 protein which result in inhibition of tumor cell. 5) Mir-29, mir-29 3p, mir-148a target and act on Wnt/ β -catenin signaling pathway causes the phosphorylation of β -catenin protein which lead to ubiquitin of β -catenin protein that inhibits the migration and proliferation of tumor cells.

2.2.9. Targeting tumor suppressing miRNA to LC

MiRNA consist of 20-22 nucleotide single stranded non-coding molecules. These molecules are of higher molecular weight, delivering only the miRNA through intranasal route to lungs, would result in poor adsorption and internalization of these miRNAs to the tumor site of LC. So, in the advancement of technology and drugs delivery system nanoparticle formulation (NPs) has been developed in delivery the lipid-based miRNA NPs for an effective delivery of miRNA formulation to target to the tumor site in LC. NPs enhance the distribution of miRNA within tissue and facilitate its precise localization to the tumor site. NPs are ideally circulated until they reach the desired site and provide an effective delivery of miRNA to the targeted site of LC. Therefore, some of the application of aerosols miRNA loaded nanoparticles in management of bronchial inflammation, COPD, and lung cancer have been discussed below.

2.2.10. Aerosolized miRNA Nanoparticle formulation targeting : Bronchial Inflammation, COPD, and Lung Cancer

Vencken et al. 2019 : The study was conducted to administer miR-17 to bronchial epithelial cells (BECs) utilizing nebulized lipid-polymer hybrid nanoparticles (LPNs) as a therapeutic approach for Bronchial Inflammation. The main goal was to lower levels of interleukin (IL)-8, a pro-inflammatory chemokine that is targeted by miR-17's. In their study, they examined how well the nebulized lipid-polymer hybrid nanoparticles (LPNs) can deliver miR-17 to BECs and can suppress the expression of IL-8. MiR-17 mimics were added via a double emulsion solvent evaporation technique to LPNs made of poly(DL-lactic-co-glycolic acid) (PLGA) and the cationic lipid 1,2-dioleoyloxy-3-(trimethylammonium)propane (DOTAP) and then inhaled using an

Aerogen Solo nebulizer. LPNs containing miRNA mimics (miR-17) exhibited a z-average size of 208.0 ± 16.7 nm, with a polydispersity index (PDI) of 0.148 ± 0.030 and a zeta potential of 42.4 ± 8.4 mV. The geometric standard deviation (GSD) and the mass median aerodynamic diameter (MMAD) of the nebulized LPN dispersions were assessed at a flow rate of 15 ± 0.5 L/min. LPNs loaded with miR-17 loading downregulated the LPS-induced IL-8 production in BECs by more than 40% were identified (Vencken et al., 2019).

Mohamed et al. 2019 : The aim of the study was to treat COPD by inhaling nanocomposite microparticles (NCMPs) that contained miR-146a. L-leucine and mannitol were used to create nanocomposite microparticles (NCMPs) containing microRNA (miR-146a) that incorporated PGA-co-PDL nanoparticles (NPs) for dry powder inhalation. miR-146a has the ability to downregulate the IL-1 receptor-associated kinase (IRAK1) and tumor necrosis factor (TNF) receptor associated factor (TRAF6), that are the two key players in the IL-1R and Toll-like receptor (TLR) signaling pathways. Target gene IRAK1 and TRAF6 were silenced by NCMPs loaded with miR-146a. Next Generation Impactor (NGI) was used to evaluate the NCMPs' *in vitro* aerosol dispersion characteristics. The range of mass median aerodynamic diameters (MMAD) was 4.20 ± 0.15 to 6.03 ± 1.08 μm (Mohamed et al., 2019).

Zhang et al. 2021 : The study was performed to examine the effectiveness of aerosolized miRNA Let-7b therapy in preventing LC. The specially designed collision type atomizer was used to atomize the let-7b miRNA mimic into droplets. Aerosolized miR Let-7b was administered to mice. The efficacy of miRNA administered as aerosol to the lungs was compared with its intravenous administration via tail vein. It was examined that the accumulation and distribution of neutral lipid emulsion (NLE) delivered miR-Let-7b in the lung when administered intravenously through the tail vein. When compared to tail vein delivery, miR Let-7b loaded aerosols resulted in delivery of higher amounts of miR Let-7b in the lungs, showing that aerosol delivery promoted more effective deposition of miR Let-7b in lungs. Let-7b therapy inhibited PD-L1 expression in lung tumor cells as well as PD-1 expression in CD8⁺ T cells. Let-7 miRNAs negatively regulated multiple oncogenes including RAS, MYC, and HMGA2, as well as cell-cycle progression regulator genes such as CDC25A, CDK6, and cyclin D2 in the treatment of LC (Q. Zhang et al., 2021a).

Zhang et al. 2023 : In a recent study related to LC. It has been investigated that the combined efficiency of aerosolized miR-138-5p and miR-200c miRNA mimics were helpful in preventing the LC. Thus, these miRNA mimics (16 nmol/ml in NLE) were atomized into a droplet using the

custom-made collision-type atomizer and it has been shown that miR-200c and miR-138 -5p both specifically target the PD-L1 expression. Treatments with miR-138-5p or miR-200c decreased the expression of PD-L1 in LC cells. It is interesting that miR-138-5p and miR200c together completely blocked the production of PD-L1 in lung carcinoma cells (Q. Zhang et al., 2023a).

2.2.11. Clinical studies

To overcome several challenges the nanocarrier (lipoid-based) formulation of miRNA has developed as a potential role in treatment of LC. Clinical research is done on miR-34a, and Let-7, and clinical research has been already completed on such as Let-7b and miR-138-5-p as shown in Table 2.3.

Table 2.3 : Clinical studies

Clinical studies completed	MiR-138-5p	Aerosols containing miR-138-5p neutral lipid emulsion (NLE) are designed to target PD-L1 by interacting with its 3'-untranslated region. This interaction leads to a reduction in PD-L1 expression within lung cancer cells.	(Q. Zhang et al., 2023b)
	MiR-Let-7b	Aerosols containing Let-7b miRNA directly target CD8+ T-cells, leading to a decrease in PD-1 expression, while it also reduce the expression PD-L1 in lung tumor cells.	(Q. Zhang et al., 2021b)
Ongoing clinical trails	MiR- 34a	- miR-34a induces apoptosis, inhibits surviving expression and downregulates mitogen activated protein kinase pathway (MAPK) that plays a key role in during cancer invasion and growth of tumor cells. - miR-34a delivered by the GC4-targeted nanoparticles downregulate the surviving expression in the metastatic tumor and reduce the tumor load in LC.	(Chen et al., 2010)

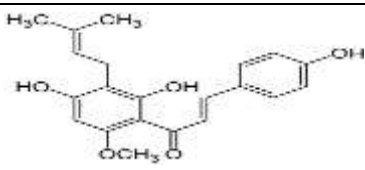
2.3. Xn

Xn is a type of prenylated chalconoid, a chemical compound, found in the female flower clusters of the plant *Humulus lupulus*, commonly known as hops.

2.3.1. Chemical and Biological Properties of Xn

Xn, scientifically known as 1-(2,4-dihydroxy-6-methoxy-3-[3-methylbut-2-en-1-yl] phenyl)-3-(4-hydroxyphenyl) prop-2-en-1-one, typically appears as solid yellow crystals. These crystals have a melting point of 172 °C, indicating the temperature at which they change from solid to liquid form (Pubchem CID 639665) <https://pubchem.ncbi.nlm.nih.gov/compound/Xanthohumol> reported at National Center for Biotechnology Information (2025) as shown in Table 2.4.

Table 2.4 : Physiochemical properties of Xn

Property	Outcome
Description	Yellow needles or yellow powder.
IUPAC name	2',4,4'-Trihydroxy-6'-methoxy-3'- (3- methylbut-2-en-1-yl) chalcone
CAS Number	6754-58-1 (2',4',4'-Trihydroxy-6'-methoxy-3'-prenylchalcone)
Source	It is a prenylated chalcone found in the hop plant (<i>Humulus lupulus</i>).
Molecular formula	C ₂₁ H ₂₂ O ₅
Molecular structure	
Molecular Weight	354.40 g/mol
Melting point	172°C
Solubility	Poor solubility in water (1.3 mg/L at 23 °C)
Log p	3-5
BCS class	Xn belongs to BCS-II
Pka	7.2 – 9.9
λ max	370nm
Uses	As a potential anti-cancer agent, an antioxidant and anti-inflammatory, and a treatment for conditions like acne, diabetes, and aging. Its potential applications are wide-ranging, with research showing effects against various cancer cells, bacteria, fungi, and viruses.

In recent years, significant research efforts have focused on exploring the anticancer capabilities of Xn, aiming to understand and utilize its potential effectively. As shown in literature survey related to Xn is shown in Table 2.5.

Literature review

Table 2.5: Literature survey related to studies conducted on Xn

Xn inhibited	Literature review	Ref.
STAT3 Signaling Pathway	Xn effectively inhibited the STAT3 pathway by obstructing the phosphorylation process of STAT3 proteins and decreasing the activity of kinases like JAK. STAT proteins are typically activated by a variety of extracellular signals, including growth factors, cytokines, and hormones.	(Krajka-Kuźniak et al., 2024)
MMP2, MMP9, FAK, NFκB1/2, Bcl-2	Xn effectively inhibited invasion and disrupted cell cycle progression in cancer cells by targeting MMP2, MMP9, FAK, and P53 genes within three-dimensional breast and lung carcinoma cell cultures. The compound activated P53 and elevated the levels of p21 and BAX proteins, resulting in cell cycle arrest. Additionally, it reduced the expression of NFκB1/2, Bcl-2, and survivin proteins, thereby facilitating cancer cell apoptosis. Cell cycle progression was evaluated through the expression of P21 and P53, as well as PI flow cytometry assays. The treatment with Xn demonstrated IC ₅₀ values of 1.9 μM and 4.74 μM in two-dimensional cultures, and 12.37 μM and 31.17 μM in three-dimensional cultures of MCF-7 and A549 cells.	(Gholizadeh Siahmazgi et al., 2023a)
AKT-FOXO3a signaling	Xn activated p53 and elevated p21 protein levels to induce cell cycle arrest, while concurrently decreasing Bcl-2 and survivin protein expression, thereby facilitating apoptosis in cancer cells. The inhibition of AKT-FOXO3a signaling played a pivotal role in Xn-induced PUMA expression in A549 and H385 cell lines, which were treated with Xn concentrations of 0, 5, 10, and 20 μM over durations of 0, 24, 48, and 72 hours.	(X. Li et al., 2022)
Cholesterol metabolism	The study had evaluated the impact of Xn on the ApoE isoform on cognitive deficits brought on by a high-fat diet. High fat diet consumption linked to metabolic syndrome and cognitive impairments and increased risk for Alzheimer's disease (AD). Sterol and sphingolipid	(Kundu et al., 2022)

Literature review

	metabolism dysfunction occurred in the brain of people with AD. ApoE (apolipoprotein E) participated in cholesterol metabolism. Xn had a beneficial effect on cholesterol metabolism. In ApoE ⁻ mice that ate diets containing Xn (300mg/kg body weight/day for 8 weeks, triglyceride and cholesterol content decreased.	
Transcription factor NF-κB	Xn effectively inhibited the angiogenesis and the activity of the transcription factor NF-κB in pancreatic cancer. Treatment with Xn led to a substantial reduction in NF-κB activation and downregulated the expression of VEGF and IL-8 in Paca-2 and BxPC-3 pancreatic cancer cell lines. These cells were exposed to varying concentrations of Xn, ranging from 0 to 50 μm/L, over a period of 72 hours.	(Hou et al., 2021)
Akt, ERK, NF-κB, and STAT3	Xn inhibited the growth and proliferation of cancer cells by modulating several signaling pathways, including Akt, ERK, NF-κB, and STAT3, as well as influencing various proteins like Notch1, caspases, MMPs, Bcl-2, cyclin D1, oxidative stress markers, tumor suppressor proteins, and miRNAs.	(Girisa et al., 2021b)
Tau protein and β-amyloid	The study aimed to evaluate the inhibitory effects of Xn on tau protein and its aggregation in Alzheimer's disease (AD), a progressive neurodegenerative condition characterized by plaques formed by β-amyloid and intracellular neurofibrillary tangles constituted by tau protein. Xn demonstrated neuroprotective properties by countering oxidative stress-induced neural cell damage and inhibiting the phosphorylation of Akt and NF-κB pathways. The research findings revealed that Xn effectively inhibited tau protein and tau fibril formation in AD. To assess the impact of Xn on apoptosis, N2a cells were treated with 10μM Xn for 24 hours, followed by the replacement of the media with one containing 1.5μM tau k18 oligomer.	(Long et al., 2019; M. Zhang et al., 2019)
Chromatin	The study examines the cytotoxic effects of Xn and its combination with cisplatin on human	(Long et al.,

Literature review

condensation and apoptosis	metastatic lung cancer cells (H1299). H1299 cells were subjected to treatments with Xn at concentrations of 6.25, 12.5, and 25 μ M, cisplatin at 12.5, 25, and 50 μ M, and a combined treatment of both agents for 24 hours. Xn facilitated chromatin condensation and apoptosis and enhanced the efficacy of cisplatin compared to cisplatin alone. Notably, the combination of Xn at 6.25 and 12.5 μ M amplified the cisplatin-induced activation of caspase-3. Furthermore, Xn at 25 μ M significantly elevated the expression of cell cycle regulatory genes p21 and p14.	2019)
Transcription factor NF-kB	In pancreatic cancer, nuclear factor-kB (NF-kB) activation was a key factor for angiogenesis. In vitro and in vivo, the researchers investigated whether Xn inhibited angiogenesis in pancreatic cancer by inhibiting NF-B activity. In tumor tissues, Xn significantly reduced both NF-kB p65 activation and VEGF and IL-8 expression.	(C. H. Jiang et al., 2018)
Angiogenesis and NF-kB	In pancreatic cancer, Xn inhibited angiogenesis and NF-kB. Xn substantially reduced NF-kB activation as well as the expression of VEGF (vascular epidermal growth factor) and IL-8 (interleukin-8) in pancreatic cancer Paca-2 and BxPC-3 cells. The cells were treated for 72 hours with Xn (0-50 μ m/L).	(Saito et al., 2018)
Apoptosis and S phase cell cycle arrest in A549 non-small cell LC cells	Xn triggered apoptosis and S phase cell cycle arrest in A549 non-small cell LC cells. Apoptosis in A549 cells was detected by observing three distinct cellular changes following treatment: first, the externalization of phosphatidylserine on the cell surface; second, changes in the potential of the mitochondrial membrane; and third, DNA fragmentation within the cells. Based on cell cycle analysis, Xn promoted cell accumulation in sub G1 and S phases, as well as enhanced caspase-3, -8, and -9 activity.	(W. Yong et al., 2015)
Mitochondrial electron transport chain complex I	Xn inhibited mitochondrial electron transport chain complex I, causing the production of reactive oxygen species and triggering death. Xn reduced the activity of mitochondrial complex	(B. Zhang et al., 2015b)

Literature review

	I but had no influence on complexes II, III, and IV. The inhibition of complex I by Xn resulted in the overproduction of reactive oxygen species, which were responsible for the triggering of apoptosis in cancer cells.	
DNA topoisomerase I	Xn inhibited the activity of DNA topoisomerase I (topo I), as assessed by the relaxation of supercoiled DNA. The suppression of topo I by Xn explained of the cytotoxicity against human cancer cell lines.	(S. H. Lee et al., 2007)

2.3.3. Approaches to overcome the poor solubility for delivery Xn

There are some physiochemical and biopharmaceutical issues that hinder Xn's absorption, despite its amazing anticancer potential. Among these difficulties are poor solubility in water and poor absorption from biological membranes, such as the GIT. Due to its poor bioavailability and high hydrophobicity, delivery to the target area is challenging, Xn fails to produce an effective therapeutic response to the target site of LC. Xn is challenging to conduct experiments with because of its low solubility in water (1.3 mg/L at 23°C). Therefore, to overcome the challenges Xn loaded NLCs were created to boost bioavailability and give an efficient therapeutic effect against the lung carcinoma cells in order to address these issues (Singh, Saxena, Sharma, Gupta, et al., 2024a).

2.4. Nanostructure lipid carriers

Nanoparticles (NPs), which are colloidal systems, typically measure between 10 to 100 nanometers. Their relatively larger surface area to volume ratio compared to larger particles enables multiple ligands to be attached to them at the same time. This characteristic enhances their ability to carry a higher quantity of drugs and improves their potential to interact with various molecules, such as the receptors on target cells. Novel drug delivery system has nanoscale material that is highly effective with excellent targeting abilities against LC. Solid lipid nanoparticle (SLN's) was discovered in early 1990's by Professor R.H Muller (Germany) as a novel formulation. However, SLN's have some limitations like poor drug loading capacity and drugs leakage during storage. To address the challenges associated with SLNs, scientists developed NLCs. As the second generation of SLNs, NLCs consist of a combination of solid lipids, liquid lipids, and surfactants, enhancing their stability and efficacy. NLCs are made using emulsifiers and a biodegradable as well as suitable lipid (solid and liquid). Literature survey related to NLCs formulation is given in table 2.6.

Literature review

Table 2.6: Literature survey related to NLCs

NLCs	Literature review	Ref.
Gefitinib-loaded NLCs	The study aimed to prepare Gefitinib-loaded NLCs by the ultrasonic melt-emulsification method for the treatment of metastatic LC. GEF-NLCs formulation showed a diameter of <300 nm, PDI <0.2%, and zeta-potential (-20mV with entrapment efficiency >90 % in A549 cell lines.	(Sherif et al., 2023)
Hyaluronic acid modified kaempferol loaded NLCs	Hyaluronic acid modified kaempferol loaded NLCs (HA-KA-NLCs) had been prepared by the methods melting ultrasonic and electrostatic adsorption to determine the preparation's anti-tumor efficacy on A549 cell lines. HA-KA-NLCs had been about 74.3 ± 2.5 nm diameter and zeta potential ($+ 7.8 \pm 0.2$ mV with low polydispersity index (PI) and the entrapment efficiency of HA-KA-loaded NLCs had been about $89.22 \pm 2.33\%$ and the drug loading had been found to be $2.96 \pm 0.56 \%$.	(Ma et al., 2022)
Arginly-glycyl-aspartic acid loaded NLCs	The study aimed to prepare arginly-glycyl-aspartic acid (RGD) by using the hot homogenization method to improve the bioavailability of galangin and enhance the cytotoxicity effect of doxorubicin (DOX). Delivering Galangin by NLCs-RGD made an effective adjuvant to increase the efficacy of chemotherapeutic agents in cancer treatment. It downregulated ABCB1, ABCC1, and ABCC2 genes. The optimum formulation had a mean size of 119.4nm, 0.23 PDI, and a zeta potential of 15mV. The entrapment efficiency of Galangin-loaded NLCs-RGD was $83.1 \pm 4.3\%$, with a drug loading capacity of 59.30 ± 3.0 mg/g.	(Hajipour et al., 2021)
Resveratrol (RES) loaded NLCs	The study aimed to prepare resveratrol (RES) loaded NLCs hydrogel by using the ultrasonic emulsification method to enhance anti UV-irradiation and antioxidant efficacy. UV-irradiation was a major cause of skin damage and cancer by generation of free radicals and ROS. The average particle size was 175.6 ± 11.2 nm, PDI (0.179 ± 0.007) and zeta potential	(Miao et al., 2021)

Literature review

	(-54.3 ± 3.2 mV, respectively.	
Folate based modified – cisplatin-loaded NLCs	The study aimed to prepare Folate based modified – cisplatin- loaded NLCs for the treatment of cervical cancer. FA-CIS-NLCs had been prepared by emulsification methods and in vitro cytotoxicity of FA-CIS-NLCs had been tested in human cervix adenocarcinoma cell lines (Hela cells). FA-CIS-NLCs had had particle size 143.2 nm and a zeta potential of (+ 25 ± 2.3 mV) , with an entrapment efficiency of 87.5 ± 3.2 % and drug loading capacity 9.3 ± 0.6%.	(G. Zhang et al., 2016)
Paclitaxel (PTX) and Doxorubicin (DOX) loaded NLCs	The study aimed to prepare NLCs co-delivery of drug paclitaxel (PTX) and doxorubicin (DOX) for synergistic effect on LC. PTX- and DOX-loaded NLCs had been prepared by melted Ultrasonic dispersion method. The diameter had been around 120-130 nm of PTX-DOX loaded NLCs with (+ 27 mV) zeta potential to ensure stability with an entrapment efficiency of 82-84% and the drug loading capacity 13- 10 % respectively in NCL-H460 cell lines.	(Y. Wang et al., 2016)
PEGylated loaded with 10-hydroxycamptothecin NLCs	The study aimed to prepare PEGylated NLCs using a melt-emulsification and homogenization process and then load them with 10-hydroxycamptothecin (HCPT), which has an anti-tumor effect on LC. HCPT-loaded PEG-NLCs and NLCs had a diameter of about 100 nm and a low polydispersity index (PI) formulation (<0.3). The entrapment effectiveness of HCPT in PEG-NLCs and NLCs was more than 80%.	(X. Zhang et al., 2010)

2.4.1 Fabrication of NLCs

NLCs consist of a mixture of solid lipids, liquid lipids, emulsifiers, and water. The formulation of NLCs involves several methods, including emulsification combined with sonification, high-pressure homogenization, micro-emulsion techniques, and solvent diffusion methods. These techniques help create NLCs with desirable properties for various applications (Gomaa et al., 2022). NLCs provide several advantages over conventional carriers in drug therapy, including increased solubility, the capacity to improve drug storage stability, enhanced permeability and bioavailability, a longer half-life, and tissue target delivery. NLCs play a significant role in determining the particle size, the efficiency of entrapment, and the drug release profile, which are essential for achieving the desired characteristics. Fabrication of NLCs by herbal drug, synthetic drug and by protein coated molecules are given in Table 2.7.

2.4.2. Methods for Preparing NLCs

The high-pressure homogenization method is the most frequently employed technique, involving the application of both high temperature and high pressure. Another is the low-temperature, high-pressure homogenization method other approaches include high-pressure homogenization, ultrasonic emulsion evaporation, solvent dispersion, the film-ultrasonic method, high-temperature emulsion evaporation–low temperature curing, microemulsion, and melt emulsification methods.

2.4.2.1. High-Pressure Homogenization Method

Methods for preparing NLCs are well-developed and suitable for mass production, offering dispersing techniques that avoid the use of organic solvents. These preparation methods can be categorized into two main homogenization protocols: one that operates at high temperature and high pressure, and another at low temperature and high pressure. The high-temperature, high-pressure homogenization method is the more commonly adopted and involves the melting of solid lipid materials first before mixing them with liquid lipid and drugs. After mixing, the molten liquid disperses throughout the surfactant-containing aqueous phase. The mixture is stirred to form the beginning of an emulsion. Fluid droplets are then broken down into nanoparticles via high-speed homogenization, extremely high shear strain. Generally, high temperatures reduce the viscosity of the mixed liquid, decreasing the particle size but increasing the probability of degrading the drug and the carrier. This method can be

successfully used for insoluble drugs and lipophilic ones but is not entirely suitable for hydrophilic drugs. The advantages include the absence of organic solvents and large-scale production. The NLCs particle size is also correlated with the homogenization pressure and time intervals (Al-Kattan et al., 2010). When the high-pressure homogenization method is used to prepare NLCs (Al-Kattan et al., 2010; Piao et al., 2011), the encapsulation efficiency of coenzyme Q₁₀ NLCs is the highest. El-Salamouni et al. prepared NLCs using a modified high-shear homogenization technique with GMS as the solid lipid, castor oil as the liquid lipid, and Poloxamer 188 as the surfactant. In this procedure, autoclaving at 121 °C for 15 min increased the surface potential and encapsulation efficiency of the drug, thereby generating stable formulations and nanoparticle sizes under 500 nm (El-Salamouni et al., 2015). In order to develop and analyze three lipid nanoparticles—SLN, NLCs, and a quercetin nano emulsion—Aditya et al. prepared NLCs by high-speed homogenization method using Imwitor 900 K and soybean phosphatidylcholine as the solid lipid, medium-chain triglyceride (MCT) as the liquid lipid, and Tween 80 and Span 20 as surfactants. The size of the NLCs was measured as 34 ± 6 nm, and the encapsulation efficiency of quercetin was 91% (Aditya et al., 2014a). The three distinct lipid nanoparticles in this study were prepared using the same technique, but the lipids' composition and physical state were different. These studies demonstrated that various lipid compositions and physical states can be utilized to create different lipid nanoparticles.

2.4.2.2. Ultrasonic Emulsion Evaporation Method

In this method, the solid lipid, the liquid lipid, and the drug mixture as oil phase are added and dispersed in an aqueous surfactant solution by probe ultrasonication. The sample was then cooled down and solidified to form NLCs. When a stable emulsion is formed, the oil phase is evaporated by heating under reduced pressure, or by evaporation while stirring continuously. Avoidance of heat during the preparation is the most important advantage of this method. Toxicological problems may result from solvent residues from the product obtained by this method.

Ranpise et al. employed GMS as the solid lipid, labrafil and linseed oil as the liquid lipids and lutrol[®] F68 and polysorbate 80 as the surfactants to entrap lercanidipine hydrochloride (Ranpise et al., 2014). However, Uprit et al. prepared NLCs by ultrasonic emulsion evaporation method using solid lipid (soy lecithin) and liquid lipids (oleic acid (OA) and tristearin). Their

findings indicated that the NLCs achieved a high entrapment efficiency for minoxidil, reaching 86.09%, and resulted in particles measured at a size of 280 nm (Uprit et al., 2013).

Shao et al. prepared NLCs by microemulsion using soya, lecithin GMS as the solid lipid, oleic acid as the liquid lipid, and Tween-80 as the surfactant to entrap paclitaxel. The particle size obtained was 79 nm, and the encapsulation efficiency was $87.1 \pm 2.1\%$ (Z. Shao et al., 2015).

2.4.2.3. Solvent Dispersion

In this type of procedure, the solid lipid, liquid lipid, and drug are combined in a are dissolved in an appropriate organic solvent. Then, the organic solution is slowly mixed into the water that contains the emulsifier, resulting in the formation of NLCs. The solvent dispersion method has several advantages, including speed, simplicity, and the low requirements of the instrument. Solvent residues can cause toxicological issues, which is one of the method's limitations. Additionally, NLCs are developed using the solvent dispersion technique (Gu et al., 2011; X. Y. Zhang et al., 2013). Emami et al. utilized the solvent dispersion method to formulate NLCs incorporating solid lipid (cholesterol) and liquid lipid (oleic acid), alongside surfactants poloxamer 188 and polysorbate 80, for the encapsulation of paclitaxel. The resulting entrapment efficiency of paclitaxel within the NLCs was measured at $72 \pm 11.6\%$ (Emami et al., 2012).

2.4.2.4. Film-Ultrasonic Method

In the film-ultrasonic method, the solid lipids, liquid lipids, and drugs are dissolved in an appropriate organic solvent, which is later removed by vacuum evaporation. A layer of mixed lipid films is created by adding an aqueous solution containing a surfactant. Next, an ultrasound probe for ultrasonic dispersion is used to create small, homogeneous NLCs. This method is most often used due to its simplicity and practicality, and its yield of small, uniform particles. However, solvent residues from the product produced using this procedure could lead to toxicological issues.

In the study of Ajiboye et al. utilized the flim-ultrasonic method to formulate NLCs incorporating solid lipid (Compritol), liquid lipid (Miglyol 812), and surfactant (Poloxamer 188 (P188) and tween 80, to entrap olanzapine. The Olanzapine NLCs showed a particle size measuring 118 nm, while the zeta potential varied between (-23 and -11 mV) (Ajiboye et al., 2021).

2.4.2.5. High-Temperature Emulsion Evaporation—Low-Temperature Curing

This technique involves separately heating both the organic and aqueous phases to the same temperature. Once both phases reach the desired temperature, the organic phase is combined with the aqueous phase, which contains an emulsifier, to create an emulsion. Heating evaporates the volatile organic solvent, resulting in a concentrated liquid that is quickly dispersed in ice water (0–4°C). Thus, a dispersion solution of NLCs is produced. This method has the advantage of being quick and easy to do. The method poses significant limitations, primarily its inadequacy for industrial-scale production and the presence of residual organic solvents, which are concerns that must be addressed.

Yanqiu Li et al. developed celecoxib NLCs by using high-temperature emulsion evaporation followed by low-temperature curing method. NLCs were composed of solid lipid (cetyl alcohol palmitate), liquid lipid (Miglyol 812), and surfactants (soybean phospholipid and Solutol HS15). The resulting NLCs demonstrated a particles size of 103.5 ± 32.6 nm (Patlolla et al., 2010).

Luan et al. prepared NLCs by using solid lipids (polyethylene glycol stearate (PEG-SA) and GMS), liquid lipid (caprylic/capric triglyceride (CCT)) and surfactants (F68 and soybean lecithin) for the encapsulation of amoitone B. The resulting NLCs demonstrated a particle size of 2.257 ± 1.36 nm and achieved an encapsulation efficiency of $68.17 \pm 0.94\%$ (Luan et al., 2014).

2.4.2.6. Microemulsion Method

Using a microemulsion approach, the lipid carrier is heated and melted, and then drugs, emulsifier, auxiliary emulsifier, and deionized water are added to yield a mixture with a transparent appearance and a thermodynamic stability similar to that of oil-in-water (O/W)-type microemulsion. The microemulsion is quickly dispersed in ice water (0–4°C), forming an NLCs dispersion system. The size of nanoparticles and particles derived from microemulsion, and dilution is closely related to the temperature difference between the cold water and the microemulsion, which is pivotal in the preparation of small-sized nanoparticle lipid carriers (NLCs). Rapid cooling and solidification are essential to prevent the aggregation of certain particles. The advantages of this method include its low drug content and simplicity, while the disadvantages are the abundance of auxiliary emulsifier and emulsifier required.

Shao et al. prepared NLCs by microemulsion using soya, lecithin GMS as the solid lipid,

oleic acid as the liquid lipid, and Tween-80 as the surfactant to entrap paclitaxel. The particle size obtained was 79 nm, and the encapsulation efficiency was $87.1 \pm 2.1\%$ (Z. Shao et al., 2015).

2.4.2.7. Melt Emulsification Method

In this protocol, solid and liquid lipids are combined through heating and mixing, followed by the addition of drugs to create an organic phase. This organic phase is then incorporated into a water phase that contains surfactant and is stirred to produce a coarse emulsion. The coarse emulsion is subjected to high-pressure homogenization to yield NLCs. This method is beneficial as it eliminates residue of organic solvents, prevents any initial burst release, and results in dispersions with a high lipid concentration. The disadvantages of this method are that it is not entirely suitable for industrial production and there is residual organic solvent. Zhiqiang Tian et al. formulated NLCs through a melt emulsification technique, utilizing solid lipid (Precirol ATO 5) and liquid lipid (Captex 100) for fenofibrate encapsulation. The resulting NLCs achieved a particle size of 227.5 nm and demonstrated an encapsulation efficiency of 8.5% (Tian et al., 2013).

Table 2.7 : Fabrication of NLCs

Fabrication of NLCs loaded with Herbal drug							
Drug	Treatment of Diseases	Solid lipid	Liquid lipid	Surfactant	Size (nm)	Research outcomes	References
Baicalin	Pneumonia, Viral Hepatitis, Cancer	GMS	MCT	Soya lecithin	244.7	The study revealed that using drug-loaded NLCs significantly improved the pharmacokinetic profile of the drug, with a 1.9-fold increase in the Area Under the Curve (AUC) and a 1.7-fold rise in mean residence time, compared to the drug in suspension form. This enhancement indicates a more efficient absorption and prolonged presence of the drug in the body when delivered via NLCs.	(Luan et al., 2015)
Biochanin A	Cancer	GMS	MCT	Soya lecithin, Tween-80	148±2.88	Biochanin loaded PEGylated nanostructured lipid carriers demonstrated increased cytotoxicity in vitro against the MCF-7 cell line, surpassing the effectiveness of the drug when administered in suspension form.	(Q. Wang et al., 2013)

Literature review

Etoposide	Lung carcinoma	GMS	Soyabean oil	Soya lecithin	91.2-125.9	The drug-loaded NLCs exhibit enhanced cytotoxic effects against human epithelial lung carcinoma cells.	(T. Zhang et al., 2011)
Luteolin	Cancer & hypertension	Precirol TM ATO 5	Labrasol®	Cremophor	43.89±4.5	The encapsulation of luteolin in NLCs enhanced its oral bioavailability.	(Y. Liu et al., 2014)
Quercetin	Protect against Heart disease & Cancer	Imwitor900 k	MCT	Tween-80, Span-20	34-47	The highest accessibility was observed with NLCs and lipid nano-emulsion, as opposed to SLNs and the drug solution.	(Aditya et al., 2014b)
Lutein	Macular disease	Precirol TM ATO 5	Corn oil	Fluronic F68	130	Encapsulation of lutein in NLCs led to enhanced aqueous solubility, and a sustained release of lutein was successfully attained.	(Lacatusu et al., 2013)
Curcumin	Anti-bacterial, anti-cancer, anti- microbial	Precirol TM	Labrafilm TM	Poloxamer188	66.8 ± 2	The enhanced NLCs exhibited stability in ex-vivo conditions at 4°C and significantly improved curcumin permeation across the rabbit cornea, achieving a 2.5-fold increase compared to the control.	(Lakhani et al., 2018)
Fabrication of NLCs loaded with Synthetic drug							
Drug	Treatment of	Solid lipid	Liquid lipid	Surfactant	Size (nm)	Research outcomes	References

Literature review

	Diseases						
Docetaxel oleate	Cancer	GMS	Oleic acid	Poloxamer F68	100	Docetaxel oleate was efficiently incorporated into nanostructured lipid carriers, achieving notably higher loading efficiency. In comparison to the drug suspension, it demonstrated a 2.06-fold increase in effectiveness.	(B. Sun et al., 2016)
Nystatin	Candida albicans	Campritrol 888 ATO	Miglyol®812 or Squalene	Poloxamer188	68-141.8	Nyst-NLCs formula tested for anti-fungal activity showed better results compared to drug solutions.	(Khalil et al., n.d.)
CoQ10	Anti-aging	Stearic acid	Oleic Acid	Tween-80, Span-80	184- 356	CoQ10 NLCs formulation tested for anti-aging showed better results as compared to drug suspension.	(Aryani et al., 2021)
Raloxifene Hydrochloride	Osteoporosis	GMS	Campul MCM C8	Polyvinyl alcohol	32.50±5.12	The bioavailability of the poorly soluble drug has improved significantly, showing a 3.75-fold increase compared to the plain drug suspension.	(Shah et al., 2016)

Literature review

Miconazole	Fungal diseases	Gelucine®	Miglyol®812	Tween-80	200	The drug-loaded NLCs demonstrated comparable therapeutic efficacy to the marketed formulation, despite having a 17-fold lower drug dose.	(Mendes et al., 2013)
Fabrication of NLCs loaded with Protein coated molecule							
Drug	Treatment of Diseases	Solid lipid	Liquid lipid	Surfactant	Size (nm)	Research outcomes	References
NLCs-piperine-BSA	Cancer	GMS	Thistle oil	Tween -20 Phosphatidylcholine	150	NLCs-pip-BSA exhibited an elevenfold increase in apoptosis across various cell lines, including colon (LoVo), breast (MCF-7), ovarian (SKOV-3), and adenocarcinoma, highlighting significant effects on these cancer cell types.	(Tincu et al., 2023)
Fibronectin coated gold-curcumin-NLCs	Stomach cancer	Glyceryl tristearate	Soyabean oil	Tween-80, Span-80	11-50	The fibronectin modified nanoparticle-ITO composite exhibited signal enhancements of four times over the bare electrode devoid of extracellular matrix (ECM), and 1.4 times greater than the composite coated with collagen.	(Angeline et al., 2020)

Literature review

Pectin coated Piperine-NLCs	Hepatocellular carcinoma	Campritol 888 ATO (glyceryl dibehenate)	Caproyl 90® Propylene glycol	Pluronic F-127	150.28±2.51	Pectin-coated piperine NLCs demonstrated a substantially significant reduction in wnt3a expression (p<0.001) compared to the free pectin group (p<0.05).	(Shehata et al., 2022)
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GMS: Glyceryl monostearate; **MCT**: Medium chain triglyceride

2.4.3. Excipients used in formulating NLCs

Generally, NLCs are composed of lipids of (solid as well as liquid), surfactants and some organic solvent. The excipients used for formulating NLCs are shown in Table 2.8.

Table 2.8 : Excipients used in formulating NLCs

Ingredient	Examples
Solid lipid	Triglycerides: Tristearin ; Fatty acids: Stearic acid, Palmitic acid ; Waxes: Beeswax, Carnauba wax 2442, Cetyl palmitate ; Sterols: Cholesterol; Specific commercial products: Precirol® ATO 5, Compritol® 888 ATO, Dynasan® 11, Dynasan® 118, Softisan® 154, Cutina® CP, Imwitor® 900 P, Geleol®, Gelot® 64
Liquid lipids (oils)	Sunflower oil, Olive oil, Glyceryl monooleate, Linoleic acid, Castor oil, Oleic acid, Medium chain triglycerides α -Tocopheryl acetate, Labrafac-PG soluble, Lauro glycol-90, Lauro glycol-FCC, Caproyl PGMC, Palm oil, Olive oil, Paraffin oil, and Coconut oil.
Emulsifying agents (Surfactants)	Hydrophilic Surfactants (Non-ionic are most common) : Poloxamers (e.g., Pluronic F68, Poloxamer 188, Poloxamer 407); Polysorbates (Tweens, e.g., Tween 20, Tween 40, Tween 60, Tween 80); Solutol

Literature review

	HS15; Polyvinyl alcohol; Sodium deoxycholate; Sodium glycocholate; Sodium oleate; PEG-ester and PEG-ether surfactants (e.g., Kolliphor RH 40, Brij L23); PG-ester surfactants (e.g., Polyglyceryl-4 Caprate, Polyglyceryl-6 Caprylate). Lipophilic Surfactants :Spans (sorbitan esters, e.g., Span 20, Span 40, Span 60, Span 80) ; Myverol 18-04K. Amphiphilic Surfactants (Lecithins); Egg lecithin; Soya lecithin; Phosphatidylcholines; Phosphatidylethanolamines; Phospholipon 90G .
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2.4.4. Characterization of NLCs

2.4.4.1. Particle size measurement and PDI

The particle size of NLCs is often measured using photon correlation spectroscopy (PCS) with a Zetasizer, which applies Mie theory. PCS, also referred to as dynamic light scattering or quasi-elastic light scattering, works by analyzing fluctuations in scattered light caused by the random motion of particles, known as Brownian motion. This method provides data on the average particle size, known as the z-average, and assesses the polydispersity, which indicates the range of particle size distribution within the system. The assessment is based on the diffraction pattern that reveals the size and shape of particles, as explained by Fraunhofer theory. By utilizing a combination of laser diffraction and polarization intensity differential scattering technology, particle sizes can be measured as small as 10 nanometers. A high span number denotes a high degree of polydispersity and a broad size distribution. Theoretically, monodisperse populations indicate $PI = 0$ (Kim et al., 2019).

2.4.4.2. Zeta potential

The zeta potential (ZP) is an important component in determining the stability of nanoparticles. The electrophoretic mobility of particles in an aqueous media serves as the principles for the ZP measurement. Zeta Potential refers to the electrical charge present on the surface of particles and gives insight into their long-term stability. A higher Zeta Potential value indicates stronger electrostatic repulsion, which minimizes the likelihood of particles clumping together, thereby enhancing stability. In contrast, lower Zeta Potential values result in weaker repulsion, which can lead to particles grouping or flocculating, potentially diminishing their stability (Kim et al., 2019). To achieve effective electrostatic stabilization of NLCs, the zeta potential of the dispersion must be maintained at values less than -30 mV or greater than +30 mV. To determine the zeta potential, a Malvern ZetaSizer Nano ZS, employing the technique of laser Doppler electrophoresis, can be utilized (Kim et al., 2019). When an electric field is applied to a sample, particles possessing a zeta potential will move toward an electrode of opposite charge. The speed at which these particles migrate is directly linked to the strength of zeta potential. This movement speed can be quantified through a technique called Laser Doppler velocimetry, also referred to as Laser Doppler anemometry. The composition of the reagents, pH, and electrical conductivity are some of the variables that affect zeta potential.

2.4.4.3. Entrapment efficiency

The influence on release characteristics plays an important role in determining the amount of drug loaded in NLCs. Entrapment efficiency (% EE) is a measure that calculates the proportion of a drug successfully encapsulated within a NLC relative to the total amount initially incorporated into the dispersion. To determine this, the ultrafiltration-centrifugation method is employed, allowing researchers to precisely quantify the drug encapsulated per unit weight of the NLCs. To analyze the concentration of the free drug in a prepared NLC dispersion, the mixture is first placed in a centrifuge tube with an ultrafilter and subjected to centrifugation. This process helps separate different components based on their density. Once this is completed, the diluted supernatant is tested using HPLC to precisely measure the amount of free drug present (Kim et al., 2019).

The % EE in the NLCs is calculated by the following equation.

$$\%EE = [(Q_i - Q_r) / Q_i] \times 100 \dots\dots\dots(1)$$

where Q_i = amount of the drug used for the preparation of formulation; Q_r = amount of the drug present in the supernatants.

2.4.4.4. Drug release studies and release kinetics models

Drug release through a membrane occurs primarily via passive diffusion, driven by a concentration gradient from high to low, where the drug moves across a barrier like a cell membrane. Key factors influencing this process include the drug's lipid solubility, size, and charge (which determines its un-ionized form's ability to cross the membrane), as well as the properties of the membrane itself, such as its thickness and pore size. Key factors influencing this process include the drug's lipid solubility, size, and charge (which determines its un-ionized form's ability to cross the membrane), as well as the properties of the membrane itself, such as its thickness and pore size. Different kinetic models are used to investigate the in-vitro release pattern of NLCs. Release pattern of NLCs are analyzed using statistical and mathematical methods with different types of kinetic models such as: Zero order kinetic model, First order kinetic model, Korsmeyer-Peppas model, Hixson-Crowell model and Higuchi model. The highest correlation coefficient (r) value represents the actual mode of release vitro (Duong et al., 2019). The Korsmeyer-Peppas equation, sometimes referred to as the power law, is used to analyze the mechanism of drug release from nanostructured lipid carriers (NLCs). By fitting the experimental drug release data to this model, researchers can determine a diffusional release exponent (n) that indicates the drug release mechanism, such as “Fickian diffusion ($n \approx 0.45$)” or “non-Fickian transport ($0.45 < n < 0.89$)” (Ahmed et al., 2019;

Duong et al., 2019; Gorle et al., 2023).

2.4.4.5. NLCs morphology

The surface morphology of NLCs can be examined using atomic force microscopy (AFM) and transmission and scanning electron microscopy (TEM, SEM). These methods are tested and true for characterizing the dimensions and structural properties of NLCs. SEM analyses are carried out by positioning the freeze-dried or tray-drying of NLCs on a metal specific holder and gold coating was further applied preceding the microscopical analysis under different magnifications (Varela-Fernández et al., 2022a). Different sample preparation techniques for TEM, such as negative staining, freeze-fracture, and vitrification by plunge freezing, can provide distinct insights into colloidal particles. For enhanced contrast during electron microscopy, the sample is positioned on a grid made from gold or copper, which has a designated mesh size, and treated with a solution containing heavy metal salts. After the material has fully dried, it is subjected to analysis through an electron microscope. Within this microscopic examination, the nanoparticles are distinctly visible as bright elements contrasted against the darker backdrop of the stain (Varela-Fernández et al., 2022a).

DSC is used for determination of crystallinity/amorphousness of the NLCs (Gill et al., 2010). The thermograms appearing in DSC are measured based on thermal properties of materials. A relation between temperature and specific physical property of substances such as melting point and enthalpy change associated with the process can be determined using DSC. A sharp crystalline peak with specific melting point is achieved in case some part of drug remains undissolved in the mixture of NLCs. In contrast to this absence of any sharp peak indicates complete solubility of drug is completely soluble in the NLCs that was adsorbed on the surface of the carrier.

PXRD is a powerful and non-destructive technique used to characterize NLCs. It utilizes the principle of Bragg's law. The PXRD pattern is unique for every compound, and it is considered as fingerprint of periodic atomic arrangements for that compound (Arczewska et al., 2013a). It shows highly crystalline peaks, if the drug is not completely solubilized in the mixture of NLCs. Whereas, if the drug is completely soluble in the mixture of NLCs and that isotropic mixture is completely adsorbed on the surface of the carrier then the crystalline peaks of drug will be absent indicating the amorphous nature (Patel et al., 2017).

2.5. Anti-cancer activity assays on A549 cells

2.5.1. Trypan blue assays

A trypan blue assay is a method used to determine viability by selectively staining non-viable cells with blue dye, while viable cells remain unstained. To assess the viability of A549 cells, 1×10^5 cells per well were seeded in a 96-well plate. After then, the cells were treated various concentrations, over a 24-hour period. The cells were detached using a trypsin-EDTA solution and centrifuged at 1200 g for 4 minutes. Afterward, the cells were combined with a 0.4% trypan blue solution in equal parts to distinguish live cells from dead ones. Using a light microscope at $10\times$ magnification, the live cells were counted, and the total number of cells was determined.

2.5.2. MTT assay for cell viability

MTT, “chemically known as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide or Thiazolyl Blue, is a tetrazolium salt that is soluble in water”. Cellular dehydrogenase enzymes catalyze the cleavage of the MTT tetrazolium ring, leading to the formation of an insoluble purple formazan. This formazan precipitate is subsequently dissolved using solvents like isopropanol, and its concentration is quantified spectrophotometrically by assessing optical density.

An MTT assay was used to investigate Calycosin's (Cal) inhibitory effect on A549 cells' viability. During the logarithmic growth phase, A549 cells were detached by using 0.1% trypsin. Further, the A549 cells (0.6×10^4 cells/well) were seeded in a 96-well plate with DMEM containing 10% FBS.

The cells were treated with different concentrations of Cal (10-90 μ M) and incubated at 37°C for a duration of 24 h. After the incubation period, 10 μ l of MTT solution (5 mg/ml) was added to each well and incubated for an additional 4 hours at 37°C. Subsequently, the supernatant from each well was removed, and 100 μ l of dimethyl sulfoxide (DMSO) was added to each well. The optical density (OD) at 570 nm was measured with a Spectra Max 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The OD value was used to quantify cell viability, which was then expressed as a percentage of viable cells (Cheng et al., 2015).

A549 cells (3000 cells each) were seeded in a 96-well plate, and the effect of mitomycin C on cell viability was assessed by MTT assay. After treatment, take off the supernatant and add 270 μ l of new DMEM medium and 20 μ l of MTT. After 4 hours of incubation, the tetrazolium crystals were dissolved with 120 μ l of DMSO, and the absorbance at 570 nm was measured using a multi-well plate reader (Tecan, Maennedorf, Switzerland) (An et al., 2015).

A549 cells (1×10^4 cells/well) were seeded in a 96-well plate, and the effects of gallic acid (GA) on cell viability was assessed by MTT assay. The cells were treated with different concentrations of GA (100-400 μ M) for 24 and 48 hours. After the treatment 4 hours before the incubation period ended, 10 μ l of MTT (10 mg/ml) was added to each well. After incubation, 100 μ l of solubilization buffer (10% SDS with 0.01 N HCl) were added to each well and read the color at 550 nm using a Bio-Tek microplate reader (Maurya et al., 2011) .

2.5.3. Rhodamine 123 Assay

Changes in mitochondrial membrane potential ($\Delta\Psi_m$) (MMP) can be used to analyze mitochondrial function, which is an important sign of a healthy cell. To evaluate these changes in mitochondrial membrane potential ($\Delta\Psi_m$) within A549 cells following treatment, rhodamine 123 retention was utilized as a measurement tool. This cationic dye binds to metabolically active mitochondria.

The retention of rhodamine 123 was used to detect alterations in mitochondrial membrane potential in A549 cells (after treatment with *Nepeta paulsenii* Briq. extract). A549 cells were incubated overnight in 6-well plates. Cells were treated with extracts at 25 μ g/mL for 6 and 24 hours before being fixed in 4% paraformaldehyde for 20 minutes. Untreated cells were thought to be negative (-) controls. To stain mitochondria, cells were treated with 5 mgmL⁻¹ of Rhodamine 123 dye and incubated for 30 minutes. To detect fluorescent signals, the wells were imaged using an EVOS fluorescent digital microscope at 20 x magnification. The study reported that the untreated cells exhibited a high fluorescence intensity, indicating that there were a lot of cells that were alive in the group. However, the fluorescence signal reduced significantly in the cells treated with the extracts, indicating a loss of mitochondrial membrane potential in the treated cells (Szliszka et al., 2009c) .

To examine MMP, A549 cells were stained with rhodamine 123. Rhodamine 123 is released from the mitochondria into the cytosol to increase intracellular fluorescence when MMP is lost. In healthy cells, rhodamine 123 clumps and accumulates in mitochondria. After 48 hours of Aloin treatment (0 to 400 μ M) and incubation, 5×10^5 A549 cells were collected and resuspended in 200 μ l PBS with 10 μ g/ μ l rhodamine 123 at 37°C for 30 minutes in the dark. After labeling, the A549 cells were rinsed three times with PBS before being examined by flow cytometry with ModFit software v2.0.0 (Verity Software House, Inc.). The level of mitochondrial depolarization was determined using the percentage of cells that fluoresced with rhodamine 123. The mitochondrial

membrane potential (MMP) and the levels of released mitochondrial calcium ions (Ca^{2+}) were analyzed through flow cytometry. Compared with the control group, which received no treatment (0 μM), the administration of Aloin at concentrations of 200, 300, and 400 μM led to a notable disruption in the MMP and an elevated release of mitochondrial Ca^{2+} , as evidenced by the increased percentage of rhodamine 123 (Wan et al., 2017).

2.5.4. DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) staining - ROS detection

Reactive oxygen species, naturally generated during cellular metabolism, serve a vital anti-tumor function by enhancing various signaling pathways linked to processes such as cell apoptosis, necrosis, and autophagic cell death. Elevated ROS levels can initiate signaling pathways mediated by ROS, leading to modifications in the levels of proteins associated with the cell cycle. The hydrolysis of the DCFH-DA probe by esterase results in the formation of DCFH within the cell membrane, where intracellular reactive oxygen species convert the non-fluorescent DCFH into the fluorescent compound DCF. Consequently, the intensity of DCF fluorescence provides a measure of ROS levels within the cell.

A549 cells were treated with 30 μM 10-Hydroxy-2-decenoic acid (10-HDA) for 3, 6, 12, and 24 hours. Half an hour before starting 10-HDA treatment, the ROS inhibitor NAC was introduced. The cells were centrifuged for 5 minutes at 5,000 rpm and washed twice with PBS. After treating cells with DCFH-DA for 30 minutes at 37°C, they were twice washed with PBS. To measure intracellular ROS levels, the cell suspension was moved to a flow cytometer. The study on 10-HDA treatment demonstrated notable effects on intracellular reactive oxygen species (ROS) levels in different human cell lines. In human LC cells, ROS levels significantly rose from 40.94% to 70.16% over time, indicating an increase in oxidative stress. Conversely, in IMR90 human normal lung cancer cells, ROS levels significantly reduced from 59.08% to 34.39% as time progressed, suggesting a decrease in oxidative stress in these cells (Lin et al., 2020).

The DCFH2-DA probe was used to identify the production of intracellular ROS. After 30 minutes of DCFH2-DA probe pretreatment, cells were incubated with 15 μM Chelerythrine (CHE) for 1.5 hours, either with or without N-acetyl cysteine (NAC) (5 mM, 1h), posttreatment cells were washed with PBS. The production of reactive oxygen species (ROS) was analyzed using flow cytometry equipment from “Becton Dickinson FACS Canto, located in Franklin Lakes, NJ”. CHE increases the generation of ROS in A549 and NCI-H1299 cells was detected by using DCFH2-DA probe. CHE increased the generation of ROS in both cell lines after treatment for 1.5 h, and

pretreatment with NAC (5mM, 1h) decreased the CHE-induced ROS generation (Tang et al., 2017).

2.5.5. Wound healing (cell migration) assay and Boyden's chamber assay

Wound healing assay measures collective cell migration in a 2D monolayer by creating a "scratch" and observing how quickly cells move to fill it, while the Boyden chamber (or transwell) assay quantifies single-cell migration through a porous membrane towards a chemoattractant, enabling analysis of chemotaxis.

Wound healing assays was performed to evaluate the effect of multi-fraction irradiation on cell motility. A549 cells were initially placed in 12-well plates at a density of 1×10^5 cells per well. Once the cells reached confluency, a scratch was made using a 10 μ l pipette tip, and then the area was washed with DMEM containing 1% FBS to remove any cellular debris. Images were taken after 30 and 48 h using a camera connected to an inverted light microscope (Olympus IX-71, Olympus Corporation, Japan). As a result, it shows that the scratched wound of the A549/R cells closed more rapidly than that of the A549 cells (L. Liu et al., 2021).

A wound healing migration assay was conducted using modified Boyden chambers equipped with 8 μ m pore filter inserts within 24-well plates, supplied by (Corning Life Sciences, Corning, NY, USA) on wild-type (WT) and BIS-knockout (BIS-KO) A549 cells. Cells were initially plated in a 35 mm dish and cultured overnight to achieve confluence. A wound in the cell monolayer was induced by scratching it with a 200 μ L pipette tip. Following a 48-hour incubation period, cell migration from the leading edge was recorded. A cell suspension containing 5×10^4 cells in 200 microliters of serum-free RPMI 1640 medium was placed into the upper chamber. Meanwhile, the lower chamber was filled with RPMI 1640 medium, enriched with 10% fetal bovine serum (FBS), to facilitate cellular growth or cell migration study. After 24 hours of incubation, the membrane was gently removed from the chamber. Any cells attached to the upper surface were cleared using cotton swabs. The cells that had moved to the lower surface through the filters were fixed using 50% methyl alcohol and then stained with a 0.1% solution of crystal violet for further analysis. Digital images of the membranes were captured, from which four random fields were selected to quantify the cells that traversed the filters. The migratory and invasive capabilities of A549 cells were inhibited following the depletion of the Bcl-2-interacting cell death suppressor (BIS) in vitro (J. Lee et al., 2018).

2.5.6. Gene expression (q-RT-PCR) and detection of apoptosis- Flow cytometry analysis

Quantitative PCR (qPCR), also known as real-time PCR, has had a tremendous impact on the field of molecular biology by allowing for precise and quantitative measurements of gene expression.

The process of qPCR gene expression analysis involves several important steps:

- 1) extraction of high-quality RNA,
- 2) reverse transcription to generate complementary DNA (cDNA), and
- 3) the amplification and detection of target sequences using fluorescent dyes or probes.

RT-qPCR is an additional step of reverse transcription of RNA to cDNA before performing qPCR.

RT-qPCR refers to the quantification of RNA after reverse transcription into cDNA. Some studies related to gene expression and flow cytometry analysis of cell cycle arrest have been given below:

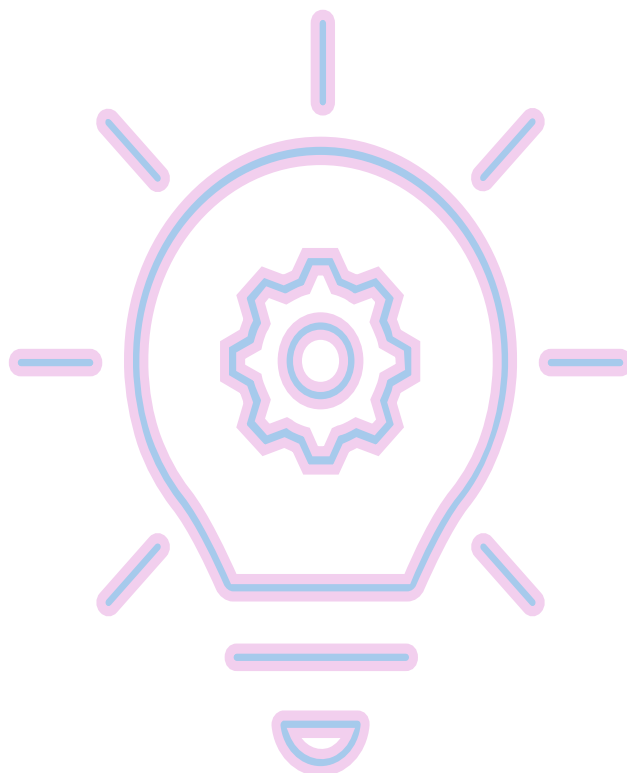
The gene expression and cell cycle arrest in MCF-7 and A549 cell lines by Xn were assessed using q-RT-PCR and flow cytometry analysis, focusing on genes such as P53, P21, BAX, MDR1, NFkB1, NFkB2, SURVIVIN, FAK, MMP2, MMP9, MCL1, ABCG2, and BCL2 (Gholizadeh Siahmazgi et al., 2023b). For normalization, β -ACTIN was used as the reference gene. The result showed that Xn treatments decreased cancer-related gene expression in MCF-7 cells ($p < 0.001$), while increasing P21 and P53 expression ($p < 0.001$) compared to the control. Under these conditions, the BAX gene was upregulated ($p < 0.05$) while the ABCG2 and NFkB1 genes were downregulated ($p < 0.05$). In A549 cells treated with Xn, expression levels of NFkB2, FAK, MDR1, SURVIVIN, and ABCG2 genes decreased ($p < .01$), whereas BAX gene expression increased ($p < 0.01$). P53 expression increased ($p < 0.05$), however MMP2, NFkB1, and BCL2 gene levels decreased ($p < 0.05$) (Gholizadeh Siahmazgi et al., 2023b). Increased Sub-G1 cell cycle arrest ($p < 0.001$) was seen in MCF-7 and A549 cells treated with Xn at the determined IC₅₀. Additionally, flow cytometry revealed a decrease in the number of cells in the G1 and S phases (Gholizadeh Siahmazgi et al., 2023b).

Cucurbitacin B effectively hinders cell proliferation and promotes apoptosis through the inhibition of the STAT3 pathway in A549 lung cancer cells (M. Zhang et al., 2014). For the experiment, the cells were initially seeded into six-well plates at a density of 5×10^5 cells per well, permitted to adhere overnight, and subsequently treated with either 0.1 or 1 $\mu\text{mol/l}$ of Cucurbitacin B for 24 hours before being collected. For the purpose of cell cycle analysis, cells were fixed in 70% ice-cold ethanol and stored at 4°C overnight. Subsequently, these cells were washed using PBS, treated with RNase, and stained with Propidium Iodide (PI) at a concentration of 100 $\mu\text{g/ml}$ in the absence

of light for a duration of 30 minutes at ambient temperature. The samples underwent examination utilizing a FACScan flow cytometer from (BD Biosciences, Franklin Lakes, NJ, USA). The study results indicated a substantial increase in the percentage of cells in the G2/M phase with escalating doses of CuB. Specifically, while the control group exhibited a G2/M phase percentage of $3.26 \pm 1.02\%$, the group receiving a high dose of CuB showed a significant rise to $31.78 \pm 3.69\%$. The results demonstrated that CuB induced G2/M arrest significantly (M. Zhang et al., 2014).



Chapter 3



Rationale of the Study...

CHAPTER 3

RATIONALE OF THE STUDY

3.1. Hypothesis

The Global Observatory Cancer (GLOBOCAN) 2020 report indicates that approximately 19.3 million new cancer cases were diagnosed in 2020, with nearly 10.0 million resulting in fatalities. Looking ahead, GLOBOCAN predicts a significant rise in cancer cases, estimating that their number will reach 28.4 million by the year 2040 (Sung et al., 2021b).

Lung cancer (LC) has a higher prevalence rate and mortality. The estimated number of LC up to 2020 was 2.2 million and 2 million people died among them. LC typically arises from various environmental and lifestyle factors, including smoking, inhaling second-hand smoke, and exposure to radiation, radon gas, asbestos, or other carcinogenic substances. It is categorized into two primary types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC accounts for the prevalence rate of 10-15 % and causes early widespread metastasis. It is most common among heavy smokers. NSCLC represents about 85% of all lung cancer cases, highlighting its predominant presence among different types of lung cancer (Rosell & Karachaliou, 2016b). NSCLC affects active as well as passive smokers. It mainly arises in bronchi and peripheral lung tissue. Metastasis constitutes a significant factor in mortality among patients with malignant tumors, responsible for approximately 90% of cancer-related fatalities (Bielenberg & Zetter, 2015b; Nemeth et al., 2003). NSCLC is categorized into three main types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Adenocarcinoma is the most prevalent form, accounting for about 40% of NSCLC cases. This type of cancer initiates in the epithelial cells, which are the cells that form the outer lining of the lungs. Squamous cell carcinoma starts in squamous cells. Squamous cells, which are flat in shape, form the lining inside the lungs. This type of LC, known as squamous cell lung carcinoma, accounts for approximately 25 to 30 percent of all NSCLC cases. Large cell carcinoma is a rare subtype of NSCLC, comprising approximately 10% of all NSCLC cases.

The currently available conventional methods that have been used for treatment of LC include surgery, chemotherapy, radiotherapy, immunotherapy and by various nanoparticle mediated formulation targeting the LC. Chemotherapy utilizes the use of synthetic drugs such as cisplatin, carboplatin, paclitaxel, etoposide, docetaxel. While radiotherapy utilizes gamma radiation.

Chemotherapy and radiotherapy have many sides effect like hair loss, mouth sore, nausea and vomiting. They also affect normal cells along with cancer cells. Chemotherapy has the capability to affect healthy cells in the body, including those in the blood, skin, and stomach lining. This occurs because chemotherapy drugs target rapidly dividing cells, a characteristic shared by both cancer cells and some healthy cells, leading to potential side effects in these areas. While surgery for cancer patients is highly painful, and immunotherapy is too costly to be achieved by patients for treatment of LC. Therefore, the use of plant-derived bioactives, which are essentially secondary metabolites, has gained popularity in the treatment of LC due to their superior safety and inhibitory activity on several LC signaling pathways. Among them Xn and its formulation have been found effective in treating cancer.

3.1.1. Xn

Xn is a prenylated chalconoid that is found in the female inflorescences of *Humulus lupulus*. It is also known as hops. First, Verzele et al. identified the structure of Xn (O et al., 1957b). Its molecular structure is shown in (Fig.3.1).

In recent years, much research has been carried out to harness the anticancer potential of Xn. These studies indicated that Xn is effective against leukemia (C. M. Wang et al., 2019), NSCLC (Gao et al., 2020), and breast cancer (Gao et al., 2020; Gieroba et al., 2020). The study indicates that Xn possesses remarkable qualities, including the ability to combat oxidative stress due to its strong antioxidant properties. Additionally, it offers benefits in reducing inflammation and shows promising potential in the fight against cancer (M. Liu et al., 2015). Xn initiates the cell cycle arrest in cancerous cell by activating p53 and elevating the levels of the p21 protein. Xn treatment promoted apoptosis in cancer cells by decreasing the levels of Bcl-2 and Survivin proteins. Xn has been found to inhibit the growth and spread of cancer by influencing various signaling pathways such as MAPK, EGFR/AKT, WNT/-CATENIN, ERK, NF- κ B, and STAT3. Additionally, it affects several proteins like caspases, Bcl-2, Cyclin D1, oxidative markers, and tumor suppressor genes (Girisa et al., 2021a).

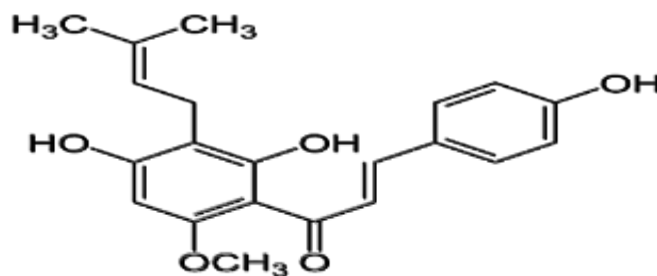


Fig. 3.1 : Structure of Xn

3.1.2. Anti-cancer activity of Xn

Research has shown that Xn possesses potent anticancer properties, proving to be effective in combating various forms of cancer. These include ovarian, thyroid, and pancreatic cancers, as well as leukemia, hepatocellular carcinoma (HCC), breast cancer, glioma, colon cancer, cervical cancer, melanoma, and NSCLC (Benelli et al., 2012; B. Zhang et al., 2015a). Numerous research efforts have been conducted to assess the anticancer properties and effectiveness of Xn in combating cancer.

3.1.3. Challenges regarding delivery Xn

Despite having wonderful anticancer potential of Xn, it has certain physiochemical/biopharmaceutical challenges that impedit its bioavailability. These challenges include poor aqueous solubility and poor absorption from biological membranes including GIT. Delivering Xn to the target site presents several challenges due to its highly hydrophobic nature. This characteristic significantly reduces its bioavailability, preventing it from effectively generating a therapeutic response at the LC target site. Additionally, the low solubility of Xn in water, measured at 1.3 mg/L at 23°C, complicates experimental efforts, further hindering its application and study. Therefore, to overcome the challenges Xn loaded NLCs were created to boost bioavailability and give an efficient therapeutic effect against the lung carcinoma cells in order to address these issues.

3.1.4. Role of nanostructure lipid carriers

Novel drug delivery system has nanoscale material that is highly effective with excellent targeting abilities against LC (Kaur et al., 2022; Kazmi et al., 2023; Khursheed et al., 2022; Kumari et al., 2023). Solid lipid nanoparticle (SLNs) was discovered in early 1990's by Professor R.H Muller (Germany) as a novel formulation. However, SLNs have some limitations like poor drug loading capacity and drugs leakage during storage. To tackle these challenges, researchers created lipid

carriers designed with nanostructures. NLCs are the 2nd generation of SLNs which consists of the composition of solid, liquid lipid and surfactant. In comparison to SLNs, NLCs exhibit a less compact arrangement of lipids and a matrix structure that is intentionally imperfect. This configuration creates vacant spaces within the matrix, allowing for a greater quantity of the drug to be integrated. Consequently, NLCs offer enhanced drug encapsulation efficiency and improved stability throughout extended storage periods (Almurshedi et al., 2021). NLCs are made using emulsifiers and a biodegradable as well as suitable lipid (solid and liquid).

3.1.5. Mechanism of Xn loaded NLCs in treating LC

LC occurs due to mutation in genes in MAPK, AKT pathway, Wnt-signaling pathway, ALP (Anaplastic lymphoma kinase), EGRK, ROS-1, NF-kB and STAT3 signaling. Various signaling pathways that are involved in inhibition of LC by the action of drug Xn loaded NLCs include MAPK, AKT pathway, Wnt-signaling pathway, ALP (Anaplastic lymphoma kinase), EGRK, ROS-1, NF-kB. These pathways are involved in the promotion of pro-apoptotic proteins (bax/bad) and caspases 3, -9 proteins for the apoptosis. The mechanism too has been observed while evaluating the anti-cancerous of Xn loaded NLCs on A549 cell lines. The schematic diagram for overall mechanism / process involved in treating LC via Xn loaded NLCs is shown in (Fig 3.2).

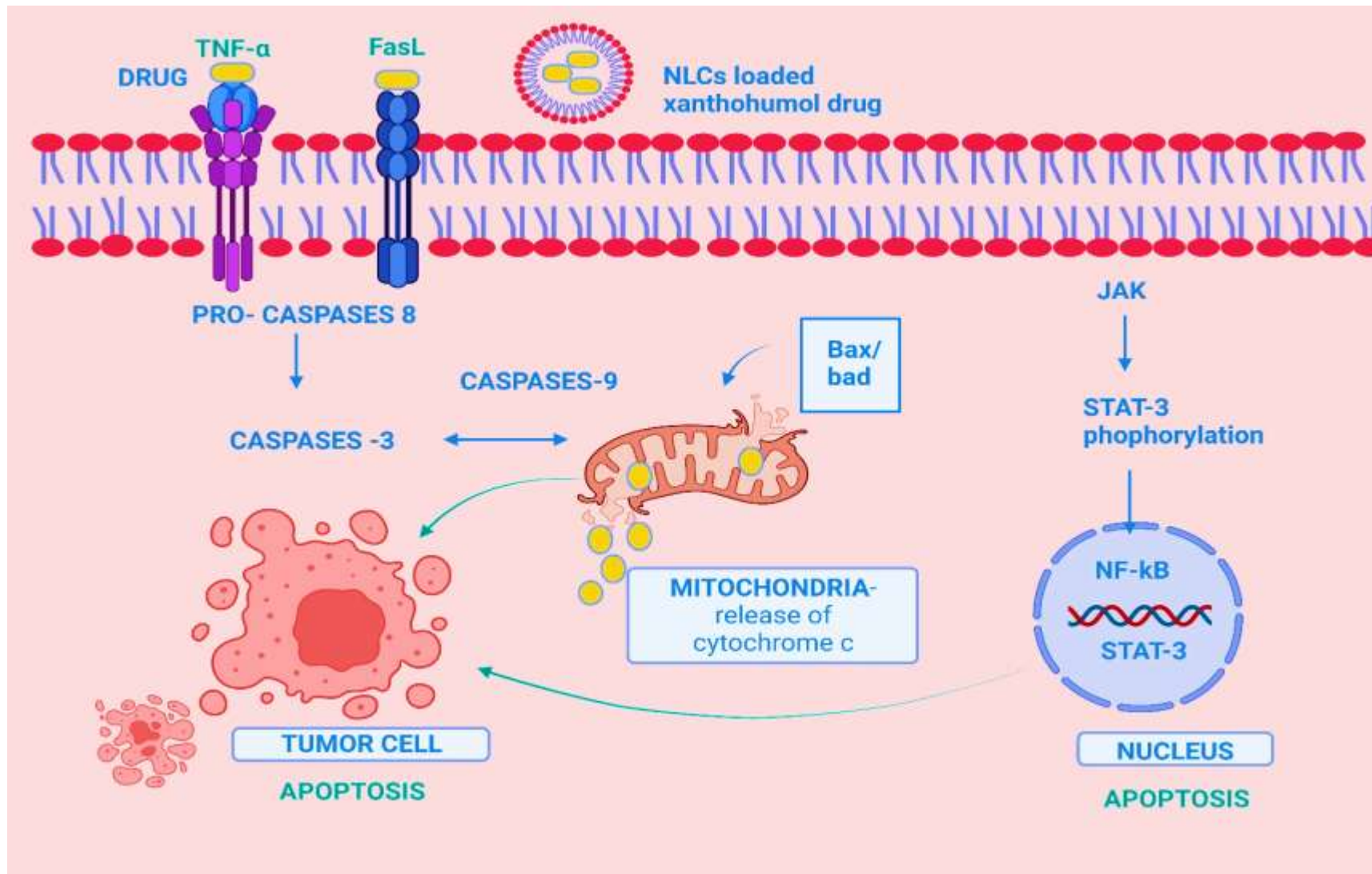


Fig. 3.2 : NLCs loaded with the Xn cause apoptosis in cancer cells.

3.1.6. Mechanism involved in pulmonary delivery of Xn-NLCs to treat LC

1. Technique for pulmonary drug delivery

The effective delivery of drug to the lungs relies on the combined interaction of the inhaler device, the properties of the drug formulation, and the inhalation technique employed by the patient. Presently, the devices available for administering pharmaceutical aerosols in clinical pulmonary therapy constitute nebulizers, pressurized metered dose inhalers (pMDIs), and dry powder inhalers (DPIs) (Laube et al., 2011) are given in (Fig.3.3). However, much effort has been put into the development of new inhalers and formulation to perfect the pulmonary delivery system for local or systemic drug targeting.

2. Drug delivery to lungs (Particle deposition)

Upon inhaling aerosols, human studies have demonstrated that ultrafine particles, specifically those with a diameter of 100 nanometers, rapidly transition from the lungs into systemic circulation within five minutes. The primary mechanisms for particle deposition within the respiratory tract include inertial impaction, sedimentation, and diffusion.

3. Transport of drug (passive or active methods)

In lung epithelial cells, most of the drug is absorbed by the action of mechanism of passive diffusion, active transport, carrier mediated transport and by vesicle mediated transport (Currie, 2018). In 90% of cases drug is absorbed by the action of passive diffusion and rest by active transport or by the vesicle mediated transport. Rate of adsorption increases with increase with a lipophilicity of compound or drug. However, in the case of Xn it is difficult to deliver Xn to the target site due to the substance's high hydrophobicity and low oral bioavailability. As a result, Xn does not result in a therapeutic response that is effective at the target site. As a result, Xn loaded NLCs can be developed to boost bioavailability of Xn in lungs and to give an efficient therapeutic effect against the lung tumor cells in order to overcome these difficulties. The schematic diagram of pulmonary delivery of Xn via NLCs is shown in (Fig.3.4).

Xn loaded NLCs targeting the lungs tumor cell

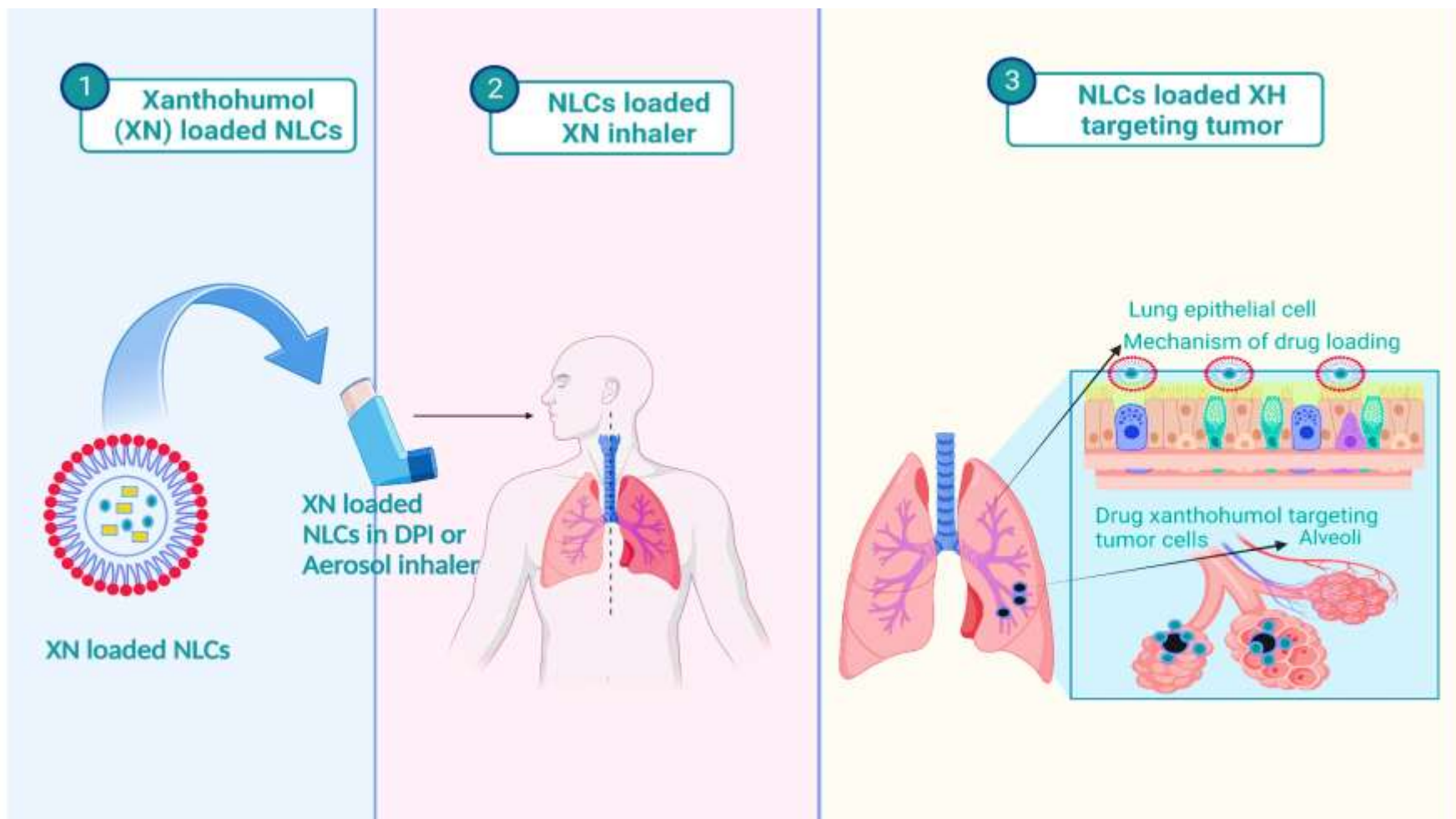


Fig. 3.3 : Mechanism involved in pulmonary delivery of Xn loaded NLCs.

Local Pharmacological Action of NLCs

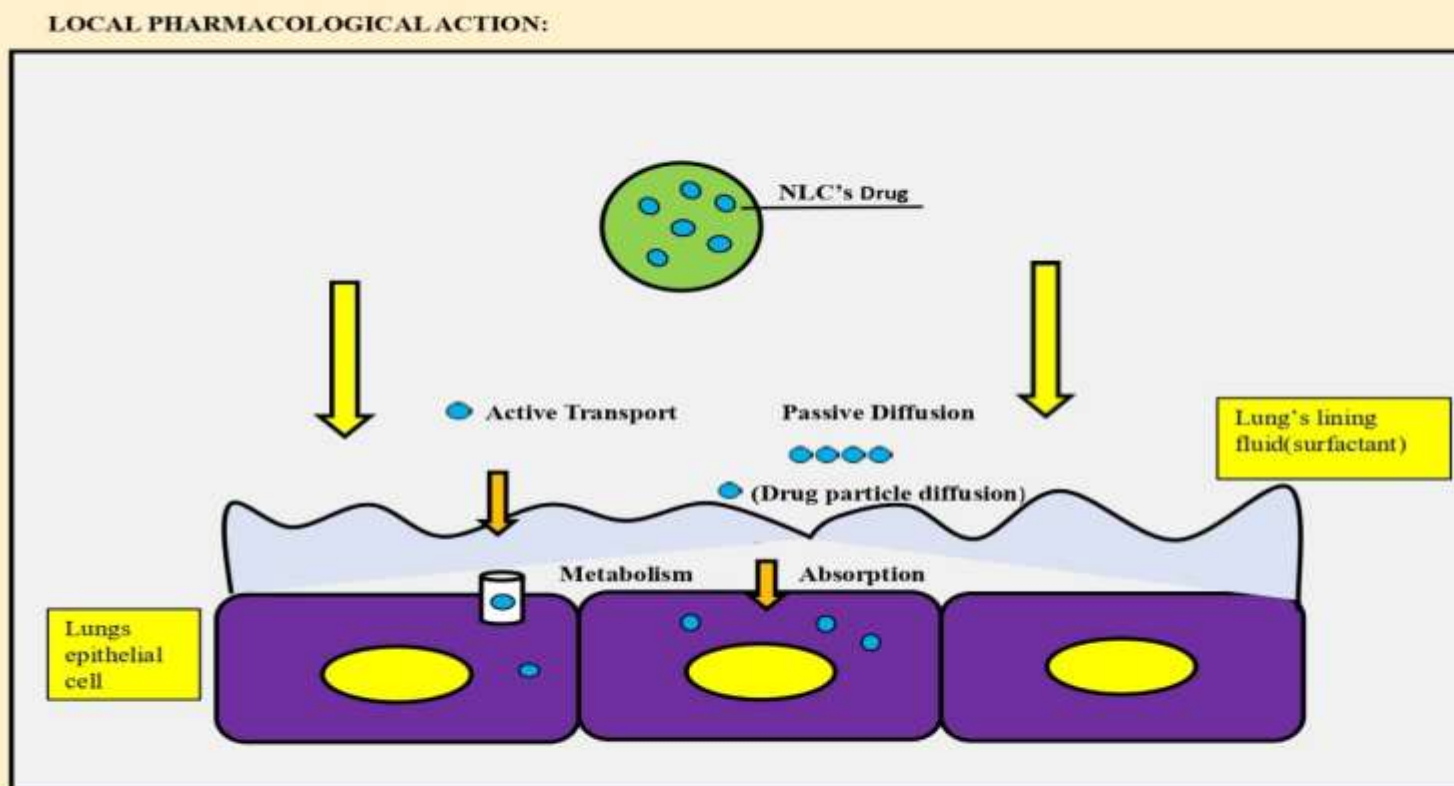


Fig. 3.4 : Local pharmacological action of drug loaded nanostructure lipid carriers NLCs reaching lungs epithelial cell

3.2. Aim

Formulation and optimization of xanthohumol loaded nanostructured lipid carriers for the treatment of lung cancer.

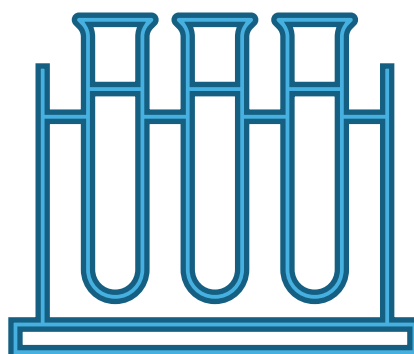
3.3. Objective

The objectives of present study are:

- ❖ Formulation and Optimization of Xn loaded nanostructure lipid carriers (NLCs).
- ❖ Characterization of Xn loaded NLCs.
- ❖ In vitro toxicity and anticancer efficacy evaluation of Xn-NLCs on healthy (BciNS1.1) and lung cancer (A549) cell lines.
- ❖ Gene expression profiling of tumor suppressor genes associated with lung cancer.



Chapter 4



Experimental work...

CHAPTER 4

EXPERIMENTAL WORK

4.1. Materials and Methods

4.1.1 Materials

Table 4.1. List of materials used in study

Chemicals	Manufacturers
Methanol	Sigma-Aldrich Pvt. Ltd., India
Acetonitrile HPLC Grade	Sigma-Aldrich Pvt. Ltd., India
HPLC grade Water	Loba Chemie Pvt. Ltd., India
Aerosil 200	Central Drug House Pvt. Ltd., India
Beeswax	BRM Chemical Pvt. Ltd., India
Glyceryl monostearate	BRM Chemical Pvt. Ltd., India
Compritol 888	BRM Chemical Pvt. Ltd., India
Sunflower oil	ASESCHEM Pvt. Ltd., India
Olive oil	ASESCHEM Pvt. Ltd., India
Glyceryl monooleate	ASESCHEM Pvt. Ltd., India
α -Tocopheryl acetate	ASESCHEM Pvt. Ltd., India
Labrafac-PG	Gattefosse India Pvt. Ltd., India
Lauroglycol-90	Gattefosse India Pvt. Ltd., India
Lauroglycol-FCC	Gattefosse India Pvt. Ltd., India
Caproyl PGMC	Gattefosse India Pvt. Ltd., India
Transcutol P	Gattefosse India Pvt. Ltd., India
Poloxamer-188	Gattefosse India Pvt. Ltd., India
Span 80	ASESCHEM Pvt. Ltd., India
Span 20	ASESCHEM Pvt. Ltd., India
Transcutol P	Gattefosse Pvt. Ltd., India
Tween (80, 20)	ASESCHEM Chemical Pvt. Ltd., India
Dialysis membrane	Hi-media, Mumbai, India
CMC	BRM Chemical Pvt. Ltd., India
Potassium phosphate monobasic (KH_2PO_4)	LABOGEN'S Chemical Pvt. Ltd., India
Sodium phosphate dibasic (Na_2HPO_4)	LABOGEN'S Chemical Pvt. Ltd., India
Potassium chloride (KCl)	LABOGEN'S Chemical Pvt. Ltd., India
Sodium chloride (NaCl)	LABOGEN'S Chemical Pvt. Ltd., India

Chemicals	Manufacturers
Table 4.1 (Continued...)	
Human lung adenocarcinoma cells (A549)	NCCS, Pune
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Merck Pvt. Ltd., India
Fetal Bovine Serum (FBS)	Sigma-Merck Pvt. Ltd., India
1% penicillin/streptomycin	Sigma-Merck Pvt. Ltd., India
MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)	Sigma-Merck Pvt. Ltd., India
Rhodamine 123	Sigma-Merck Pvt. Ltd., India
DCFH-DA	Sigma-Merck Pvt. Ltd., India
Dimethyl sulfoxide (DMSO)	Sigma-Merck Pvt. Ltd., India
Propidium iodide (PI)	Sigma-Merck Pvt. Ltd., India
DAPI (4',6-diamidino-2-phenylindole)	Sigma-Merck Pvt. Ltd., India
TRIzol reagent	Invitrogen, Germany
cDNA Synthesis Kit	Bio-Rad iScript™
iTaq™ Universal SYBR green	BioRad, Hercules, CA, USA
Primers	Barcode Biosciences Pvt.Ltd, Bangalore, Karnataka

Table 4.2. List of equipment used in the study

Equipment	Manufacturer
Centrifuge	REMI RM-12C, Remi Elektrotechnic. Ltd, Vasai, India, Mumbai, India
Differential scanning calorimeter	1. DSC Q200 TA, Universal instrument, Bangalore, India 2. DSC 6000 PerkinElmer, USA
Digital camera	Olympus, Japan
Disintegration apparatus	LabIndia, Analytical Instruments, Pvt. Ltd., Mumbai, India
Dissolution apparatus	DS 8000 (Manual) Lab India, Mumbai, India
Electronic weighing balance	CY360, Shimadzu Co. Ltd., Kyoto, Japan
High performance liquid chromatography	1. HPLC LC-20AD, Shimadzu Co. Ltd., Kyoto, Japan 2. Autosampler HPLC LC-20AD, Shimadzu Co. Ltd., Kyoto, Japan
Hot air oven	Cadmach Drying Oven, Cadmach Machinery Ltd., Ahmadabad, India
Homogenizer	Glass-Teflon potter homogenizer, Thomas Scientific, USA
Magnetic stirrer	Remi 5MLH, Vasai, Mumbai, India
Melting point apparatus	Popular, India
Optical microscope	KYOWA- Getner Instruments Pvt. Ltd., Japan
Particle size analyzer	Malvern Zetasizer, Nano ZS90, UK
pH meter	Phan, Lab India, Mumbai, India
Powder x-ray diffractometer	Bruker D8 Advance, USA
Scanning electron microscope	Jeol JSM-7610F Plus, Japan
Transmission electron microscope	Hitachi H-7500, Japan 120 kV
Shaking water bath	LabFit, India

Spray dryer	JISL Spray Mate, Jay Instruments, Navi Mumbai, India
Table 4.2 (Continued...)	
Stability chamber	Remi CHM 10S, Remi Sales & Engineering Ltd., Mumbai, India
Transmission electron microscope	JEM-2100 plus Electron microscope, Jeol, Japan
Ultrasonication bath	Loba Life, Lobachemie, Mumbai, India
UV spectrophotometer	UV-1800, Shimadzu Co. Ltd., Kyoto, Japan
Vortex mixer	REMI CM101, Delhi, India
0.45 µm syringe filter	Merck, Germany
0.22 µm syringe filter	Merck, Germany
Laminar flow hood	Thermo-Fisher Scientific
Co2 incubator	Mediatech, India
EVOS FL inverted Fluorescence Microscope	Thermo-Fisher Scientific, Waltham, MA, USA
BMG Labtech microplate reader	BMG LABTECH, Germany
ImageJ software	version 1.53c, Bethesda, MD, USA
Nano Drop spectrophotometer	Thermo-Fisher Scientific, Waltham, MA, USA
Eppendorf Flexid Master cyclor	Hamburg, Germany
Nexus thermal cyclor	
CFX96 PCR system	BioRad, Hercules, CA, USA
FACS CytoFlex- Beckman Coulter	Beckman Coulter Life Sciences, USA

4.2. Methodology

4.2.1. Analytical method development

4.2.1.1. Preparation of standard stock solution and sample solution

The following steps were taken: 10 mg of Xn was dissolved in 10 mL of methanol in a standard volumetric flask (100 mL) and the volume was adjusted to 100ml in order to achieve the ultimate concentration of 100 μ g/mL (Solution A). Further to obtain a concentration of 10 μ g/mL an aliquot (10 mL) was taken out and further diluted to 100 mL (solution B).

4.2.1.2. Determination of wavelength maxima

The solution B was used to determine the wavelength maxima (λ_{max}) by scanning in the range of 200 to 800 nm on double beam UV- visible spectrometer.

4.2.1.3. Method of preparation for NLCs

NLCs were developed using the hot high-pressure homogenization method (Al-Kattan et al., 2010; Piao et al., 2011), which was then followed by ultra-sonication. In this process, Xn was mixed into the lipid phase, consisting of Beeswax and Lauroglycol-90, and heated in a water bath at 80°C. Meanwhile, the aqueous phase was prepared by dissolving the surfactant Tween-80 in water. The two phases were consistently kept at a temperature exceeding the lipid's melting point of 80°C. At this elevated temperature, the liquid lipid phase was integrated into the heated surfactant phase, forming a pre-emulsion through mechanical agitation using equipment from (Remi Instruments Ltd., Mumbai, India). This stirring was performed at 2000 rpm for a duration of 30 minutes while maintaining a temperature of 80°C. The heated pre-emulsion was processed through a high-pressure homogenizer to form the NLC dispersion (Singh et al., 2025) .

4.2.1.4. Chromatographic conditions

The chromatographic conditions were analyzed by observing the peak of Xn in different solvents such as (methanol, acetonitrile, acetic acid, and water). While selecting the chromatographic condition for the method development different solvent were used in different ratio in order to determine the peak of Xn. A chromatogram was seen at a wavelength of 370 nm (Singh et al., 2025). The aforementioned observations led to the determination of a certain ratio for the mobile phase, as well as the retention time and flow rate. Analytes with a detection wavelength of 370 nm were performed using an analytical column C18, 5 μ m, 250 $\times$ 4.6mm. The temperature chosen for operation was 40°C. Throughout the procedure, an injection volume of 20 μ L was consistently maintained, along with a flow rate of 1.0 mL/min. The duration of the run was ten minutes

4.2.2. Method Validation

The International Conferences on Harmonization (ICH) Q2 (R1) guidelines followed the validation of the developed HPLC method. The specificity, linearity and range, accuracy, precision, sensitivity, drug stability, and robustness were the method's validation parameters. An additional method of evaluating the system's performance was to measure appropriate metrics such tailing factor, LOD, LOQ, peak purity index, and theoretical plate and height similar to theoretical plate height (HETP) (Singh et al., 2025).

4.2.2.1. Specificity

Specificity is one of the important steps that is to be performed to analyze the analytical method's capacity to distinguish the analyte from the other elements of the complex mixture (Batrawi et al., 2017). The method's specificity was assessed by injecting 20 µL of the standard solution of nanostructure lipid formulation (with drug) sample, the placebo formulation (without drug) separately and the MQC solution of 6 µg/mL.

4.2.2.2. Linearity and Range

A standard stock solution A (100 µg/mL) was diluted in methanol to create various standard solutions (0, 2, 4, 6, 8, 10 µg/mL) in order to assess the linearity and range of the method. Appropriate aliquots of 0, 0.2, 0.4, 0.6, 0.8, and 1 µg/mL were obtained from solution A and were diluted using methanol into six distinct 10 mL containers. The sample's final concentration was, in parts per million, or ppm, 0, 2, 4, 6, 8, and 10 µg/mL. The six injections of each concentration were examined using an identical set of parameters. Each concentration's six injections were analyzed using the same set of parameters. The calibration curve was plotted between the concentration and the mean peak area of each concentration. The regression equation and coefficient have been generated using the calibration curve data.

4.2.2.3. Stability study of Xn solution

Placing the sample at ambient temperature for 7 days and were allowed to assess the stability of the Xn standard MQC solution (6 µg/mL). The concentration of samples was analyzed for six days by injecting them into HPLC.

4.2.2.4. System suitability and estimation of LOD and LOQ

To validate the system's appropriateness, the peak purity index, LOD, and LOQ were recorded. These parameters were used to calculate standard deviation of response (sigma) and slope of calibration curve (S). The formulae to calculate LOD and LOQ are shown in Equation 1 and 2.

$$\text{Limit of Detection (LOD)} = 3.3 \times \sigma / S \dots\dots\dots (1)$$

$$\text{Limit of Quantification (LOQ)} = 10 \times \sigma / S \dots\dots\dots (2)$$

4.2.2.5. Accuracy

An analytical procedure's accuracy can be used to represent how closely its results agree with the true value. To perform the test, three distinct levels of mid concentration (6µg/ml) were quantified: lower (LQC-80%), medium (MQC-100%), and higher (HQC-120%). Three separate HPLC runs were done for these concentrations. Appropriate aliquots of 4.8, 6 and 7.2 mL were obtained from solution B and put in a 10 mL volumetric flask to provide these concentrations. Methanol was used to bring the final amount down to 10 mL. The final concentrations of the three solutions are 4.8 µg/ml or ppm (LQC), 6 µg/ml or ppm (MQC), and 7.2 µg/ml or ppm (HQC). These concentrations were run in triplicate on an HPLC. Equation 3 was used to determine the absolute percentage of recovery.

$$\% \text{ Recovery} = \text{Actual concentration recovered} / \text{Theoretical concentration} \times 100 \dots\dots\dots (3)$$

4.2.2.6. Precision

The precision of the method was evaluated through the application of repeatability and intermediate precision. (Aggarwal et al., 2014; Ankit et al., 2011; Poola et al., 2002; Riley et al.). The intermediate precision was assessed by identifying the Lower (LQC-80%), Medium (MQC-100%) and Higher (HQC-120%) concentration of sample on the same days (Intra-day), different days (Inter-day), and by different analysts (Inter-analysts) under the identical experimental settings (Forootan et al., 2017). Then the mean data were recorded, and the percentage RSD was calculated by Equation (4).

$$\% \text{ RSD} = \text{Standard deviation (of peak area)} / \text{Mean peak area} \times 100 \dots\dots\dots (4)$$

4.2.2.7. Sensitivity

Analyzing different Xn solutions and determining the signal-to-noise ratio has allowed for the establishment of the Xn's limit of detection (LOD) and limit of quantitation (LOQ). The concentration at which the signal-to-noise ratio is around 3:1 is known as the limit of detection (LOD), and the concentration at which it was approximately 10:1 was known as its limit of quantitation (LOQ) (Forootan et al., 2017).

4.2.2.8. Robustness

The robustness of the analytical method was tested by examining the effects of minor internal adjustments on system suitability parameters in HPLC settings. The robustness of the technique was verified by implementing deliberate minor modifications to the experimental configurations, such as **Flow rate:** ± 0.2 mL/min and **Mobile phase composition variation:**

± 2%.

4.2.3. Characterization of active Pharmaceuticals ingredients (API)

Characterization of Xn was carried out to determine its physical nature, melting point.

4.2.3.1. Physical description of Drug

Approximately 10 milligrams (0.01 grams) of Xn were placed on a glass slide for examination. The identification of the drug's color was conducted through direct visual inspection.

4.2.3.2 Melting Point

The melting point of the drug was determined using two methods: the capillary fusion method and Differential Scanning Calorimetry (DSC).

4.2.3.3 Capillary fusion method

Capillary fusion method was used to determine the melting point of Xn. The 2 capillaries were taken and sealed at one end by heating on a flame. The sample (Xn) was placed in a thin-walled capillary tube of 10-15cm length and a diameter of 1 mm. The capillary containing the drug and a thermometer were placed in the melting point apparatus and was heated evenly.

4.2.3.4. DSC

The DSC of pure Xn was evaluated by the Perkin Elmer DSC 6000 (USA) Apparatus. Powdered sample (2-3mg) was taken in aluminum pan, heated over the range of 28-400⁰C at 10⁰C/min using a dry-nitrogen flush at rate of 19.8mL/min. The thermograph of Xn showed a sharp endothermic peak at 173.17 °C and ΔH was 104.6 J/g. It was concluded that Xn was a yellow powder in nature.

4.2.3.5. Determination of log P

Log P of Xn of was carried out in octanol-water system (Resztak & Czyrski, 2024). The studies were carried out in three different glass separating funnels (250 ml) and 25 ml water and 25 ml octanol was added in each separating funnel. The liquid was mixed homogeneously and left unshaken for 24 hours to attain the equilibrium. Xn (20 mg) was added to the separating funnel. All the flasks were shaken for 8 hours with a time gap of 60 minutes in alpha mode. Then after the sample was left unshaken for 24 hours in order to achieve clear separation of drug in the two immiscible liquids. The separated layer was filtered using a 0.45 μm membrane filter, after which the sample was diluted and injected into the HPLC system for further analysis.

$$\text{Log P} = \log_{10} (\text{P})$$

4.2.3.6. Powder X-ray diffraction (PXRD)

The physical nature of Xn was determined by the X-ray diffractometer (patterns were obtained at room temperature on a Bruker AXS, D8 Advance, USA. The physical properties of Xn were

characterized using a PXRD diffractometer with Cu-K α 1 radiation ($\lambda = 1.5406 \text{ \AA}$) and a 2D detector (point or strip detector). The sample is placed on rotating flatbed sample holder that is continuously rotated during the measurement with an 2Θ resolution of 3.12° and exposition time of 120 s per step (Arczewska et al., 2013b).

4.2.3.7. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR of Xn was determined by using Perkin Elmer Spectrum 2 by (1-2mg of drug) direct sampling method. The bands were recorded in the range of $4000\text{-}400\text{cm}^{-1}$ (Arczewska et al., 2013b)(1-2mg of drug) direct sampling method. The bands were recorded in the range of $4000\text{-}400\text{cm}^{-1}$ (Arczewska et al., 2013b).

4.2.4. Solubility studies

4.2.4.1. Screening of solid and liquid lipids and surfactant

Prior to the formulation of NLCs, components were examined in order to identify the most appropriate solid and liquid lipids that may be employed to achieve high drug entrapment and maximum drug loading into the lipid matrix.

Xn solubility was determined in different lipids including the solid lipids, liquid lipids, and surfactants. The solubility of Xn in solid lipids such as Beeswax, Compritol 888, and GMS (1000 mg) was measured. The weighed drugs were subsequently added to the test tubes maintained at 80°C on controlled temperature water bath. The drug was added to these molten solid lipids until saturation was attained. The solubility of Xn in solid lipids was assessed through physical observation. The liquid lipids, i.e., Sunflower oil, Olive oil, Glyceryl monooleate, α -Tocopheryl acetate, Labrafac-PG Soluble, Lauro glycol -90, Lauro glycol -FCC and Caproyl PGMC (1ml) were taken in micro centrifuge tube separately and the drug was added to then until saturation was attained. Vortexing was done at an interval of 10 min. Surfactants were such as Tween 80, Tween 20, Span 20, Span 80, Transcutol P and Poloxamer-188 (1ml) were taken in micro centrifuge tube tube separately and the drug was added until saturation was attained. Intermittent vortexing was done at an interval of 10 min. After 72 hours, the resultant mixtures were centrifuged at $10,000g$ for 10 minutes. The supernatant from the centrifuged solutions was extracted, diluted with methanol, and tested for solubility of Xn using HPLC (Chauhan et al., 2020b; Chu et al., 2020; Elmowafy & Al-Sanea, 2021b; Soleimanian et al., 2018).

4.2.4.2. Pseudo-ternary phase diagrams

Pseudo ternary phase diagrams which are essential in optimizing the (Lipid)_{mix} ratio was constructed using aqueous titration technique. The selected solid lipid (Beeswax) and liquid lipid (Lauroglycol-90) were melted at 75°C, to which selected surfactants (Tween-80) were added in varied ratios (to generate homogenous mixtures). The pseudo-Ternary Phase Diagram (TPD) of Xn loaded NLCs was designed using Pro Sim ternary Software. A total of 27 prototypes were generated using TPD by varying the lipid mixture in the ratio of 1:1, 1:2, 2:1. The ratio of surfactant was kept constant. The amount of drug (10 mg) was kept constant in 27 prototypes.

4.2.4.3. Method of preparation for NLCs

Formulation of NLCs was done by hot high- pressure homogenization and ultrasonication. Initially, Xn was incorporated into molten beeswax at 65-70°C, followed by the addition of lauroglycol-90. The resulting mixture was then transferred to a 50 mL glass beaker and heated to a temperature of 80°C (Chauhan et al., 2020b; Chu et al., 2020; Elmowafy & Al-Sanea, 2021b; Soleimanian et al., 2018). In this procedure, an aqueous solution of Tween-80 (100 mL) was incorporated into the heated mixture. The temperature of the mixture was maintained at 80°C, which exceeded the melting point of beeswax. The hot dispersion was stirred at 2000 rpm for 30 minutes in order to form homogeneous emulsion. This emulsion was subjected to a high pressure homogeneous, wherein it was homogenized for 3 cycles at a pressure of 700 bar. The Homogenized NLCs were subjected for ultrasonication at 37°C for 10 minutes. The NLCs were allowed to equilibrate at ambient temperature.

4.2.4.4. Box-Behnken design (BBD) : Formulation and Optimization

The formulation of the NLCs was optimized utilizing the BBD through the Design-Expert software (Design-Expert 7.0, State-Ease Inc., Minneapolis, MN, USA) (Ferreira et al., 2007; Kraisit & Sarisuta, 2018; Kudarha et al., 2015) BBD was employed using three factors and three levels. The independent variables selected were the amount of Beeswax (A, -1 = 200 mg and +1 = 300 mg), Lauro glycol 90 (B, -1 = 100 mg and +1 = 200 mg), and Tween 80 (C, -1 = 400 mg and +1 = 600 mg). The dependent variables examined in this study comprised particle size (PS), zeta potential (ZP), and drug entrapment efficiency (EE%).

4.2.4.5. Drying- NLCs

The optimized NLCs were converted into free-flowing powder (S-Xn-NLCs) by rotary evaporation followed by drying it in a tray dryer along with mannitol. In order to formulate Solid NLCs (S-NLCs) of Xn, 250 mL of liquid NLCs were taken, which contained 100 mg of

the drug. This indicated the formulation of 0.0004 mg/mL solution of Xn-NLCs. To this 51g of mannitol was added. Then, the formulation was subjected for rotavapor (ESCY IC Model:201) for the evaporation of water at 65°C-70°C for 10 minutes and was spread in the stainless steel trays and subjected for drying in the hot air oven (Remi Instruments Ltd., Mumbai, India) at 70°C for ½ h. Mannitol was used in this study as a solidifying agent because it has various advantages and physicochemical characteristics such as low hygroscopicity, inertness with respect to APIs, has no known adverse effects in humans, good compatibility, and the ability to produce extremely robust powder (Misha et al., 2018; Plabon et al., 2024).

4.2.5 Characterization of liquid Xn-NLCs and solid Xn-NLCs powder

4.2.5.1. PS, PDI and ZP

The PS, PDI, and ZP of liquid Xn-NLCs (L-Xn-NLCs) and S-Xn-NLCs were done using the principle of dynamic light scattering. Zeta-sizer Nano-ZS-90 of Malvern Instruments, UK was used for this study. The mean hydrodynamic diameter (z-average) was used to calculate PS of formulations, which was represented by the PDI, and measurements were taken at 25°C. The S-Xn-NLCs were re-dispersed in Milli-Q water at a 1% (w/v) concentration using a vortex for 30 seconds. Data collection and analysis were conducted using the Malvern DTS v5.2 program (Kim et al., 2019)

4.2.5.2. % Entrapment Efficiency

The % EE of L-Xn-NLCs and S-Xn-NLCs was determined using the centrifugation technique. NLCs dispersion (containing an equivalent to 10 mg of drug) was centrifuged at 20000g for 1 hour. The supernatant was collected. To determine the concentration of drugs in supernatants, the sample was filtered through a 0.45 µm syringe filter and analyzed using HPLC. The %EE of Xn in formulations was calculated using the following equation (5) (Kim et al., 2019)

$$\%EE = [(Q_i - Q_r) / Q_i] \times 100 \dots\dots\dots(5)$$

where Q_i = amount of the drug used for the preparation of formulation; Q_r = amount of the drug present in the supernatants.

4.2.5.3. Powder Flow evaluation of S-Xn-NLCs

S-Xn-NLCs (20g) was taken for evaluation of powder flow which includes “angle of repose, bulk density (BD), tapped density (TD), Carr’s index (CI), Hausner ratio (HR)” (Goyal et al., 2015) Testing of these parameters is crucial for understanding powder flow and compressibility. The S-Xn-NLCs were poured into the 50 mL glass measuring cylinder and the volume of powder was noted as 35 mL. The BD was calculated using equation 6. For

calculating TD, S-Xn-NLCs kept in measuring cylinder was placed on the digital tap density apparatus (Lab India Instruments Ltd., Mumbai, India). Afterward, the cylinder tapped for 100-200 tapings, and the tapped volume was noted as 32.5 mL. The TD was calculated using equation 7. CI and HR were calculated using the formula given in equations 8 and 9. In order to calculate the angle of repose, 100 ml funnel was taken and placed in stand above 2cm (height) from touching the ground surface of graph paper, then the S-Xn-NLCs were poured into the funnel and dropped from it until it reached the tip of funnel that was placed above 2cm. Then, the outline circle was drawn on graph paper with the help pencil in order to calculate the distance or radius covered by S-Xn-NLCs powder. The radius was found to be 6cm, then the angle of repose was calculated using equation 10.

Bulk Density (ρ_b) = W/V_b , where W is weight in gram and V_b is the volume.....(6)

Tapped Density (ρ_t) (g/mL) = W/V_t , where W is weight in gram and V_t is tapped volume in milliliters.....(7)

Carr's index ratio = $100 [((\rho_{\text{tapped}} - \rho_{\text{bulk}}) / \rho_{\text{tapped}})]$, where ρ is density.....(8)

Hausner's ratio = Tapped density of the powder/ Bulk density of the powder or ratio of ($\rho_{\text{tapped}} / \rho_{\text{bulk}}$), where ρ is density(9)

Angle of repose (Θ) = $\tan^{-1}(h/r)$, where h is the height and r are the radius(10)

4.2.5.4. *In vitro* drug release studies of optimized Xn loaded NLCs

Release study was carried out for L-Xn-NLCs and S-Xn-NLCs (reconstituted) using a dialysis bag. The membrane of 14 kDa molecular weight used for dialysis was purchased from Hi-media (Mumbai, India). The pre-conditioning of membrane was done by soaking it in PBS of pH 7.4 for a period of 24h. Afterward, the membrane bag was prepared in the form of a sac and 1mL of NLCs were added in the sac. Each mL of NLCs contained 10 mg of Xn. Both the ends of the sac were tied using a thread. Similarly, 1mL of 0.2% w/v carboxymethyl cellulose (CMC) suspension containing 10 mg of Pure Xn was transferred in another sac. The sample containing dialysis bags were subjected to a 250 mL glass beaker that contained equal volumes (50:50) of PBS (pH 7.4) and methanol. The magnetic bead was added in the beaker and the medium was stirred at 100 rpm. The temperature of medium was kept at 37°C. The study was conducted 24h. The solution inside bag was acting as a donor compartment and medium outside was acting as receptor compartment. To maintain sink condition, 1 mL of the sample was taken out of the receptor compartment at predetermined intervals and replaced with the same volume of fresh medium. Finally, the concentration of Xn released was quantified using HPLC system

(Shimadzu, Japan) (Brito Raj et al., 2019; Duong et al., 2019; H. J. Lee et al., 2020).

4.2.5.5. Release kinetics of NLCs formulation

Various kinetic models were employed to analyze the in-vitro release patterns of L-Xn-NLCs and S-Xn-NLCs. The model that demonstrated the highest correlation coefficient (r) value indicates the true mechanism of release in vitro (Duong et al., 2019). Release pattern of L-Xn-NLCs and S-Xn-NLCs were analyzed using statistical and mathematical methods with different types of kinetic models such as: Zero order kinetic model, First order kinetic model, Korsmeyer-Peppas model, Hixson-Crowell model and Higuchi model.

a. Zero order kinetic model

Drug release by zero order is observed when the drug release is independent of its concentration. This method is adopted to achieve pharmacologically prolonged action. Zero order kinetic is expressed by equation 11.

$$C = K_0t \dots\dots\dots (11)$$

Where, k_0 = zero order rate constant; t = time

b. First order kinetic model

The graphical representation is between concentration and time.
This kinetic model is used to describe absorption and/or elimination of drug. It can be expressed by equation 12.

$$\log C = \log C_0 - \frac{K_1t}{2.303} \dots\dots\dots (12)$$

c. Korsmeyer-Peppas model

This simple method was developed by Korsmeyer in 1973, and it relates to the drug release to the time elapsed as given in equation 13.

$$\frac{M_t}{M_\infty} = Kt^n \dots\dots\dots(13)$$

Where, K = drug release constant

t = release time

M_t/M_∞ = fraction of drug

release n = diffusion constant

d. Hixson-Crowell model

This model describes drug release with the change in surface area or particle diameter as given in equation 14.

$$W_0^{1/3} - W_t^{1/3} = K \cdot t \quad \dots\dots\dots(14)$$

Where, W_0 = Initial amount of drug in the formulation

W_t = Amount of drug left after time t

κ = Surface-volume relation constant

t = time in h

e. Higuchi model

Higuchi model is used to identify drug release from inert matrices. The model describes diffusion as drug release process based on Fick's law as given in equation 11.

$$M_t/M_\infty = K_h t^{1/2} \quad \dots\dots\dots(15)$$

Where, M_t = Amount of drug released in time(t)

M_∞ = Amount of drug released after time ∞

K_h = Higuchi release constant

4.2.5.6. DSC, PXRD, SEM and TEM

The DSC of pure Xn, Beeswax, Lauroglycol 90, Tween 80, Mannitol, L-Xn-NLCs and S-Xn-NLCs, was evaluated by the Perkin Elmer DSC Apparatus. Powdered sample (2-3mg) was taken in an aluminum pan, heated over the range of 28-400°C at 10°C/min using a dry-nitrogen flush at a rate of 19.8mL/min (Gill et al., 2010).

The physical nature of Beeswax, Pure Xn, Mannitol and S-Xn-NLCs was determined by the X-ray diffractometer (patterns were obtained at room temperature on a Bruker AXS, D8 Advance, USA). The physical properties of sample were characterized using a PXRD diffractometer with Cu-K α 1 radiation ($\lambda = 1.5406 \text{ \AA}$) and a 2D detector (point or strip detector). The sample is placed on rotating flatbed sample holder that is continuously rotated during the measurement with an 2θ resolution of 3.12° and exposition time of 120 s per step (Arczewska et al., 2013a)

The surface morphology of Beeswax, Pure Xn, Mannitol and S-Xn-NLCs was recorded using

a scanning electron microscope (Model: JSM-7610F Plus EDS: OXFORD EDS X-MaxN JEOL SEM, USA). SEM analysis provides high-resolution images of the surface of a sample. It also provides information about the sample's composition and physical features. The sample were freeze-dried and sprinkled onto piece double adhesive carbon tape that was attached to an aluminum stub coated with a gold sputter module in a high vacuum evaporator using argon (Brito Raj et al., 2019). Photographs at magnifications ranging from 10-300,000 $\times$ were taken after scanning the sample of SEM.

The surface characteristics and morphology of L-Xn-NLCs were analyzed using a high-resolution transmission electron microscope (HRTEM), specifically the Hitachi H-7500 model from Japan, operating at 120 kV and equipped with a CCD camera. In order to perform TEM analysis L-Xn-NLCs were stained 1% aqueous solution of phosphor-tungstic acid (PTA). PTA is a common negative stain used in TEM analysis. A copper grid coated with pioloform and having a mesh size of 200 microns was used for mounting the sample. The film was examined under a TEM at 40–120 kV after drying for one hour.

4.2.5.7. Stability study

Stability studies were conducted to examine the stability of the drug and its formulation. Prior to analysis, S-Xn-NLCs powder was kept in a glass vial. It was stored at 40°C $\pm$ 2°C and 70 $\pm$ 5% relative humidity for 6 months. After 6 months, S-Xn-NLCs were analyzed for EE%, PS, ZP and *in vitro* release study (Varela-Fernández et al., 2022b).

4.2.6. In-Vitro A549 cell proliferation assay

4.2.6.1. Trypan blue staining

A trypan blue assay is a method used to determine viability by selectively staining non-viable cells with blue dye, while viable cells remain unstained. To assess the viability of A549 cells, 1 $\times$ 10⁵ cells per well were seeded in a 96-well plate. Following this, the cells were treated to pure Xn and S-Xn-NLC across various concentrations, ranging from 0 to 141 μ M, over a 24-hour period. The cells were detached using a trypsin-EDTA solution and centrifuged at 1200 g for 4 minutes. Afterward, the cells were combined with a 0.4% trypan blue solution in equal parts to distinguish live cells from dead ones. Using a light microscope at 10 $\times$ magnification, the live cells were counted, and the total number of cells was determined.

4.2.6.2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay)

The anti-cancer efficacy in terms of cytotoxicity for S-Xn-NLCs was evaluated by carrying

Experimental work

MTT assay on human lung adenocarcinoma cell line, A549. The cells for the current research have been provided by the National Centre for Cell Science (NCCS), Pune. The culturing of cells was done in DMEM which containing a mixture of FBS (10% v/v) and penicillin-streptomycin (1% v/v). Cells were grown in an incubator maintained with 37°C temperatures and 5% CO₂. For MTT assay, A549 were seeded on a 96-well plate. Next day, cells were treated with pure-Xn mixed in DMSO or S-Xn-NLCs powder at five different concentrations of 0 µM, 28.21 µM, 56.42 µM, 84.63 µM, 112.84 µM and 141 µM for 24 h. For comparison, DMSO only (final concentration less than 0.1%) were taken in case of pure Xn. Similarly, placebo of NLCs powder were taken as controls for comparing the effect of S-Xn-NLCs. After 24h, 10 µl solution of 5mg/mL MTT solution prepared in PBS was added and incubated for 4 hours. After incubation, the supernatant was removed and DMSO (100µL) was added to solubilize formazan (purple color) (J. Li et al., 2025; Nagabhishek et al., 2019; Sirpu Natesh et al., 2018a, 2018b; Szliszka et al., 2009a, 2009b). The absorbance of developed purple colors in each cell was recorded at 540 nm using a microplate reader (EnSpire Perkin Elmer, USA). The control group's proliferation rate was set at 100%, and the percentage of cell viability was then calculated in relation to the cells treated with S-Xn-NLCs. The concentration that resulted in 50% inhibition of cell growth in comparison to control cells was identified by calculating the IC₅₀ value using the following formula. The cell cytotoxicity (MTT assay) assay was performed based on the previously published protocol (Moussa et al., 2025; Mustafa et al., 2025).

$$\text{(\% Inhibition)} = \frac{\text{Mean OD of untreated cells} - \text{Mean OD of treated cells}}{\text{Mean OD of untreated cells}} \times 100$$

4.2.6.3. Determination of mitochondria membrane potential ($\Delta\Psi_m$)

Changes in mitochondrial membrane potential ($\Delta\Psi_m$) (MMP) can be used to analyze mitochondrial function, which is an important sign of a healthy cell. Using the technique described by Abe and colleagues, the retention of rhodamine 123 was used to assess the changes in the mitochondrial membrane potential ($\Delta\Psi_m$) in the A549 cells (after treatment). This cationic dye binds to metabolically active mitochondria. To correlate the findings of MTT cell proliferation, we performed the rhodamine 123 assay staining of the A549 cells seeded in a cover slip inside a 6-well plate and were treated with the IC₅₀ concentrations of pure-Xn and S-Xn-NLCs was for 24h. Untreated cells were thought to be negative (-) control. After 24h, the cells were washed in PBS, fixed with 4% paraformaldehyde and stained with 2 µM Rhodamine 123 in a mounting media and inoculated for about 30 min in dark condition to stain mitochondria. After cover slipping in a microscopic glass slide, the cells were examined under

a fluorescent microscope, and images were taken at 20x magnification (EVOS FI inverted Fluorescence Microscope). The cells were examined for changes in mitochondrial membrane potential ($\Delta\Psi_m$) by using a fluorescence microscope, which operated at excitation and emission wavelengths of 505 nm and 534 nm, respectively (Bagchi et al., 2016; J. Li et al., 2025; Nagabhishek et al., 2019; Sirpu Natesh et al., 2018b, 2018a; Szliszka et al., 2009b, 2009a), respectively. Degenerated mitochondrial membrane potential is indicated by a decrease in green fluorescence. ImageJ software was used to calculate fluorescence intensity measurement.

4.2.6.4. DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) Staining - ROS detection

DCFH-DA staining is a technique used to detect reactive oxygen species (ROS) in A549 cells, a human lung carcinoma cell line. The A549 cells seeded in a six-well plate. The IC₅₀ concentration of Pure Xn and Xn-NLCs were added in the well plates and incubated for 24 h. Then, the fluorescent dye, DCFH-DA (10 μ M), was added with working solution diluted with serum-free liquid and co-incubated for 10 min at 37 °C. Following dye loading, cells were washed twice with HBSS to remove extracellular probe and immediately imaged on an inverted fluorescence microscope using a FITC filter set (Ex ~485 nm / Em ~530 nm) with a 20 $\times$ objective; exposure time and camera gain were kept identical for all groups (Bagchi et al., 2016; J. Li et al., 2025; Nagabhishek et al., 2019; Sirpu Natesh et al., 2018b, 2018a; Szliszka et al., 2009b, 2009a).

4.2.6.5. Wound healing (cell migration) assay

A549 cells were initially placed in 6-well plates at a density of 1×10^5 cells per well and allowed to adhere overnight. Once they attained confluency, a 10 μ l pipette tip was used to create scratches, and PBS was employed to wash away cellular debris. Subsequently, the cells were exposed to the IC₅₀ concentration of pure Xn and Xn-NLCs for a duration of 24 hours. The distance between the two scratched edges of the monolayer was measured using IS capture software, after which the percentage of wound closure was calculated. Photographs of the wound closure were taken at 0 hours, 24 hours and 48 hours using a microscope, and the distance between the edges was then measured with ImageJ software (version 1.53c, Bethesda, MD, USA) (Bagchi et al., 2016; J. Li et al., 2025; Nagabhishek et al., 2019; Sirpu Natesh et al., 2018b, 2018a; Szliszka et al., 2009b, 2009a).

4.2.6.6. Boyden's chamber assay

The trans-well invasion assay was carried out using specialized invasion chambers to evaluate the migratory behavior of A549 cells. This procedure utilized trans-well permeable supports

Experimental work

specifically designed for such assays, namely Ibidi Transwells 3464, equipped with a 6.5 mm insert and an 8 μm pore-sized polycarbonate membrane. To prepare the assay, the lower surface of the membranes was coated with 2.5% gelatin in a 1 M acetic acid solution for one hour. Subsequently, a concentration of 1×10^5 cells/mL was seeded into the upper chamber containing 200 μL of DMEM medium. The chamber was subsequently positioned within a well that held 600 μL of DMEM, facilitating the assay. Following a 24-hour incubation period in a humidified chamber maintained at 37°C, the cells were exposed to the IC₅₀ concentration of pure Xn and S-Xn-NLCs, subsequently allowing them to migrate for an additional 24 hours. Cells from the upper surface were carefully removed with a cotton swab, while the lower chamber's cells that migrated to the membrane's bottom surface were stained with crystal violet. Prior to staining, these cells were fixed in a 10% formalin solution to ensure preservation. The membranes were subsequently placed on microscope slides, where the invaded cells were observed and counted using a microscope at 20x magnification. Cell counts were performed in five random fields per chamber employing a phase contrast microscope, followed by statistical analysis of the data (Bagchi et al., 2016; J. Li et al., 2025; Nagabhishek et al., 2019; Sirpu Natesh et al., 2018b, 2018a; Szliszka et al., 2009b, 2009a).

4.2.6.7. Detection of apoptosis- Flow cytometry analysis

4.2.6.7.1. Apoptosis by PI and AO/EtBr Staining method

The nuclear morphology was examined using PI and AO/EtBr staining to determine if the cell death of A549 was due to apoptosis. A549 cells were seeded (2×10^5 cells/well) in a 6-well plate and cultured in a CO₂ incubator for the whole night. After the incubation period, the cells were treated with the IC₅₀ concentrations of pure Xn and S-Xn-NLCs for 24 hours. After 24 hours, cells were gently washed with PBS and stained with PI (10 $\mu\text{g/mL}$) for 5 minutes in dark conditions. The morphology of apoptotic cells was studied and photographed using an EVOS FL inverted fluorescence microscope.

The AO/EtBr staining technique is used to visualize nuclear changes and distinguish between viable, apoptotic (early or late phase), and necrotic cells. After incubation, cells underwent trypsinization, and a 25 μL portion of the cell suspension was carefully placed onto a glass slide. Following its preparation, the sample was combined with 1 μL of dual fluorescent staining solution consisting of 10 $\mu\text{g/mL}$ AO and 10 $\mu\text{g/mL}$ EB, covered with a coverslip. The morphological characteristics of apoptotic cells were analyzed and captured using an EVOS FL inverted fluorescence microscope (Bagchi et al., 2016; J. Li et al., 2025; Nagabhishek et

al., 2019; Sirpu Natesh et al., 2018b, 2018a; Szliszka et al., 2009b, 2009a).

4.2.6.7.2. Cell cycle analysis - G0-G1, S, and G2/M phases

Cell cycle analysis was conducted using FACS CytoFlex from Beckman Coulter following the cell preparation protocol outlined by Davis et al. (1990). A549 cells, at a density of (2×10^6), were exposed to an IC₅₀ concentration for 24 hours. Post-incubation, the cells underwent trypsinization with 0.05% trypsin-EDTA, followed by thorough washing with media via vortexing. The cells were subsequently centrifuged at 2000 rpm for five minutes at room temperature, and the supernatant was discarded. The pellet formed was then resuspended in ice-cold 70% ethanol and stored at -20°C for four hours. Afterwards, the cells underwent a PBS wash and were resuspended in 250 µL of PBS containing 100 µg/mL RNase A and 40 µg/mL. They were then stained with propidium iodide (PI) at a concentration of 100 µg/mL, protected from light, for 30 minutes at room temperature. The analysis of the data was conducted utilizing DIVA software to ascertain the proportion of cells present in the various phases of the cell cycle, specifically G0-G1, S, and G2/M phases (Bagchi et al., 2016; J. Li et al., 2025; Nagabhishek et al., 2019; Sirpu Natesh et al., 2018b, 2018a; Szliszka et al., 2009b, 2009a).

4.2.6.8. Gene expression analysis-Reverse transcriptase-quantitative polymerase chain reaction (q-RT-PCR)

4.2.6.8.1. Cell treatment and harvesting

A549 cells were initially placed in 6-well plates at a density of 1×10^6 cells per well and allowed to adhere overnight. On the next day, the cells were exposed to the IC₅₀ concentration of pure-Xn and S-Xn-NLCs for a duration of 24 hours. Following this treatment period, the cells were washed three times using ice-cold PBS, then lysed and collected using 500 µL of TRIzol reagent. The resulting samples were subsequently stored at -80 °C until further analysis.

4.2.6.8.2. RNA extraction

The TRIzol technique, by Invitrogen in Germany, was used to efficiently extract total RNA from A549 cells. Initially, 200 µl of chloroform was added to the cell lysate, and the mixture was incubated at 4°C for 5 minutes to allow phase separation through centrifugation at 12,000 g for 15 minutes at the same temperature. This process separated the liquid into an aqueous phase, which was then carefully collected. The aqueous phase, containing RNA, was combined with isopropanol and incubated at 4°C for 15 minutes to precipitate RNA pellets. The isopropanol was subsequently removed by centrifuging at 12,000 g at 4°C for 15 minutes, and

the RNA was washed with 1 ml of 75% ethanol. Finally, the RNA was dissolved in RNase-free water. The concentration and purity of the isolated RNA were assessed by determining absorbance at wavelengths of 260 nm and 280 nm using a NanoDrop spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA)(Bagchi et al., 2016; J. Li et al., 2025; Nagabhishek et al., 2019; Sirpu Natesh et al., 2018b, 2018a; Szliszka et al., 2009b, 2009a).

4.2.6.8.3. Reverse Transcription

The RNA samples were initially diluted to a concentration of 100 ng/μL using nuclease-free water. For each cDNA synthesis reaction, a 20 μL reaction mixture was prepared on ice using the Bio-Rad iScript™ cDNA Synthesis Kit, as follows: (4 μL of 5× iScript Reaction Mix; 1 μL of iScript Reverse Transcriptase; 1 μg of total RNA; Nuclease-free water was added to bring the final volume to 20 μL). The reverse transcription was performed in an Eppendorf Flexlid Master cycler Nexus thermal cycler (Hamburg, Germany) with the following program: denaturation (65°C, 10 min); annealing (25°C, 10 min), reverse transcription (46°C, 20 min), and reverse transcriptase inactivation (95°C, 1 min). The synthesized complementary DNA (cDNA) was preserved at a temperature of -20°C prior to its utilization in quantitative real-time PCR (qPCR) analysis (Bagchi et al., 2016; J. Li et al., 2025; Nagabhishek et al., 2019; Sirpu Natesh et al., 2018b, 2018a; Szliszka et al., 2009b, 2009a).

4.2.6.8.4. Real-Time qPCR

An aliquot of 4 μL of cDNA was precisely measured and subjected to real-time quantitative PCR analysis utilizing 10 μL of 2× iTaq™ Universal SYBR Green (BioRad, Hercules, CA, USA), along with primers in forward and reverse orientations (1 μL each) and 4 μL of nuclease-free water. This procedure was executed via the CFX96 PCR system (BioRad). The real-time qPCR involved thermal cycles of Denaturation 95°C for 30 s (1 cycle), Denaturation 95 °C for 15 s (40 cycles), and Annealing/Extension 60°C for 30 s (1 cycle). PCR data (Ct values) were initially processed using qPCR instrument software (Bio-Rad CFX Manager) and then normalizing target gene expression to a housekeeping gene (β-actin) in Excel. The β-actin gene served as the reference gene for the comparison. The relative expression using the $2^{-\Delta\Delta Ct}$ method, a common mathematical approach in quantitative studies. After determining the expression levels, they conducted statistical analyses utilizing the GraphPad Prism software to ensure precise and reliable interpretation of the data.

4.2.6.8.5. Nucleotide sequence of primers used qRT-PCR

<i>Gene Name</i>	<i>Forward sequence</i>	<i>Reverse sequence</i>
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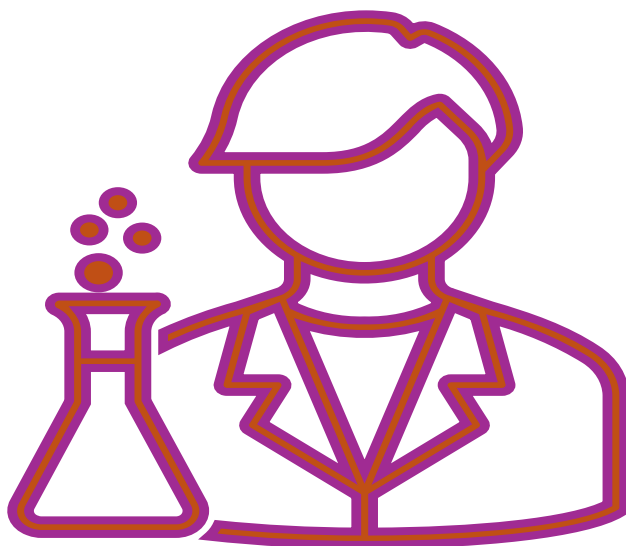
1) <i>β-actin</i>	<i>AGAGCTACGAGCTGCCTGAC</i>	<i>AGCACTGTGTTGGCGTACAG</i>
2) <i>P⁵³</i>	<i>CAGCACATGACGGAGGTTGT</i>	<i>TCATCCAAATACTCCACACGC</i>
3) <i>NF-κB</i>	<i>ATGGCTTCCTATGAGGCTGAG</i>	<i>GTTGTTGGTCTGGATTCTGTG</i>
4) <i>Caspases 3</i>	<i>CATGGAAGCGAATCAATGGACT</i>	<i>CTGTACCAGACCGAGATGTCA</i>
5) <i>BCL-2</i>	<i>GGTGGGGTCATGTGTGTGG</i>	<i>CGGTTCAGGTACTCAGTCATCC</i>

4.2.7. Statistical analysis

The statistical analysis for PS, ZP, and drug EE% was performed using analysis of variance (ANOVA). The paired t-test was used to evaluate the level of significance in saturation solubility, *in vitro* drug release, and cytotoxicity investigations, with p-values < 0.05 indicating significant results (Teaima et al., 2023). The data are presented as the mean ± SEM of 3 independent experiments. Analysis was conducted using GraphPad Prism software (GraphPad Software, USA). Statistical significance was determined at levels of p<0.05, p<0.01, or ***p<0.001, using Student's t-test or one-way analysis of variance (ANOVA), followed by the Dunnett multi-comparison test comparison and two-way ANOVA, followed by the Bonferroni post-test.



Chapter 5



Results and Discussion...

CHAPTER 5

Results and Discussion

5.1. Method development and Optimization

The Xn solution was scanned in the range of 200 to 800nm and its λ_{max} was found at 370nm (fig. 5.1b). The chromatographic conditions were analyzed by observing the peak of Xn in different solvents such as methanol, acetonitrile, acetic acid, and water. The peak of Xn was examined while different solvents were used in different ratios for selecting the chromatographic conditions for the development of the methods. The chromatogram was recorded at a wavelength of 370 nm. The aforementioned observations led to the determination of a certain ratio for the mobile phase, as well as the retention time and flow rate. Finally, the method was optimized wherein the stationary phase was an analytical column C18, 5 μ m, 250 $\times$ 4.6mm and mobile phase composition of methanol and water was ratio of 95:5 v/v. The optimized flow rate for the procedure was determined to be 1.0 mL/min, resulting in the chromatogram being recorded at a retention time of 4.53 minutes (fig. 5.1a). The 3D chromatogram was also recorded (fig. 5.1d). The peak purity index was noted for the developed chromatogram (fig. 5.1c), and it was found to be 0.99, which indicates that the developed method and chromatogram are free from any impurities. LOD and LOQ values were 0.85 and 2.6 μ g/mL (Singh et al., 2025).

5.1.1. System suitability and estimation of LOD and LOQ

To validate the system's appropriateness, the peak purity index, the theoretical plate, HETP, tailing factor, LOD, and LOQ were recorded as given in Table no.5.1. All the parameters were found within the limits. This shows that system suitability was good and sensitive (Singh et al., 2025).

Table 5.1. Results of System suitability and estimation of LOD and LOQ

S.NO	PARAMETER	VALUE
1	LOD	0.85 μ g/mL
2	LOQ	2.6 μ g/mL
3	Peak purity index	0.99
4	Theoretical Plate	6244.331 tR/W

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5	HETP	24.022
6	Tailing Factor	1.207
7	Area	1184845 (mAU*min)

5.1.2. Stability study of Xn solution

Placing the sample for one week at ambient temperature allowed us to assess the stability of the Xn standard MQC solution (6 μ g/ml). The concentration of the sample was analyzed for six days by injecting them into HPLC as shown in Table no. 5.2.

Table 5.2. Results of Solution Stability Study

Concentration (μ g/ml)		Area (mAU*min)							
Day	1	2	3	4	5	6	Mean Area (cm ²)	Stand ard deviati on ($\pm$)	%R SD
6	1175035 .00	1174082 .00	116657 4.00	118484 5.00	1140576 .00	119777 1.90	117314 7.26	19244. 53	1.6

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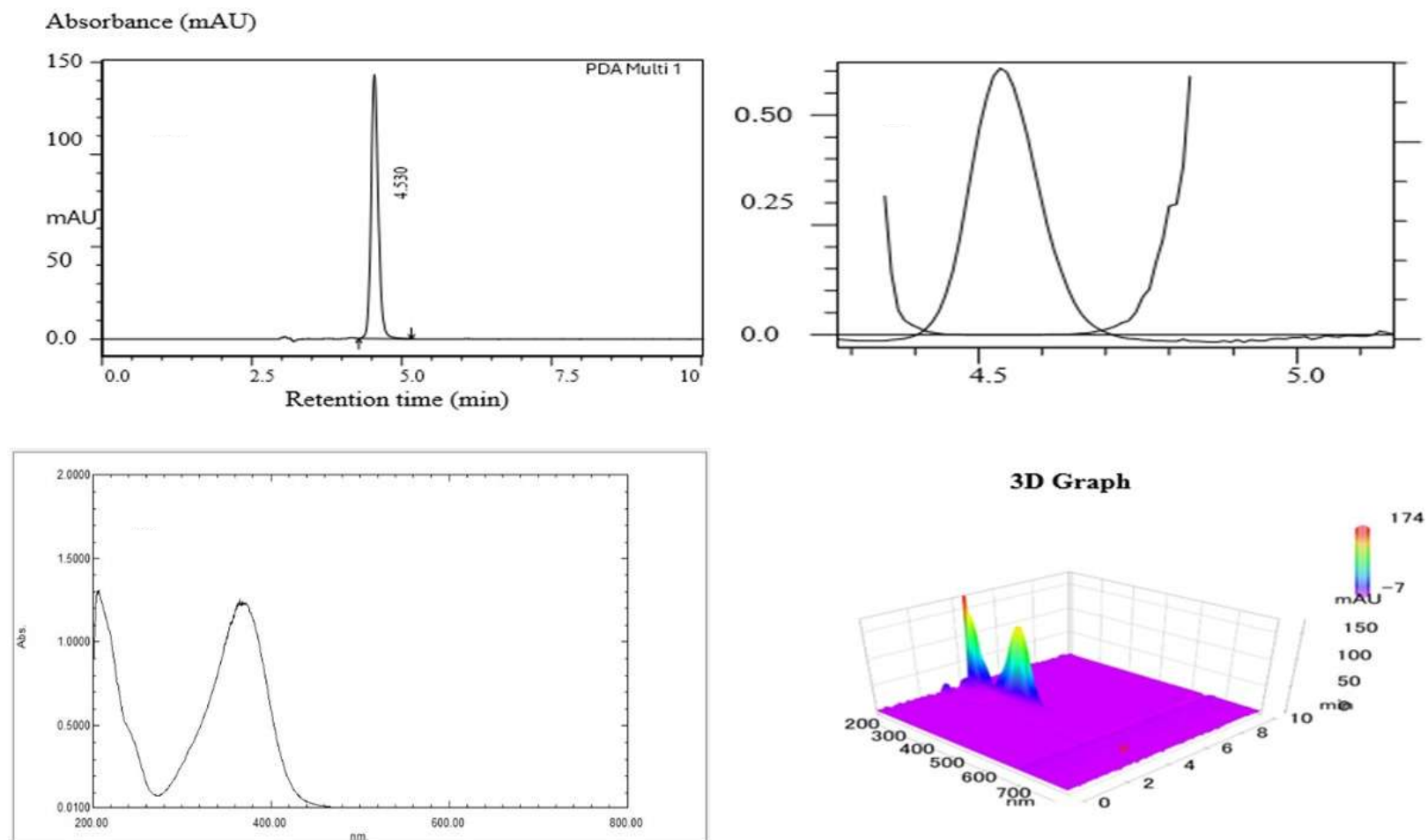


Fig. 5.1 : The figure represents **5.1 a)** HPLC- chromatograms monitored at 370 nm of Xn ; **5.1 b)** UV Spectrum of Xn ; **5.1 c)** Peak Purity index graph of Xn at Rt– 4.53 ; **5.1 d)** 3D graph of purity index of Xn at Rt – 4.530.

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5.1.3. Linearity and Range

The calibration curve was found linear in the range of 2-10 $\mu\text{g/mL}$ with R^2 value of 0.99, Slope - 192560x and intercept -25385. Using the least square linear regression method, linear regression analysis was used to evaluate the calibration curve's linearity. A calibration curve was developed between concentration and mean peak area and six injections were made into each sample in order to calculate the mean peak area as shown in fig 5.2. The curve was used to determine the regression equation and coefficient.

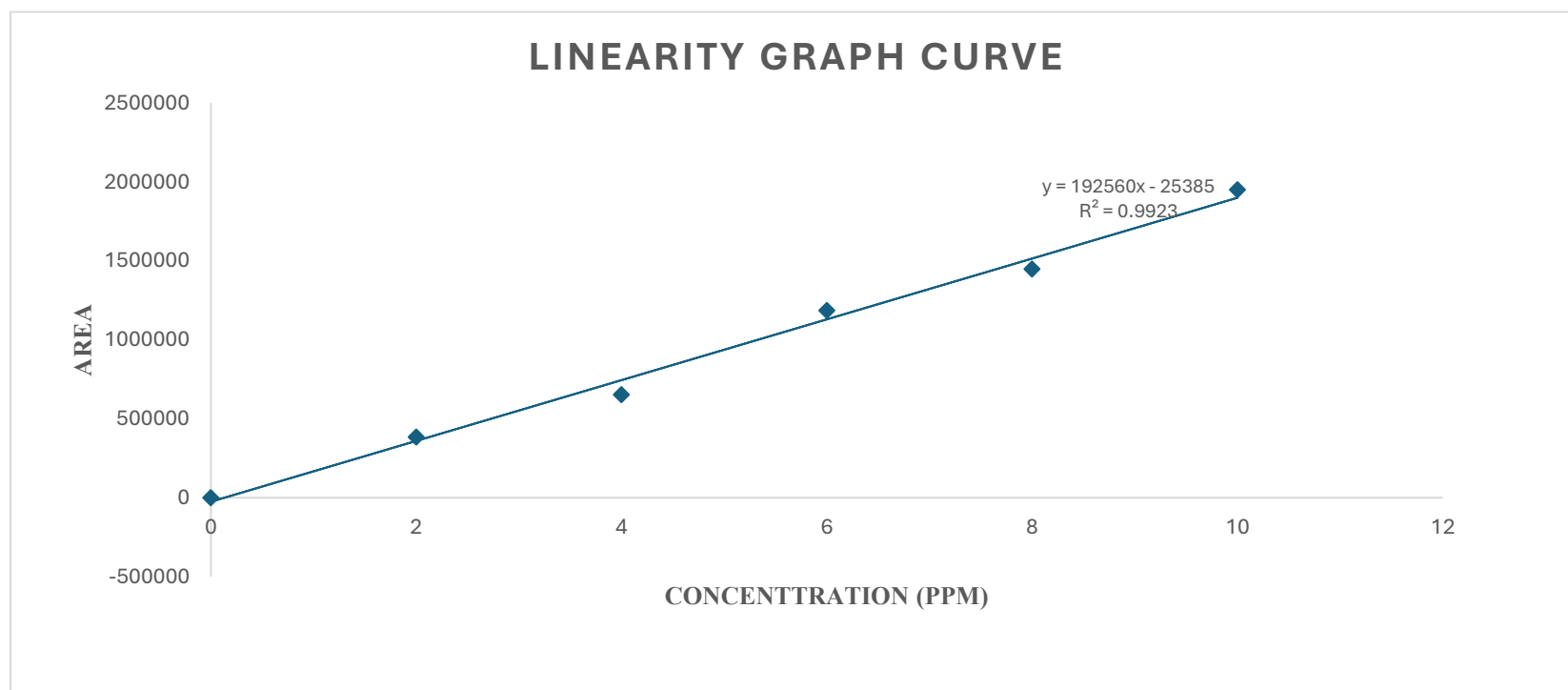
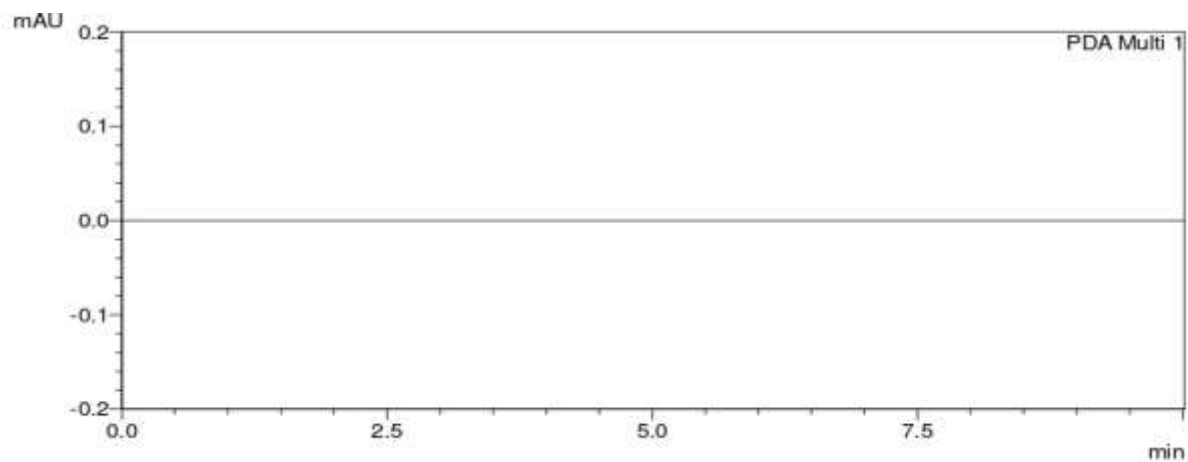


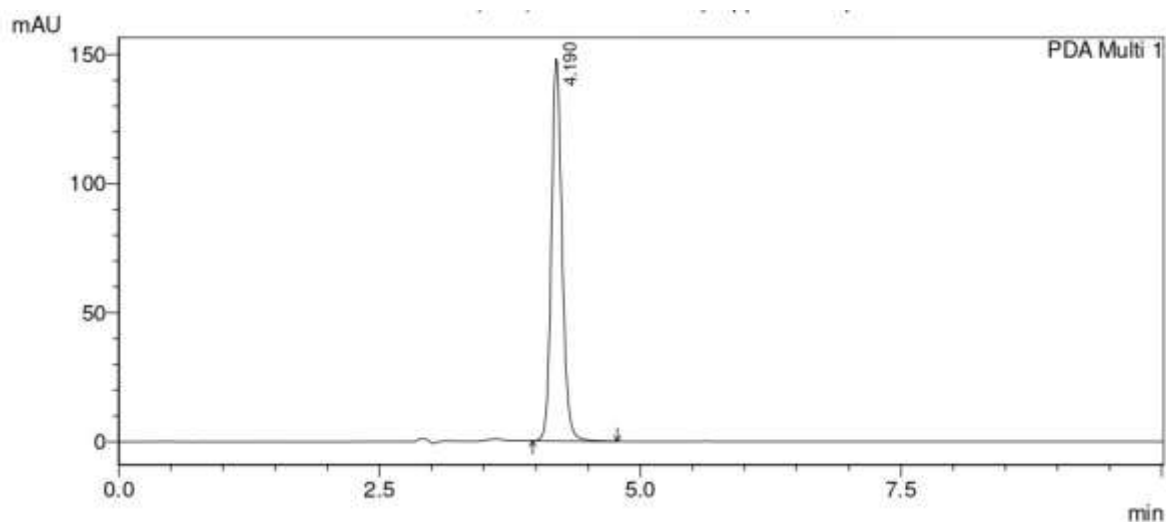
Fig. 5.2 : Linearity Graph Curve

5.1.4. Specificity

The chromatogram of pure Xn solution (6 $\mu\text{g/mL}$) is shown in fig. 5.3c, NLCs placebo and NLCs containing Xn is shown in fig. 5.3a and 5.3b. It can be observed that there is an absence of any additional peak in the placebo solution and NLCs Xn solution at the retention of Xn. This indicates that the excipients used in the formulation of NLCs do not have any interference with Xn peak indicating specificity of method.



a



b

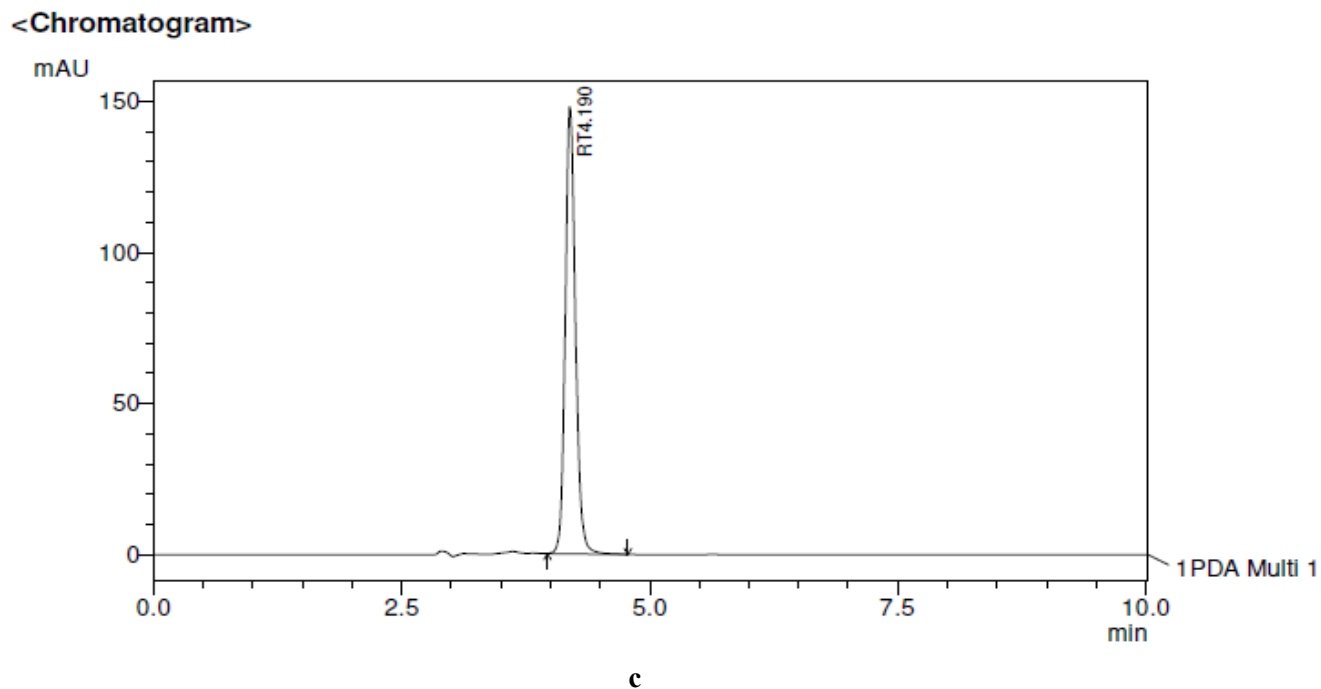


Fig 5.3 : The chromatogram represents **5.3 a)** Chromatogram of placebo formulation without drug; **5.3 b)** Chromatogram of NLCs formulation with drug; **5.3 c)** Chromatogram of standard Xn

5.1.5. Accuracy

The accuracy result shows a good stability of the standard solution of LQC , MQC , HQC which was indicated by the recovery studies. Accuracy results show a % recovery from 95 to 101 % and the mean recovery rate of 96.3% and mean percent RSD of 0.7, which was not greater than 2% as given in Table no. 5.3.

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Table 5.3. Accuracy Results

Level	Concentration($\mu\text{g}/\text{ml}$)		Area								
		1	2	3	y=Mean Area (cm^2)	m	c= Intercept	x= y- c/m	% recovery,	Mean % recovery	%RSD
LQC	4.8	847302.90	846463.00	836607.00	843457.57	187171.00	13063.00	4.43	92.29	96.32	0.7
MQC	6	1175035.00	1174082.00	1166574.00	1171897.00			6.10	101.00		0.4
HQC	7.2	1287682.00	1311004.00	1312123.00	1303603.03			6.89	95.69		1

5.1.6. Precision

The precision was assessed by calculating the Lower (LQC-80%), Medium (MQC-100%) and Higher (HQC-120%) sample on the same days (Intra-day), different days (Inter-day), and by different analysts (Inter-analyst) as shown in Table no. 5.4 (Forootan et al., 2017). The results of precision of Intraday, Inter-day and Inter-analyst were within acceptable limits and the % RSD was less than 2%. This indicates that the developed method was précised.

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Table 5.4. Results of precision studies

Parameter	Level	Concentration($\mu\text{g/ml}$)		Area							
			1	2	3	4	5	6	Mean Area (cm^2)	Standard deviation ($\pm$)	%RS D
Intraday											
	LQC	4.8	789935.00	794739.1 0	792949 .00	766902.0 0	763713. 10	795148.8 0	783897.83	14551.77	1.8
	MQC	6	1162793.20	1145416. 50	113109 7.80	1154518. 40	1121730 .00	1184845. 00	1150066.81	22696.80	1.9
	HQC	7.2	1307910.00	1272952. 00	128585 8.00	1316203. 70	1286355 .90	1247972. 50	1286208.68	24529.72	1.9
Parameter	Level	Concentration($\mu\text{g/ml}$)		Area							
			1	2	3				Mean Area (cm^2)	Standard deviation ($\pm$)	%RSD
Inter-day											
	LQC	4.8	789935 .00	795148. 80	792949 .00				792677.60	2617.47	0.33
DAY1	MQC	6	118484	1197771	115451				1179044.96	22202.59	0.1868

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			5.00	.90	8.00						
	HQC	7.2	1316203	1286355	128585				1296139.20	17378.14	1.34
			.70	.90	8.00						
	LQC	4.8	822142.	828054.	807031				819076.03	10841.70	1.3
			40	30	.40						
DAY2	MQC	6	1161045	1192977	118231				1178778.16	16256.28	1.3
			.90	.40	1.20						
	HQC	7.2	1316572	1339016	131726				1324284.50	12762.55	0.96
			.50	.00	5.00						
	LQC	4.8	809639.	794739.	822490				808956.56	13888.50	1.7
			70	10	.90						
DAY3	MQC	6	116657	1175035	114057				1160728.30	17958.10	1.5
			4.00	.20	5.70						
	HQC	7.2	1257140	1229603	121838				1235042.40	19942.94	1.6
			.60	.40	3.20						
Parameter	Level	Concentration(µg/ml)		Area							

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			1	2	3				Mean Area (cm ²)	Standard deviation (±)	%RSD
Inter-analyst											
	LQC	4.8	780375. 60	811627. 00	799846 .90				797283.17	15782.65	1.9
Analyst1	MQC	6	110545 9.00	1116669 .90	111567 0.00				1112599.63	6204.15	0.55
	HQC	7.2	125791 0.00	1271831 .60	122834 6.80				1252696.13	22206.31	1.7
	LQC	4.8	834096. 10	836607. 20	819031 .20				829911.50	9505.89	1.1
Analyst2	MQC	6	114932 5.70	1108143 .00	113027 5.00				1129247.90	20610.55	1.8
	HQC	7.2	129682 4.20	1307910 .50	131588 5.00				1306873.23	9572.64	0.73
	LQC	4.8	847302. 90	839412. 90	846462 .60				844392.80	4333.13	0.05
Analyst3	MQC	6	117408	1147742	118522				1169014.96	19246.40	1.64

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			1.90	.00	1.00						
	HQC	7.2	128768	1311003	131212				1303603.03	13799.37	1
			2.00	.80	3.30						

5.1.7. Robustness

The robustness of the analytical method was tested by examining the effects of internal minor adjustments alteration on system suitability parameters in HPLC settings as shown in Table no. 5.5. The findings of the intrinsic factor test demonstrated that the little adjustments to the chromatographic procedure parameters, like the rate of flow, and the ratio of the mobile phase, are reliable and fall within permissible bounds and the % RSD was less than 2%.

Table 5.5. Results of Robustness Study

Parameter	mL/min	Concentratio n(µg/ml)	1	Area 2	3	Mean Area (cm ²) Standard deviation (±)	%RSD
Flow Rate							
	1	6	1128588.00	1127009.00	1122902.00	1126166.23 2935.12	0.26
	0.8	6	1073607.00	1058967.00	1050884.00	1061152.33 11518.11	1
	1.2	6	909219.60	904892.70	910149.00	908087.10 2805.19	0.3
Parameter		Concentratio n(µg/ml)	1	Area 2	3	Mean Area (cm ²)	%RSD

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						Standard deviation (±)		
Mobile Phase Ratio %								
MeOH %	Water %							
97	3	6	1137547.00	1105109.00	1118252.00	1120302.36	16315.92	1.4
93	7	6	1125050.00	1117898.00	1124714.00	1122553.73	4035.83	0.35
95	5	6	1184243.00	1171705.00	1148222.00	1168056.66	18285.53	1.5

5.2. Characterization of active Pharmaceuticals ingredients (API)

Characterization of Xn was carried out to determine its physical nature, melting point.

5.2.1. Physical description of Drug

About 10 mg of (0.01g) Xn were taken and spread on a glass slide. The Xn identification of color of the drug was performed through personal inspection. The results are given in Table 5.6.

Table 5.6. Physical description of Xn

Xn			
Sr. No.	Property	Observation	Literature Report
1.	Color	Yellow	Yellow crystalline

5.2.1.1. Melting Point

The melting point of the drug was determined utilizing both the capillary fusion method and the differential scanning calorimetry (DSC) technique.

5.2.1.2. Capillary fusion method

Capillary fusion apparatus was used to determine the melting point of Xn. The 2 capillaries were taken and sealed at one end by heating on a flame. The sample Xn was placed in a thin-walled capillary tube of 10-15cm length and a diameter of 1 mm. The capillary containing the drug and a thermometer were placed in the melting point apparatus and was heated evenly. The observed melting point of Xn was found to be 172-173°C as shown in fig. 5.4 and Table 5.7.

Table 5.7. Melting point of Xn using capillary fusion method

Sr. No.	Drug	Observed value	Reported value	Reference
1.	Xn	172-173°C	173°C	(Sánchez-Aguinagalde et al., 2021)



Fig. 5.4 : Melting point determination of Xn drug by Capillary fusion method

5.2.1.3. Differential Scanning Calorimetry (DSC)

The DSC of pure Xn was evaluated by the Perkin Elmer DSC Apparatus. Powdered sample (2-3mg) was taken in aluminum pan, heated over the range of 28-400°C at 10°C/min using a dry-nitrogen flush at rate of 19.8mL/min. The thermograph of Xn showed a sharp endothermic peak at 173.17 °C and ΔH was 104.6 J/g is shown in fig.5.5 and results are given in Table 5.8. It was concluded that Xn was a yellow crystalline powder in nature.

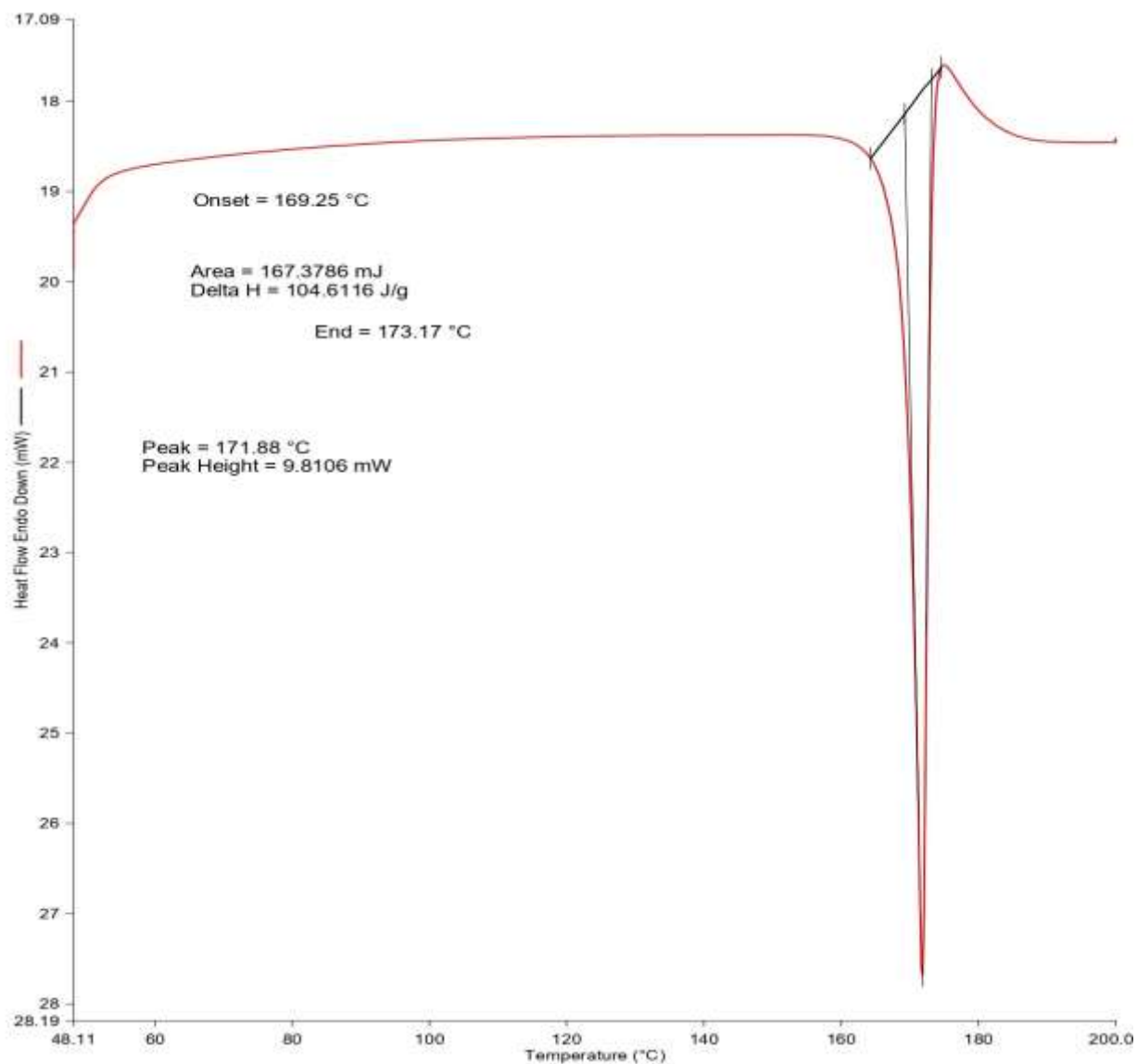


Fig. 5.5 : DSC thermogram of Xn

Table 5.8. DSC results of pure Xn

Sr. No.	Observed Endothermic peak	Reported Endothermic peak	Reference
1.	173.17°C	172-173°C	(Sánchez-Aguinagalde et al., 2021)

5.2.2. Determination of log P

The log P value of Xn was found to be 3.9. This shows that the drug is extremely hydrophobic, which results in poor aqueous solubility.

5.2.3. Powder X-ray diffraction (PXRD)

Xn showed sharp peaks at various diffraction angles, exhibiting a typical crystalline pattern. This was understood by observing diffraction peaks appeared in the diffractogram of Xn as shown in fig.5.6 and Table 5.9. For instance, appearance of sharp peaks at (2θ) (7.435, 11.661, 12.788, 13.326, 17.629, 18.295, 19.934, 22.880, 25.211) with a peak count of 1524, 791, 636, 804, 1292, 1600, 1153, 3100, 4311 respectively, indicated as sharp peaks.

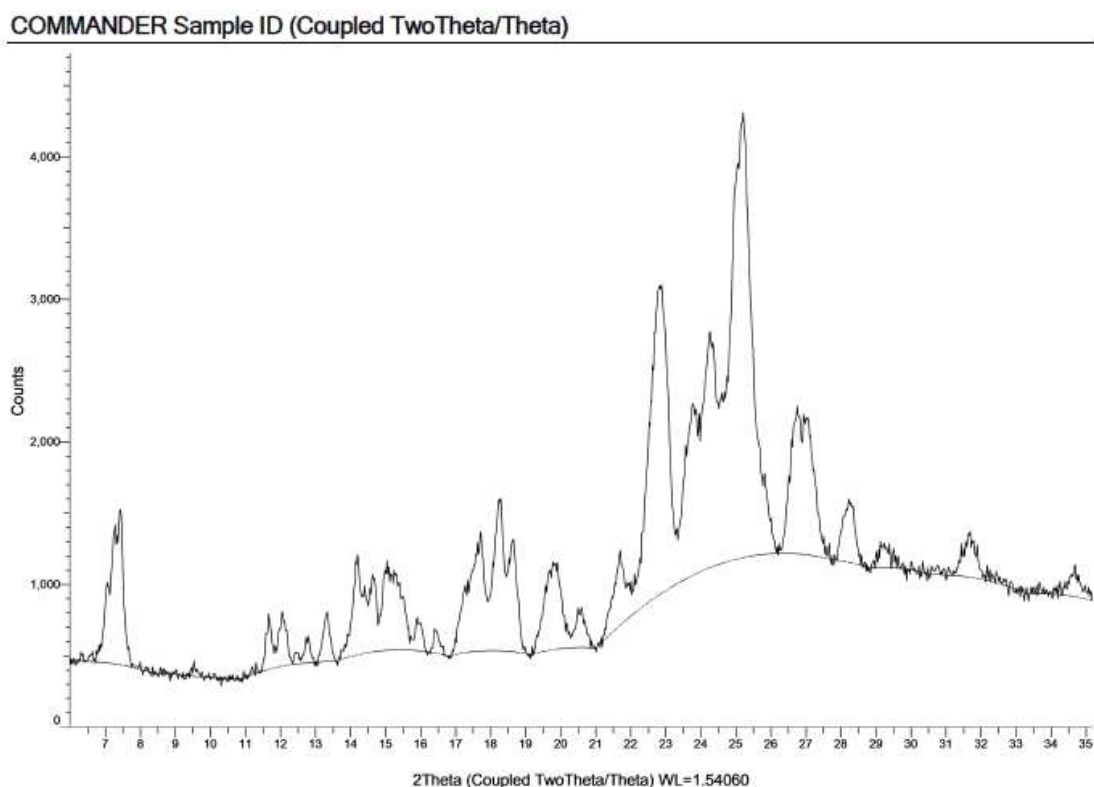


Fig. 5.6 : PXRD of Xn shows sharp peaks at (2θ) that exhibit a crystalline nature of drug

Table 5.9. PXRD results of pure Xn

Sr. No.	Observed XRD Peaks (At 2θ diffraction angle)
1.	(2θ) (7.435, 11.661, 12.788, 13.326, 17.629, 18.295, 19.934, 22.880, 25.211)

5.2.4. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR of Xn was determined by using Perkin Elmer by (1-2mg of drug) direct sampling method (Arczewska et al., 2013b). The bands that were captured covered a range of wavelengths from 4000 cm^{-1} to 400 cm^{-1} . The major functional group was identified by the FTIR and vibrational frequency cm^{-1} observed O-H (Hydroxyl group) stretching was found to be 3675.19 cm^{-1} , -OCH₃ (Methoxy group) stretching was found to be 2901.01 cm^{-1} , C=O (Carbonyl group) stretching was found to be 1600.21 cm^{-1} , C₆H₆ (Benzene) stretching was found to be 1218.89 cm^{-1} as given in

Table 5.10. All major groups were present that confirms the identification of drug as shown in fig.5.7.

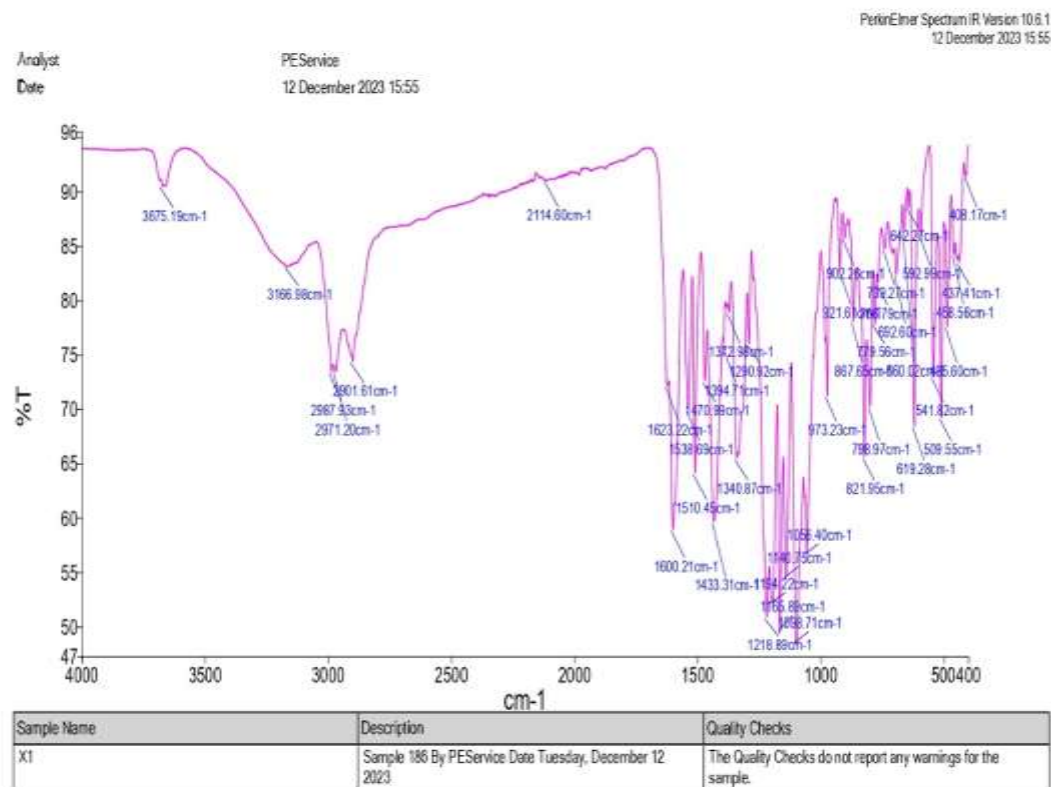


Fig. 5.7 : FTIR spectrum of Xn

Table 5.10. FTIR interpretation of Xn

Sr. No.	Vibrational frequency range cm ⁻¹	Major functional groups present	Observed vibrational frequency cm ⁻¹
1.	3400-3700	O-H stretching	3675.19
2.	2800-3100	-OCH ₃ stretching	2901.01
3.	1600-1740	C=O stretching	1600.21

4.	1600-200	Benzene	1218.89
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5.3. Solubility studies and method of preparation for NLCs

5.3.1. Screening of solid & liquid lipids and surfactant

During the screening of solid, liquid lipids and surfactants, the Xn solubility in solid lipids was found to be 34.6 ± 2.25 mg/mL in Beeswax, 24.00 ± 1.05 mg/mL in Compritol 888, 29.31 ± 1.04 mg/mL in GMS as shown in fig.5.8. While the Xn solubility in liquid lipids was found to be 4.60 ± 0.15 mg/mL in Sunflower oil, 4.30 ± 1.07 mg/mL in Olive oil, 9.0 ± 1.27 mg/mL in Glyceryl monooleate, 4.60 ± 0.13 mg/mL in α -Tocopheryl acetate, 19.0 ± 0.81 mg/mL in Labrafac-PG , 24.60 ± 1.00 mg/mL in Lauroglycol-90, 19.60 ± 0.93 mg/mL in Lauro-glycol -FCC and 14.0 ± 1.93 mg/mL in Caproyl PGMC as shown in fig.5.9. The solubility of Xn in surfactant was found to be 4.6 ± 1.25 mg/mL in Tween 20, 24.6 ± 2.55 mg/mL in Tween 80, 14.3 ± 1.0 mg/mL in Transcutol P, 4.3 ± 1.00 mg/mL in Span 20, 4.3 ± 1.56 mg/mL in Span 80 and 24.3 ± 0.25 mg/mL in Poloxamer-188. The study's findings indicated that Xn had a higher solubility in Beeswax (34.6 ± 2.25 mg/mL) among solid lipids; Lauroglycol-90 (24.60 ± 0.93 mg/mL) as liquid lipid and Tween 80 (24.6 ± 2.55 mg/mL) as surfactant as shown in fig.5.10. As a result, for the NLCs formulation, Beeswax and Lauroglycol-90 were selected as the solid and liquid lipids. Since non-ionic surfactants are pH-independent, they are highly stable, compatible, and have low toxicity (Prieto & Calvo, 2013) The solubility Xn was found to be higher in Tween 80 (24.6 ± 2.55 mg/mL) than in other ionic or non-ionic surfactants. As a result, in the NLCs formulation Tween 80 was selected as the surfactant.

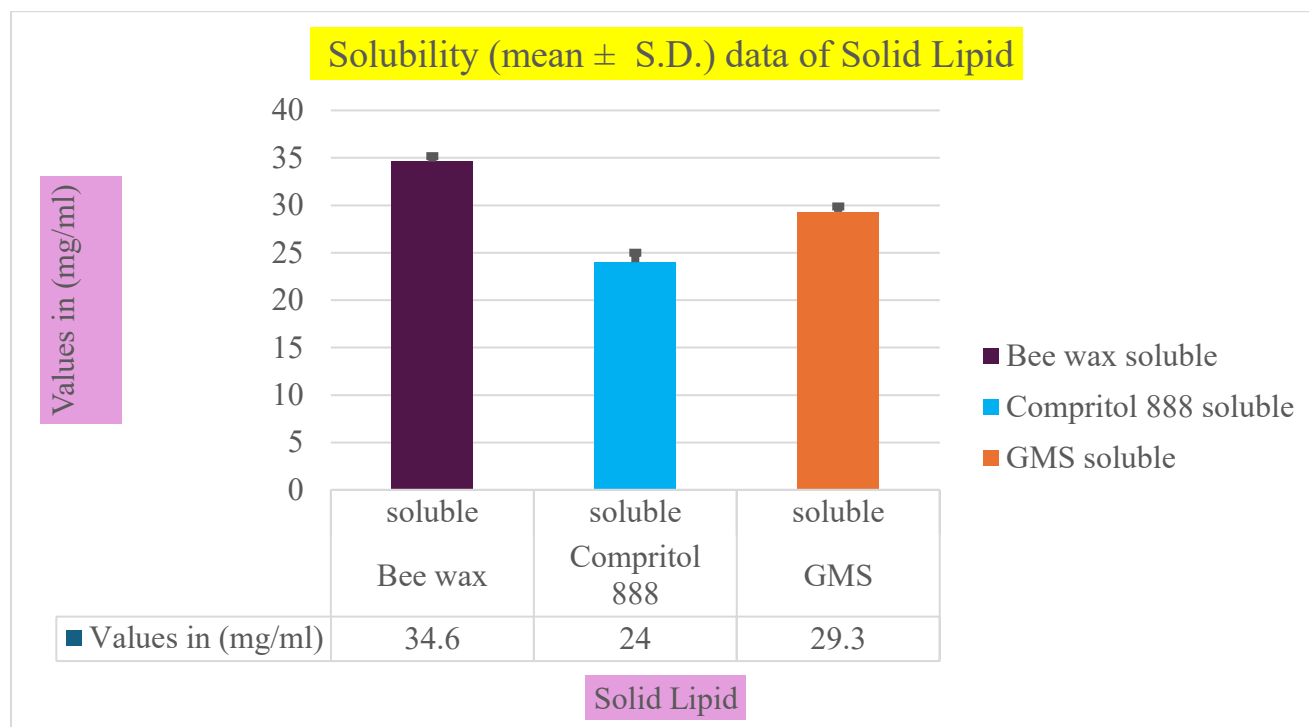


Fig. 5.8 : Solubility data (mean $\pm$ S.D; n=3) of Xn in solid lipids

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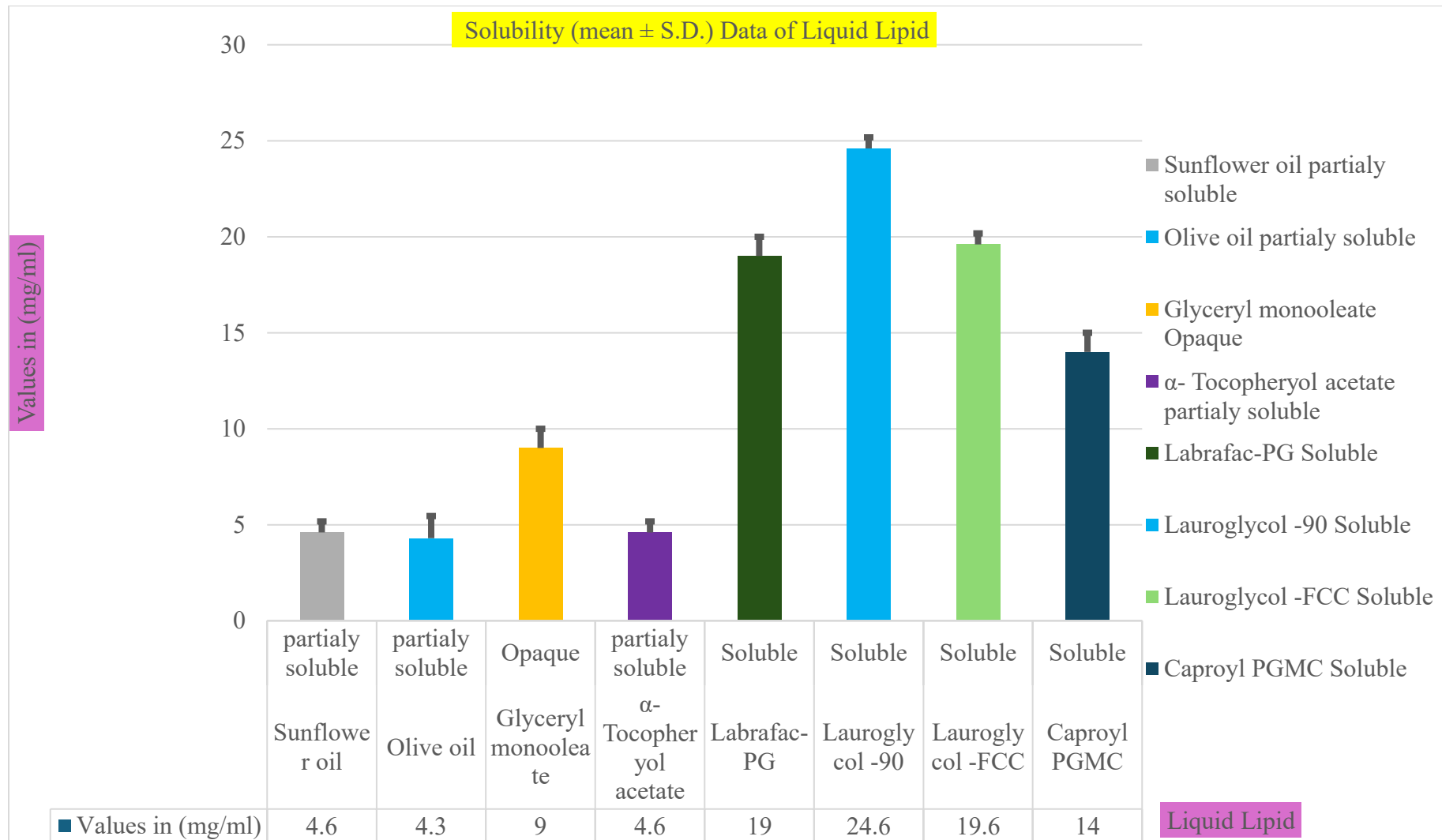


Fig. 5.9 : Solubility data (mean $\pm$ S.D; n=3) of Xn in liquid lipids

Results and discussion

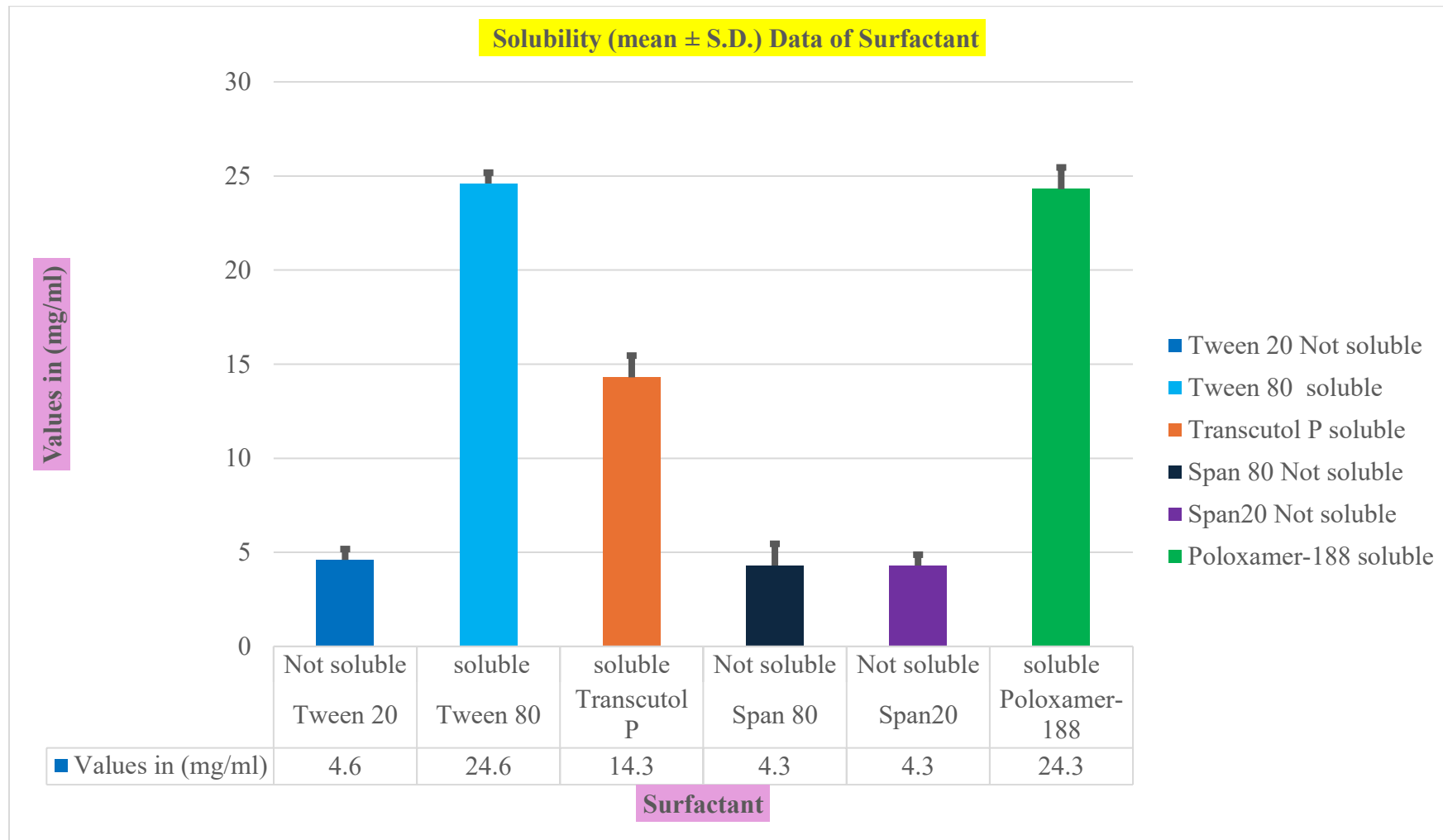


Fig. 5.10 : Solubility data (mean $\pm$ S.D; n=3) of Xn in surfactants

5.3.2. Method of preparation for NLCs

Formulation of NLCs was done by “hot high-pressure homogenization” and “ultrasonication”. Initially, Xn was incorporated into molten beeswax, followed by the addition of lauroglycol-90. The resultant mixture was subsequently transferred to a 50 mL glass beaker and subjected to heating until it reached a temperature of 80°C. In this procedure, an aqueous solution of Tween-80 (100 mL) was incorporated into the heated mixture as shown in fig. 5.11 a & b. The temperature of the mixture was maintained at 80°C, which exceeded the melting point of beeswax. The hot dispersion was stirred at 2000 rpm for 30 minutes in order to form homogeneous emulsion. This emulsion was subjected to a high pressure homogenizer, wherein it was homogenized for 3 cycles at a pressure of 700 bar. The Homogenized NLCs were subjected for ultrasonication at 37°C for 10 minutes. The NLCs were allowed to stabilize at room temperature.

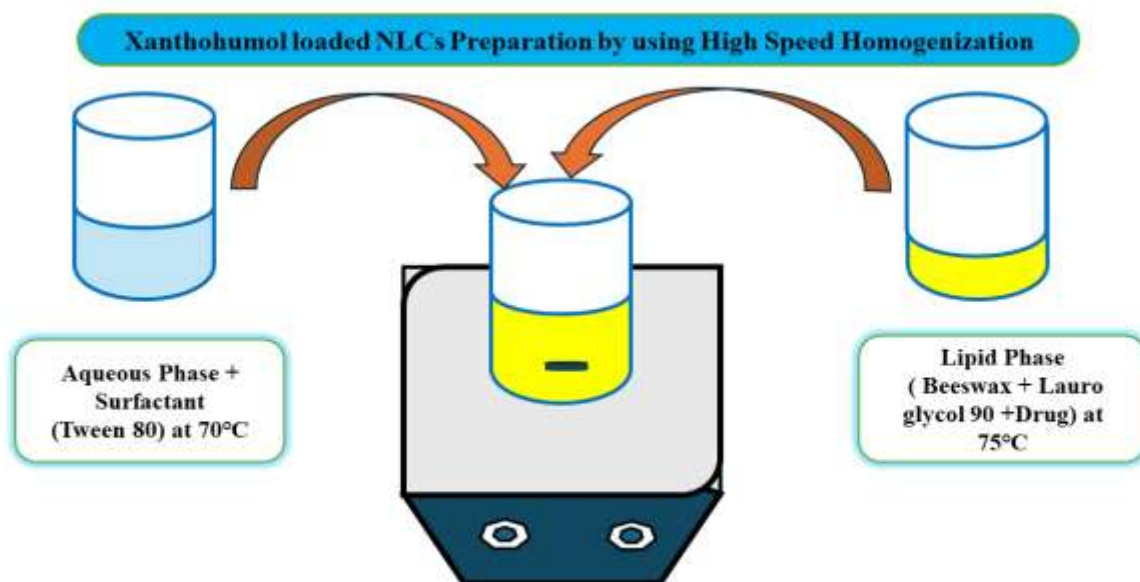


Fig. 5.11a : Xn-loaded NLCs are prepared using ultrasonication and high-speed homogenization techniques.

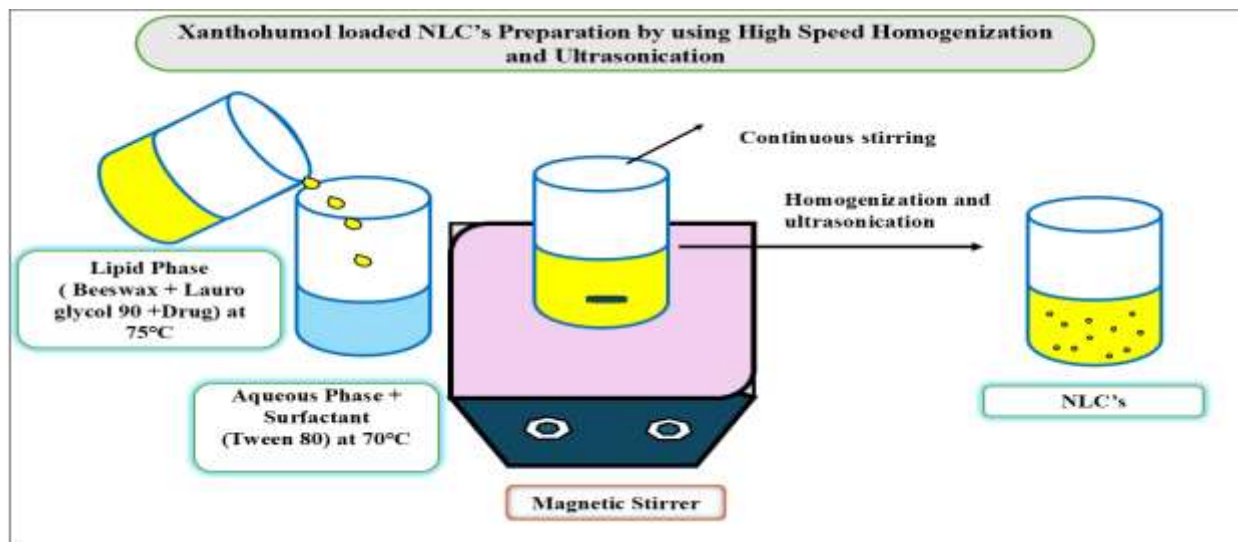


Fig. 5.11b : Xn-loaded NLCs are prepared using ultrasonication and high-speed homogenization techniques.

5.3.3. Ternary phase diagram (TPD)

The TPD was constructed wherein the solid lipid-liquid lipid to surfactant ratio varied as 1:1, 1:2, 2:1. This resulted in development of 27 NLCs formulation as shown in fig.5.12. The composition of all formulations is shown in Table 5.11. Formulation appearing transparent were considered as NLCs. The transparency was observed for formulation F1, F4, F5, F6, F10, F14, F15, F19, F20, F21, F23 and F24. In order to assess the most suitable range of formulation for further optimization using BBD. PS, ZP and EE% of these formulations were also evaluated.

Table 5.11. TPD

Batches (1:1)	Solid lipid (Beeswax) (μl)	Surfactant (Tween 80) (μl)	Liquid lipid (Lauroglycol-90)(μl)	Water (100ml)	Appearance
F1	50	900	50	100	Transparent
F2	100	800	100	100	Turbid
F3	150	700	150	100	Turbid
F4	200	600	200	100	Transparent
F5	250	500	250	100	Transparent
F6	300	400	300	100	Transparent

Results and discussion

F7	350	300	350	100	Turbid
F8	400	200	400	100	Turbid
F9	450	100	450	100	Turbid
Batches (1:2)	Solid lipid (Beeswax) (μl)	Surfactant (Tween 80) (μl)	Liquid lipid (Lauroglycol-90)(μl)	Water (100ml)	Appearance
F10	30	900	70	100	Transparent
F11	60	800	140	100	Turbid
F12	100	700	200	100	Turbid
F13	130	600	270	100	Turbid
F14	160	500	340	100	Transparent
F15	200	400	400	100	Transparent
F16	230	300	470	100	Turbid
F17	260	200	540	100	Turbid
F18	300	100	600	100	Turbid
Batches (2:1)	Solid lipid (Beeswax) (μl)	Surfactant (Tween 80) (μl)	Liquid lipid (Lauroglycol-90)(μl)	Water (100ml)	Appearance
F19	70	900	30	100	Transparent
F20	140	800	60	100	Transparent
F21	200	700	100	100	Transparent
F22	270	600	130	100	Transparent
F23	340	500	160	100	Transparent
F24	400	400	200	100	Transparent
F25	470	300	230	100	Turbid
F26	540	200	260	100	Turbid

F27	600	100	300	100	Turbid
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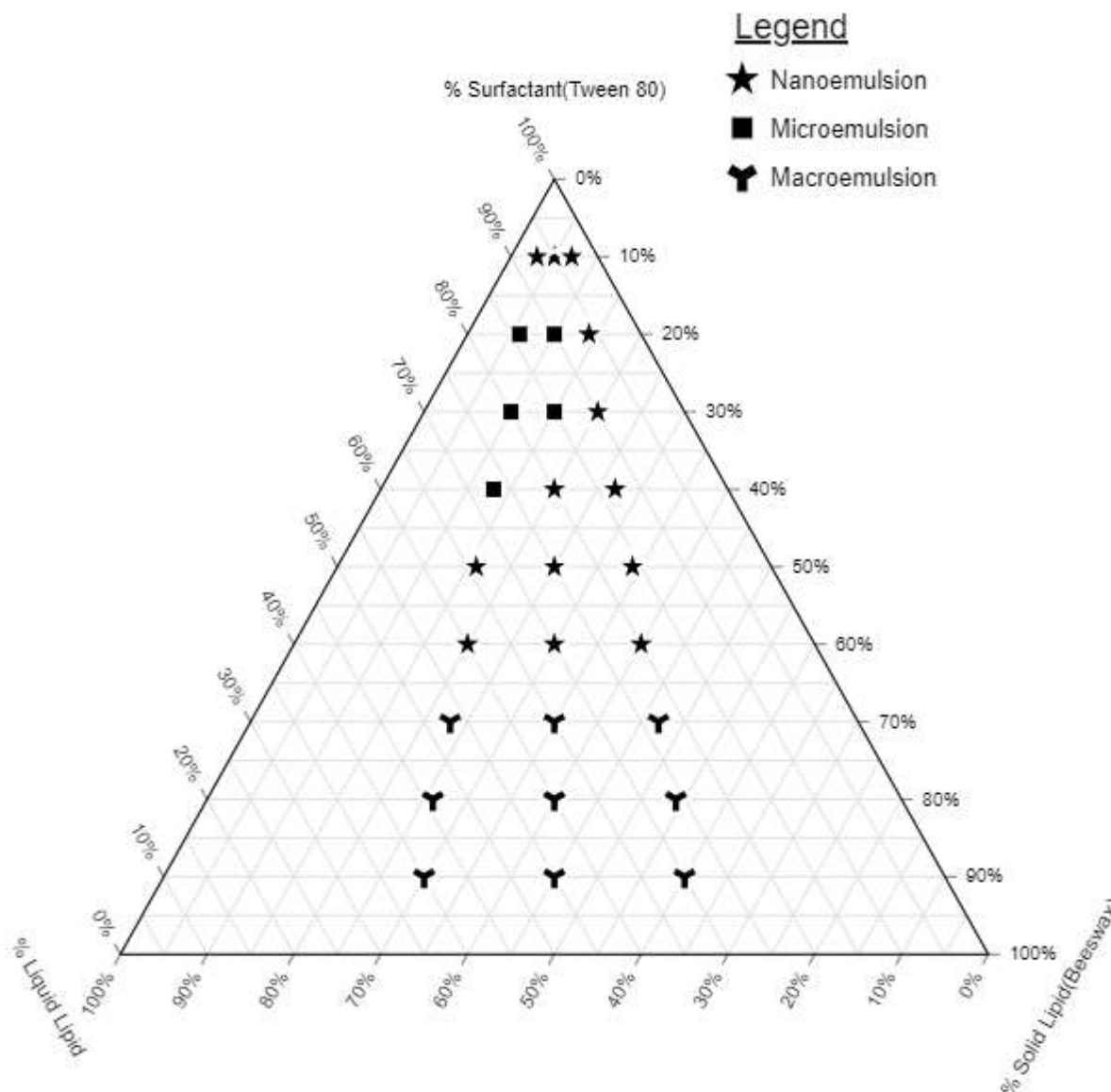


Fig. 5.12 : Ternary Phase Diagram of Xn loaded NLCs.

5.3.4. Box-Behnken design (BBD) : Formulation and Optimization

The formulation of NLCs was optimized through the application of the BBD, utilizing a 3-factor, 3-level approach (Ferreira et al., 2007). The selected independent variable(s), such as the amount of Beeswax (A), Lauroglycol-90 (B), and Tween-80 (C), had a direct effect on the dependent variables i.e. PS, ZP, and EE%. Three-dimensional (3-D) graphs have been generated for each of

the three responses (R1, R2, and R3). The responses observed from the 17 generated runs are presented in Table 5.12 & fig.5.13.

Table 5.12. NLCs formulation by BBD

Std	Run	Factor 1 A:Beeswax mg	Factor 2 B:Lauroglycol mg	Factor 3 C:Tween 80 mg	Response 1 Zeta Potential mV (R1)	Response 2 Particle Size nm (R2)	Response 3 Drug Entrapment % (R3)
10	1	250.00	200.00	600.00	-15	118	99.1
1	2	300.00	100.00	500.00	-19	138	89.2
9	3	250.00	100.00	400.00	-21.5	149	86
11	4	300.00	150.00	600.00	-13.4	128	89.7
6	5	200.00	150.00	400.00	-25.3	151	94.9
5	6	250.00	150.00	500.00	-20	138	86.2
4	7	250.00	200.00	400.00	-20.7	140	99.8
2	8	250.00	150.00	500.00	-19.2	159	85
7	9	200.00	200.00	500.00	-18.5	147	85
13	10	250.00	150.00	500.00	-19.35	146	85
14	11	200.00	150.00	600.00	-15	134	91.8
3	12	300.00	150.00	400.00	-21.8	118	94.7
17	13	300.00	200.00	500.00	-19.2	108	97.3
15	14	250.00	150.00	500.00	-18.6	154	96.7
8	15	250.00	150.00	500.00	-19.8	159	89.4
16	16	200.00	100.00	500.00	-19	131	79.6
12	17	250.00	100.00	600.00	-14	141	85.5

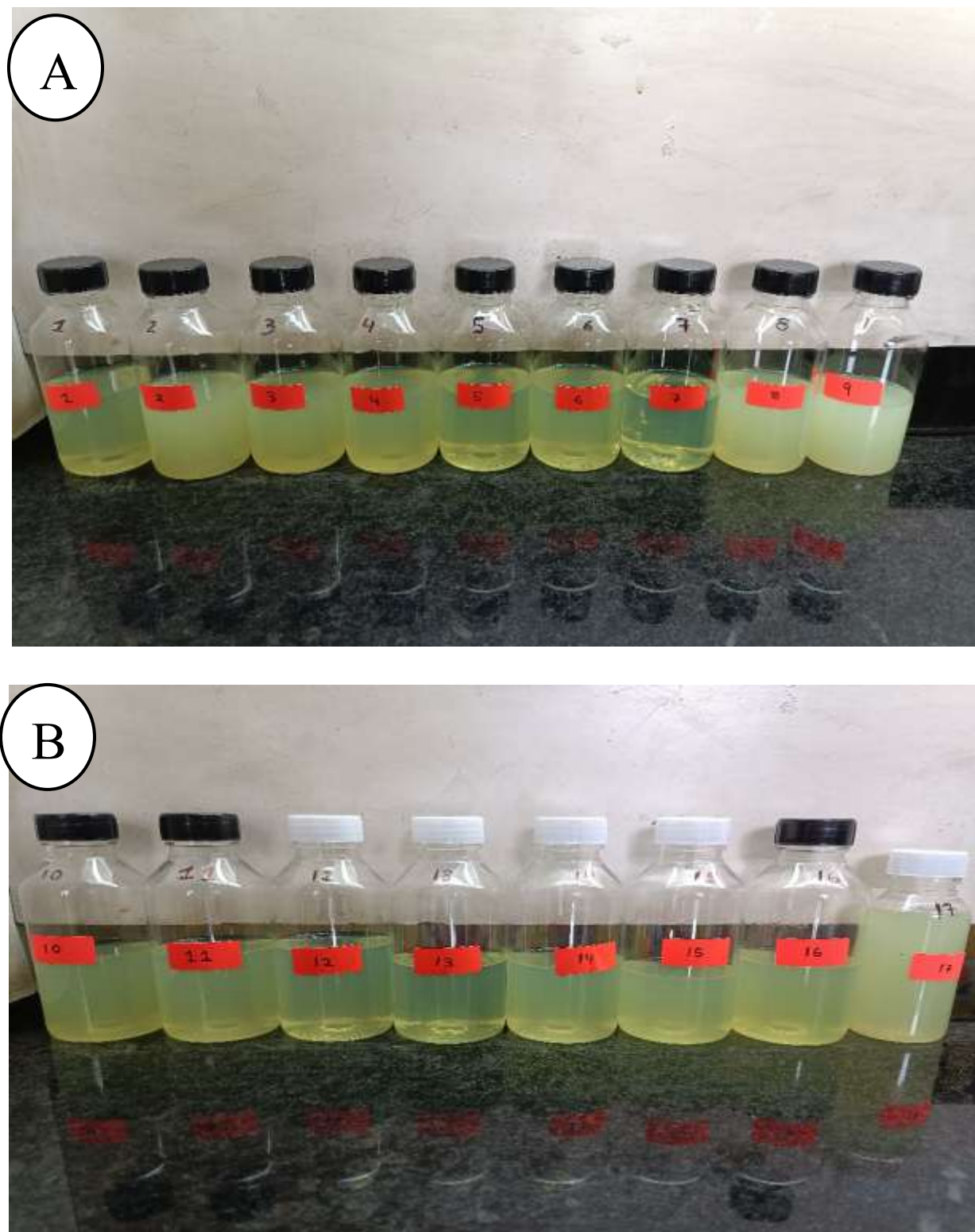


Fig. 5.13 : A and B Represents the Formulation of NLCs of DOE batches (Box-Behnken design).

5.3.4.1. The effect of independent variables on ZP (R1)

ANOVA and polynomial equation for the quadratic model's response surface showed that the model terms were significant in response to ZP, as indicated by the model F-value of 11.58 and probability > F values less than 0.0500, implying that the model was significant. The Design-Expert program determined the final mathematical model (polynomial equation) based on coded factors shown in Eq.1. These equations were used to generate different perturbation plots and 3-D response surface plots for different independent variables (Ferreira et al., 2007; Kraisit & Sarisuta, 2018; Kudarha et al., 2015) The R² value of 0.9371 indicated a very good correlation between ZP and variables. “An adequate precision value of 11.943 obtained by ANOVA indicates an adequate signal-to-noise ratio (S/N > 4 is desirable)”.

$$\text{ZP} = -19.39 + 0.55 \text{ A} + 0.013 \text{ B} + 3.99 \text{ C} - 0.17 \text{ AB} - 0.49 \text{ AC} - 0.45 \text{ BC} - 0.30 \text{ A}^2 + 0.77 \text{ B}^2 + 0.82 \text{ C}^2 \dots\dots\dots(1)$$

The perturbation plot in fig.5.14a showed that the ZP of Xn-loaded NLCs was negatively impacted by the independent variables i.e. such as Beeswax (A), Lauroglycol-90 (B), and Tween 80 (C). The negative value of ZP got increased with all the 3 variables. According to the perturbation plot factor B (Lauroglycol-90) has shown maximum effect on ZP followed by factor A (Beeswax) and then factor C (Tween 80). The 3D response surface plot of factors AB, AC, BC represented the interaction effect of independent variable on ZP as shown in fig. 5.14b,5.14c,5.14d.

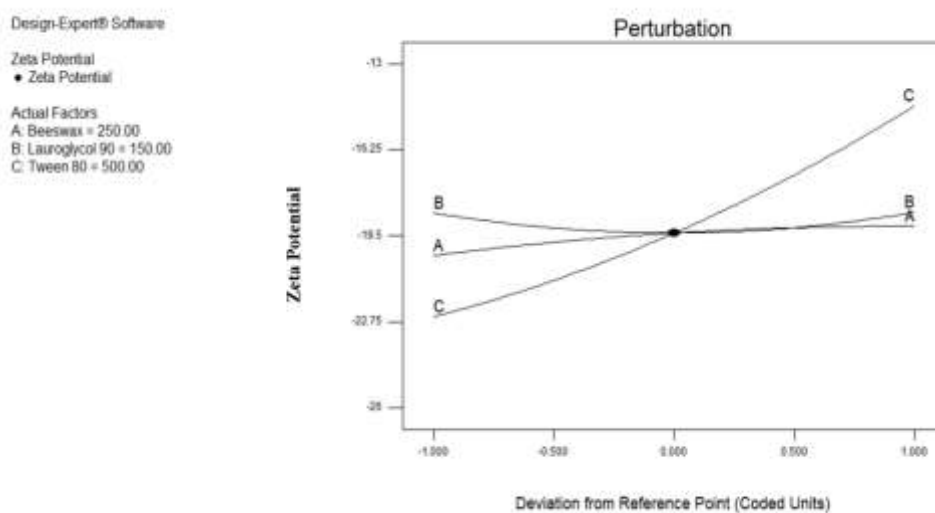


Fig. 5.14. (a) : Perturbation plot showing Independent variable effect on ZP

Design-Expert® Software

Zeta Potential



X1 = A: Beeswax
X2 = B: Lauroglycol 90

Actual Factor
C: Tween 80 = 500.00

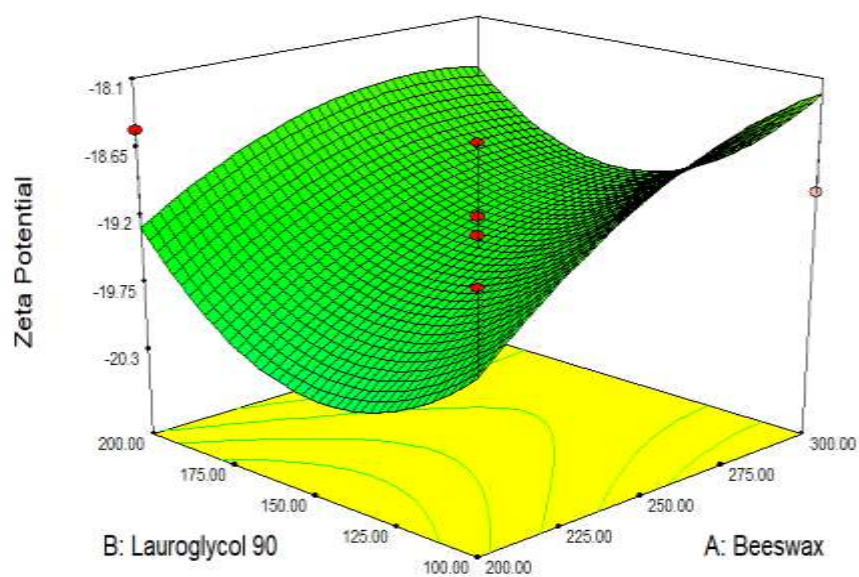


Fig. 5.14. (b) : 3D plot of factor (AB) represents the effect of independent variable on ZP

Design-Expert® Software

Zeta Potential



X1 = A: Beeswax
X2 = C: Tween 80

Actual Factor
B: Lauroglycol 90 = 150.00

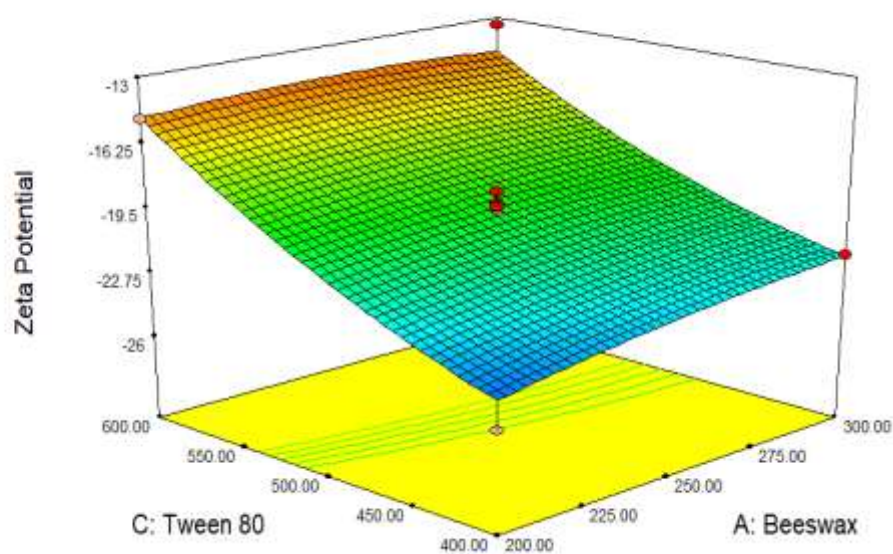


Fig. 5.14. (c) : 3D plot of factor (AC) represents the effect of the independent variable on ZP

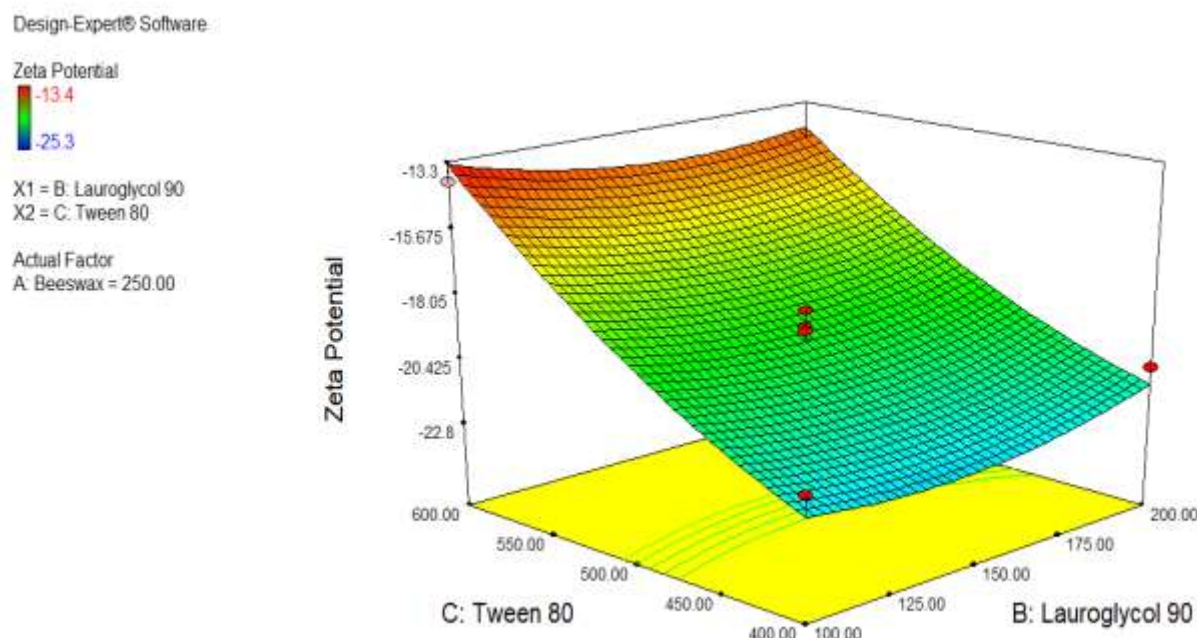


Fig. 5.14. (d) : 3D plot of factors (BC) represents the effect of independent variable on ZP

5.3.4.2. The effect of independent variables on PS (R2)

ANOVA and polynomial equation for the quadratic model's response surface showed that the model terms were significant in response to PS, as demonstrated by the model's F-value of 5.26, which suggests that the model is significant, and the values of probability > F less than 0.0500, which also suggests that the model is significant. The Lack of Fit F-value of 0.45 suggests that the lack of fit is not significant in relation to the pure error, which is good because it indicates that the model fits well. The Design-Expert program determined the final mathematical model (polynomial equation) based on coded factors shown in Eq.2. These equations were used to generate different perturbation plots and 3-D response surface plots for different independent variables (Ferreira et al., 2007; Kraisit & Sarisuta, 2018; Kudarha et al., 2015). The R^2 value of 0.8771 indicated a very good correlation between PS and variables. "An adequate precision value of 7.883 obtained by ANOVA indicates an adequate signal-to-noise ratio ($S/N > 4$ is desirable)".

$$PS = +151.20 - 8.88 A - 5.75 B - 4.63 C - 11.50 AB + 6.75 AC - 3.5 BC - 12.23 A^2 - 7.98 B^2 - 6.22 C^2 \dots\dots\dots(2)$$

The data of perturbation predicts that the PS increases as the independent variables, i.e. such as

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Beeswax (A), Lauroglycol-90 (B) and Tween 80 (C) decrease as shown in fig.5.15a. According to the perturbation plot on increasing the factor C (Tween 80) the PS decreases, followed by the factor B (Lauroglycol-90), and then factor A (Beeswax). The 3D response surface plot of factors AB, AC, BC represented the interaction effect of independent variables on PS as shown in fig. 5.15b,5.15c,5.15d.

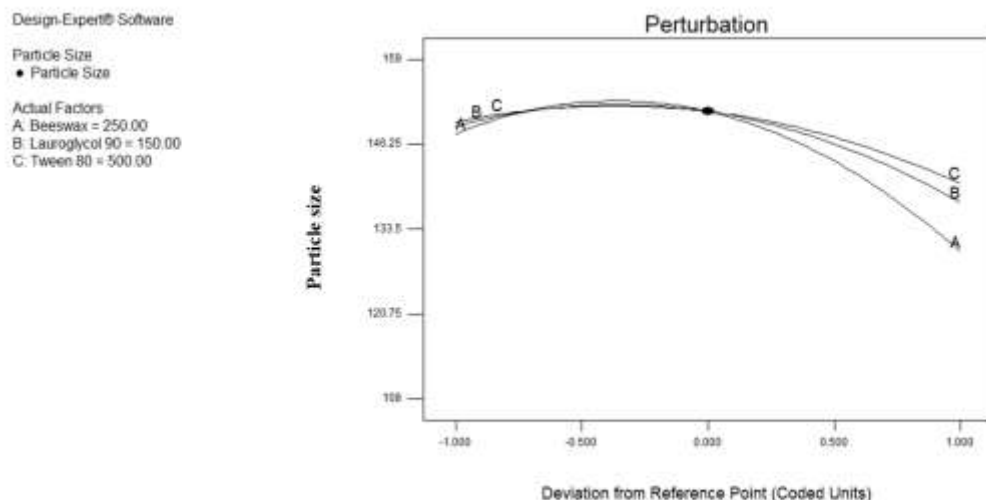


Fig. 5.15. (a) : Perturbation plot showing Independent variable effect on PS

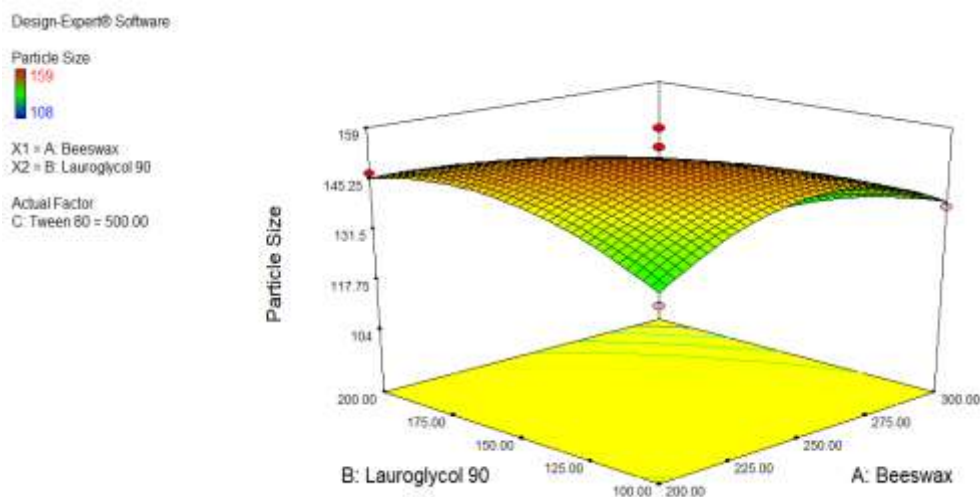


Fig. 5.15. (b) : 3D plot of factor (AB) represents the effect of independent variable on PS

Design-Expert® Software

Particle Size

159

108

X1 = A: Beeswax

X2 = C: Tween 80

Actual Factor

B: Lauroglycol 90 = 150.00

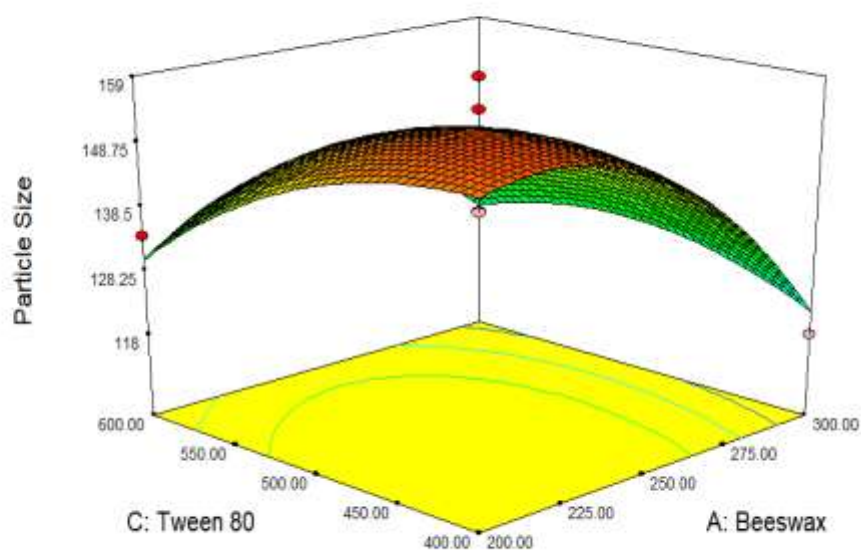


Fig. 5.15. (c) : 3D plot of factor (AC) represents the effect of the independent variable on PS

Design-Expert® Software

Particle Size

159

108

X1 = B: Lauroglycol 90

X2 = C: Tween 80

Actual Factor

A: Beeswax = 250.00

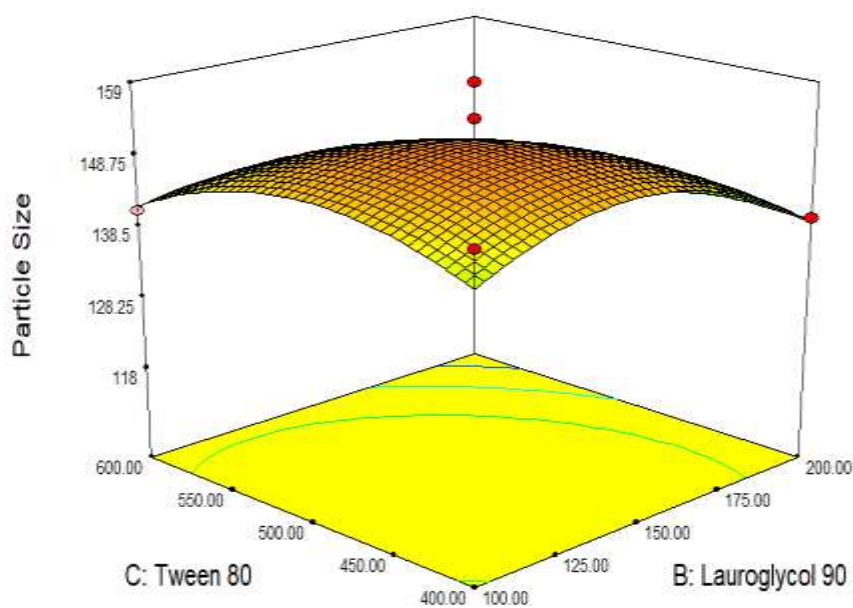


Fig. 5.15. (d) : 3D plot of factors (BC) represents the effect of independent variable on PS

5.3.4.3. The effect of independent variables on drug entrapment (R3)

ANOVA for the responses surface of the linear model indicates that the model terms were significant in response on drug entrapment. The model F-value of 3.98 and the values of probability > F less than 0.0500, which implies the model is significant, show that the model terms were significant in response to drug entrapment. The Design-Expert program determined the final mathematical model (polynomial equation) based on coded factors shown in Eq.3.. These equations were used to generate different perturbation plots and 3-D response surface plots for different independent variables (Ferreira et al., 2007; Kraisit & Sarisuta, 2018; Kudarha et al., 2015) The R^2 value of 0.5 indicated a very good correlation between drug entrapment and variables. “An adequate precision value of 6.580 obtained by ANOVA indicates an adequate signal-to-noise ratio ($S/N > 4$ is desirable)”.

$$\text{Drug Entrapment} = +90.29 + 2.45 A + 5.11 B - 1.16 C \dots\dots\dots(3)$$

The perturbation plot in fig.5.16a showed that the drug entrapment of Xn-loaded NLCs was positively impacted by the independent variables i.e. such as Beeswax (A) and Lauroglycol-90 (B). The drug entrapment of NLCs increased as the concentration of independent variables such as Beeswax (A) and Lauroglycol-90 (B) increased. On the contrary, increasing the Tween 80 (C) concentration reduced drug entrapment in NLCs. According to the perturbation plot factor B (Lauroglycol-90) has maximum effect on drug entrapment followed by the factor A (Beeswax) and then factor C (Tween 80). The 3D response surface plot of factors AB, AC, BC represented the interaction effect of independent variables on drug entrapment as shown in fig. 5.16b,5.16c,5.16d.

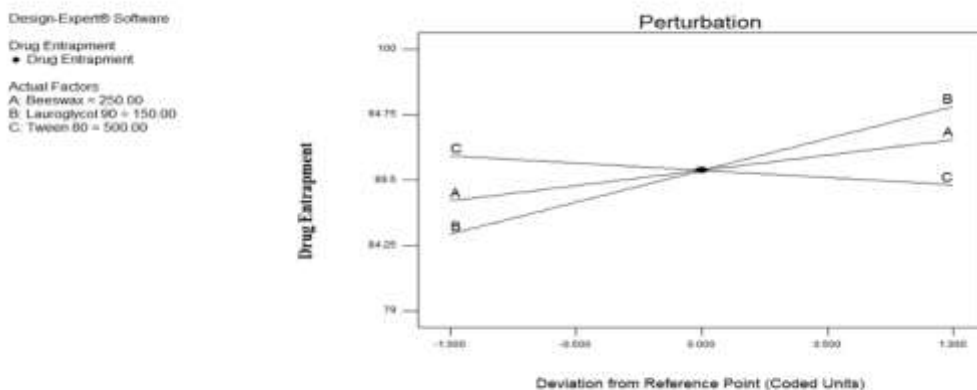


Fig. 5.16. (a) : Perturbation plot showing Independent variable effect on Drug entrapment

Design-Expert® Software

Drug Entrapment

99.8

79.6

X1 = A: Beeswax

X2 = B: Lauroglycol 90

Actual Factor

C: Tween 80 = 500.00

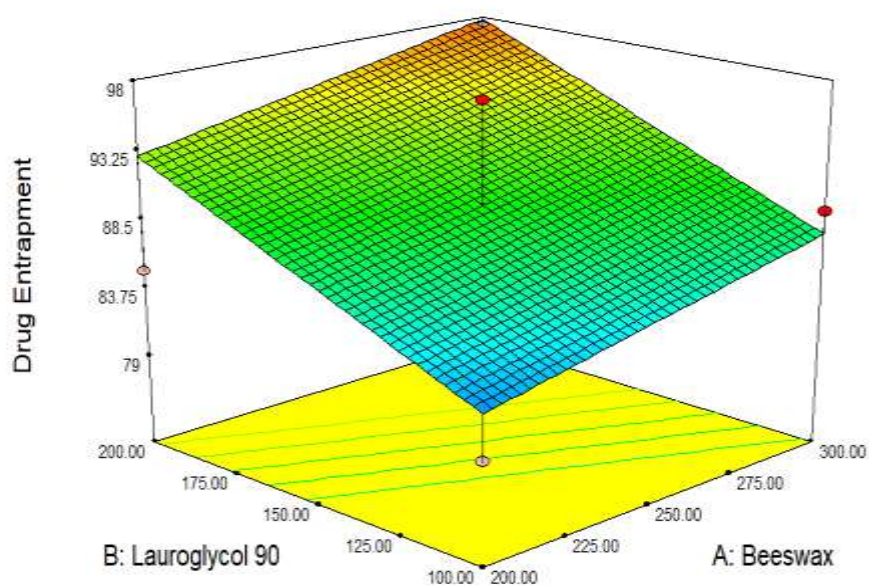


Fig. 5.16. (b) : 3D plot of factor (AB) represents the effect of independent variable on Drug entrapment

Design-Expert® Software

Drug Entrapment

99.8

79.6

X1 = B: Lauroglycol 90

X2 = C: Tween 80

Actual Factor

A: Beeswax = 250.00

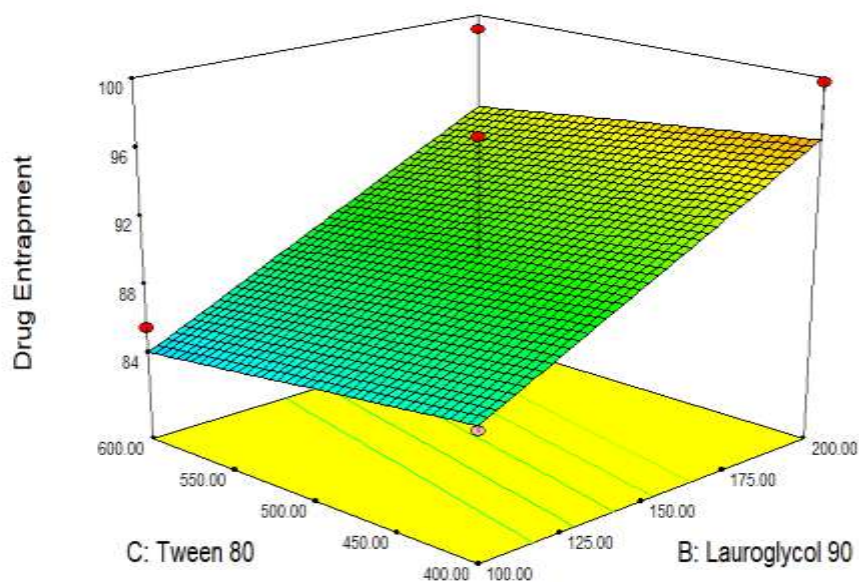


Fig. 5.16. (c) : 3D plot of factors (BC) represents the effect of independent variable on Drug entrapment

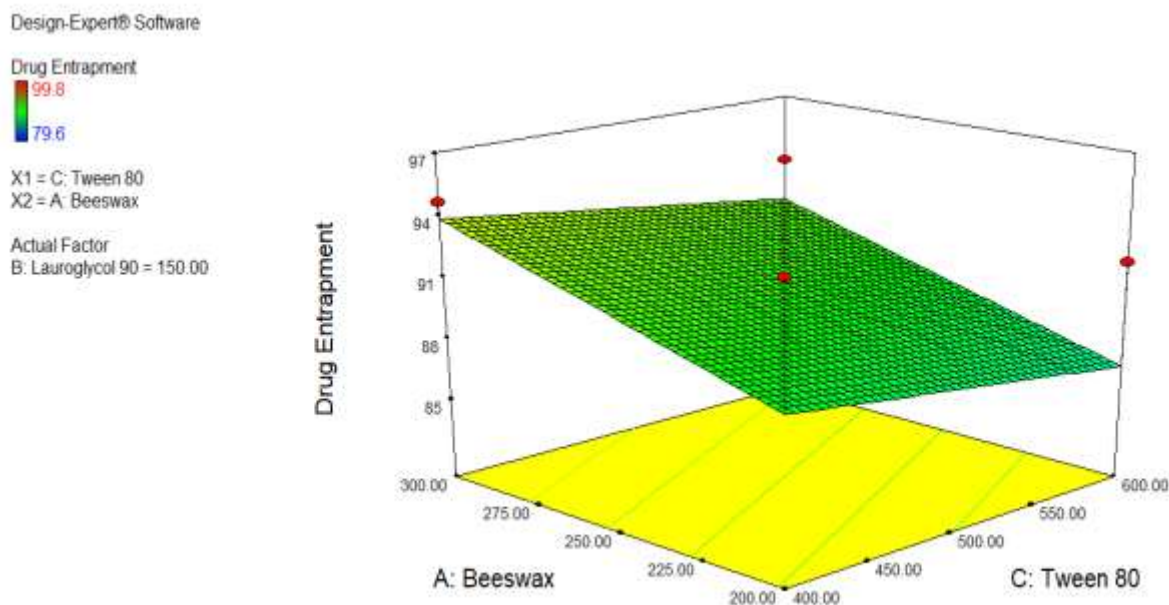


Fig. 5.16. (d) : 3D plot of factor (AC) represents the effect of the independent variable on Drug entrapment

5.3.5. Optimized Formulation Characterization

By using the design and analyzing the obtained results, it was observed that all the factors have shown significant impact on the responses. Graphical optimization method was used to finalize the design space and optimize the amount of each component's that have been utilized for preparing Xn-NLCs. The results predicted the use of 300 $\mu\text{g/mL}$ of Beeswax, 200 $\mu\text{g/mL}$ of Lauroglycol-90 and 400 $\mu\text{g/mL}$ of Tween 80 to formulate NLCs, which could result in ZP near to -21 mV, PS close to 100 nm and drug entrapment closer to 99 %. The desirability of the optimized formulation was determined to be 0.917 which shows good reliability. Using the predicted values of solubilizers, the experiment was conducted in triplicate in the lab. The observed value of ZP was -25.6 mV, PS was 101.60 ± 1.47 nm and EE% of $97.02 \pm 1.05\%$. A linear correlation was observed between obtained value and predicted value with $R^2 > 0.99$. The optimized formulation was assessed for its desirability, resulting in a value of 0.917, indicating a highly favorable outcome. Further, P-value was found above 0.005, indicating non-significant results. This indicated the suitability of the model. The overlay plot, desirability and 3-D graph of desirability is shown in 5.17 a, b, c.

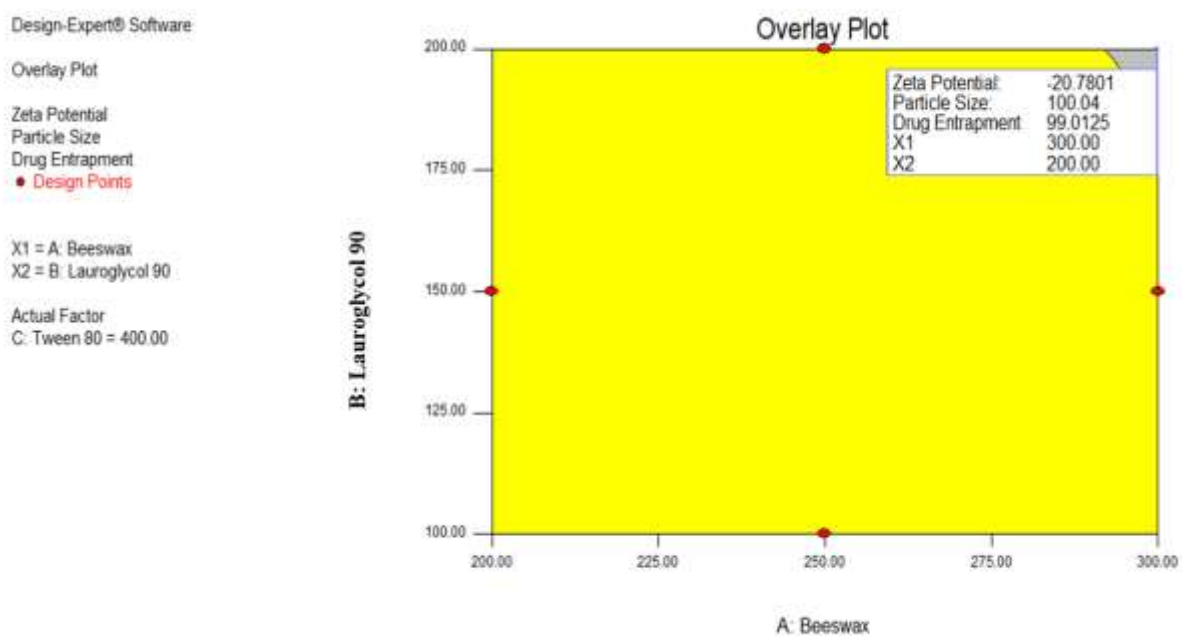


Fig. 5.17a : Overlay Plot of the optimized Xn-loaded NLCs formulation.

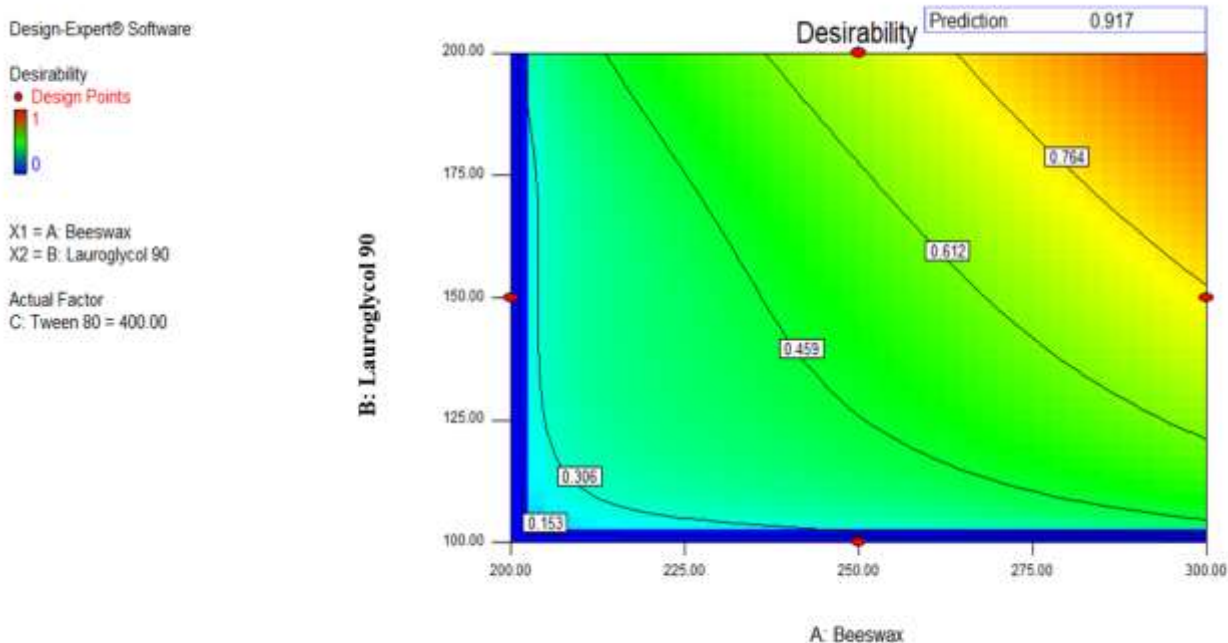


Fig. 5.17b : Desirability of the optimized Xn-loaded NLCs formulation

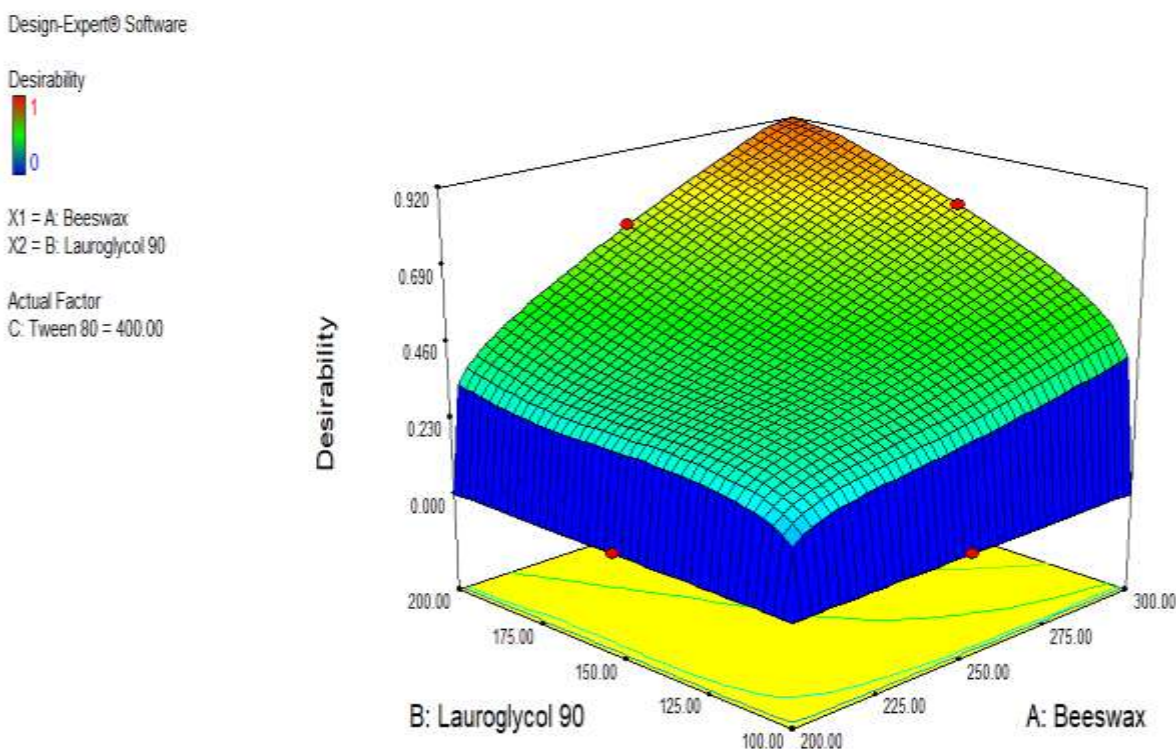


Fig. 5.17c : 3D-graph illustrates the desirability of the optimized Xn-loaded NLCs formulation

5.3.5.1 Drying- NLCs

The optimized NLCs were converted into free-flowing powder (S-Xn-NLCs) by rotary evaporation followed by drying it in a tray dryer along with mannitol as shown in fig.5.18. In order to formulate Solid NLCs (S-NLCs) of Xn, 250 mL of liquid NLCs were taken, which contained 100 mg of the drug. This indicated the formulation of 0.0004 mg/mL solution of Xn-NLCs. To this 51g of mannitol was added. Then, the formulation was subjected for rotavapor (ESCY IC Model:201) for the evaporation of water at 65°C-70°C for 10 minutes and was spread in the stainless steel trays and subjected for drying in the hot air oven (Remi Instruments Ltd., Mumbai, India) at 70°C for ½ h. Mannitol was used in this study as a solidifying agent because it has various advantages and physicochemical characteristics such as low hygroscopicity, inertness with respect to APIs, has no known adverse effects in humans, good compatibility, and the ability to produce extremely robust powder (Misha et al., 2018; Plabon et al., 2024).

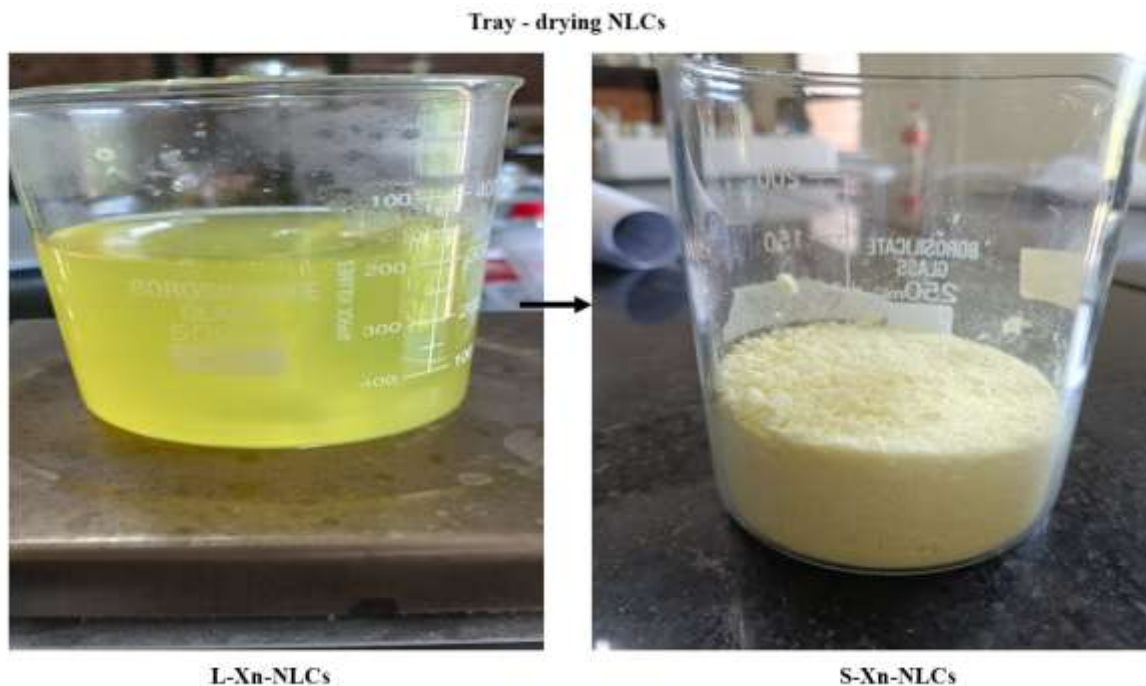


Fig. 5.18 : Tray- drying of Xn loaded NLCs formulation

5.3.5.2. Formulation of S-Xn-NLCs

The final composition of the drug (Xn) and excipients (Beeswax, lauroglycol-90, Tween-80 and Mannitol) is given in Table 5.13.

Table 5.13. Final composition of the drug and its excipients

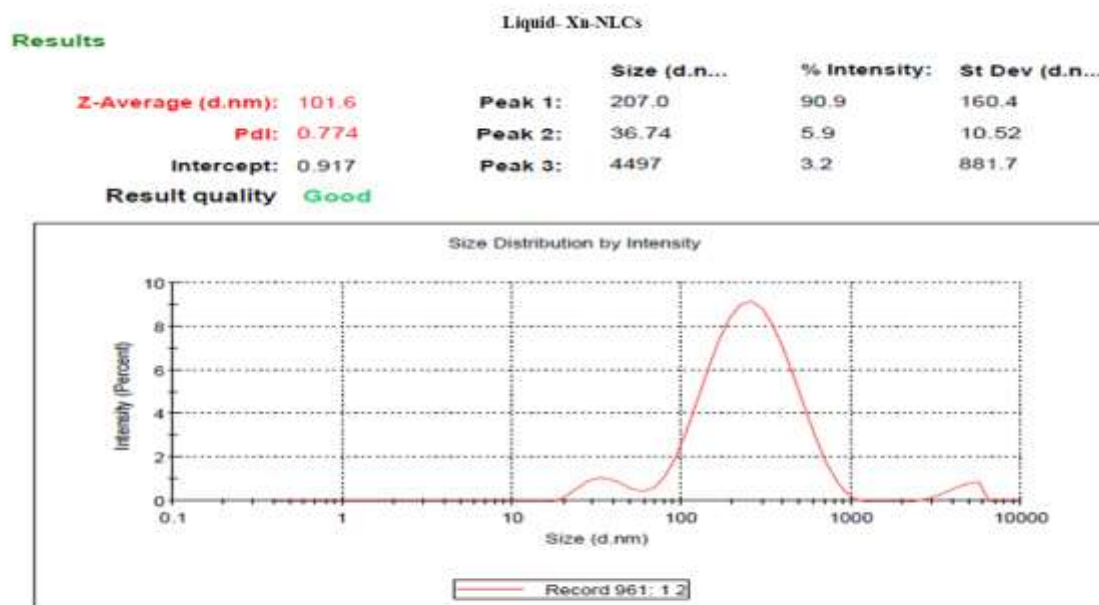
S.No.	Compound	Quantity
1	Xn	0.1 g
2	Beeswax	1.4 g
3	Lauroglycol-90	0.6 g
4	Tween-80	8 g
5	Mannitol	51 g
Total		61.1 g

5.3.5.3. ZP, PS, and Drug EE %

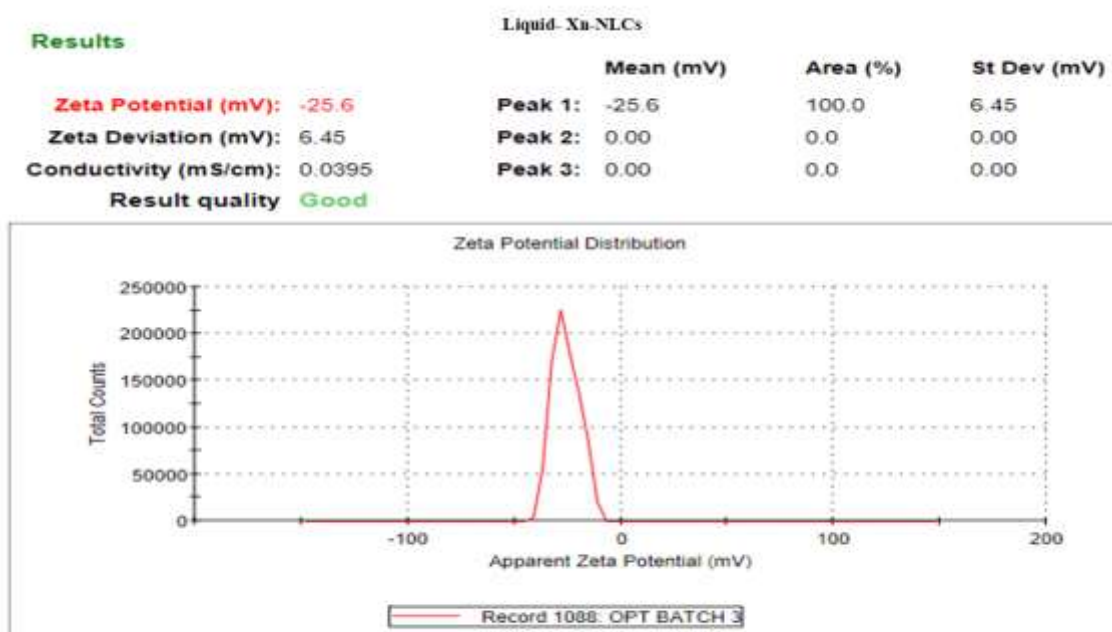
The optimized L-Xn-NLCs shows a PS of 101.60 ± 1.47 nm , PDI of 0.77 ± 0.029 and ZP of -25.6 mV, indicating particle stability. The optimized formulation obtained a drug EE % of $97.02 \pm 1.05\%$. As a result, the EE% results show that NLCs have augmented drug encapsulation

Results and discussion

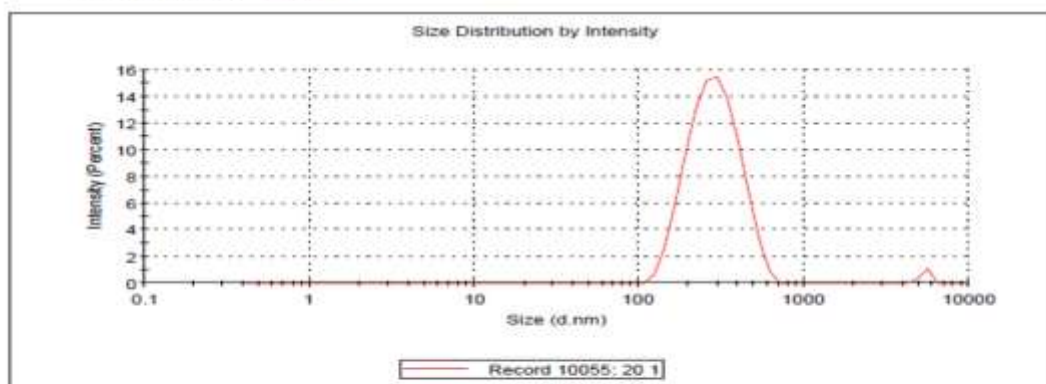
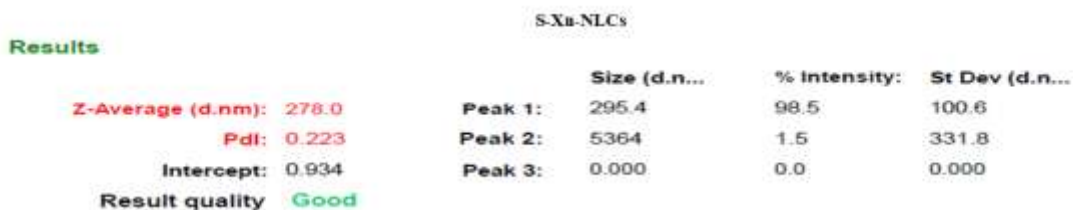
efficiency. Fig. 5.19a and 5.19b showed the PS, PDI, and ZP of the optimized L-Xn-NLCs. The reconstituted S-Xn-NLCs showed PS of 278 nm, PDI of 0.22 , ZP of -44.3 mV and EE% of 95.5 %, as shown in fig. 5.19c and 5.19d.



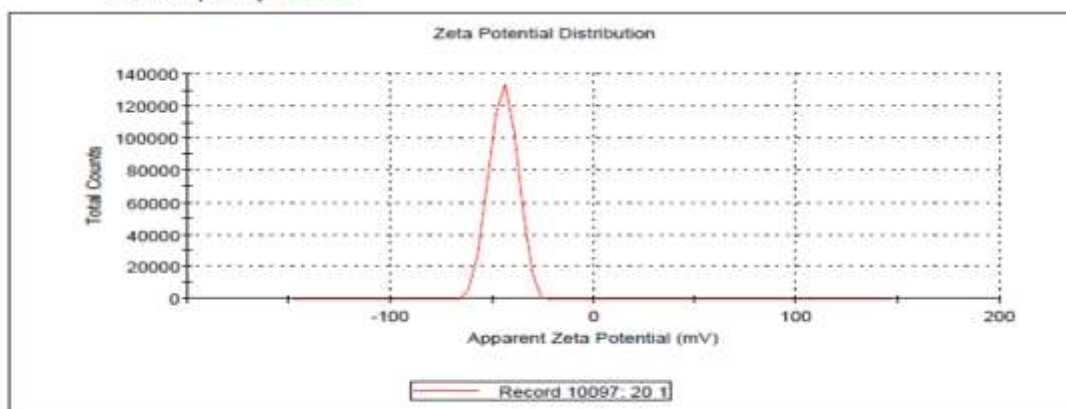
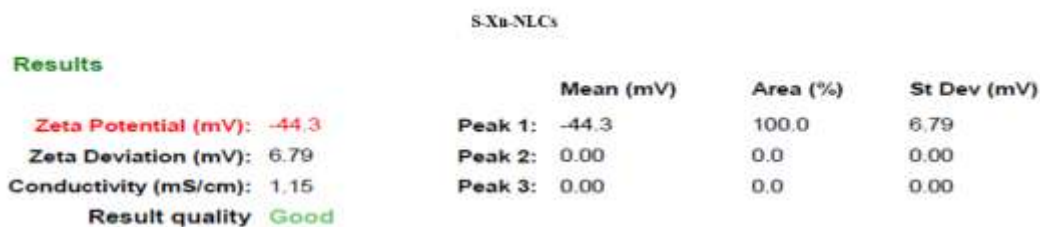
(a)



(b)



(c)



(d)

Fig. 5.19 : (a) PS of L-Xn-NLCs ; (b) ZP of L-Xn-NLCs ; (c) PS of S-Xn-NLCs ; (d) ZP of S-Xn-NLCs.

5.3.5.4. Stability study

Stability studies of L-Xn-NLCs and S-Xn-NLCs were conducted to examine the stability of the

drug and its formulation. The results of stability studied are given in Table 5.14. It was found that both L-Xn-NLCs and S-Xn-NLCs were stable after six months of stability study.

Table 5.14. Stability study

Parameters(L-Xn-NLCs)	Storage (Months)	
	0	6 th
Physical characteristics	Results	Results
PS	101.60	102
ZP	-25.6	-28.4
%EE	97.72	97.35
Parameters (S-Xn-NLCs)	Storage (Months)	
	0	6 th
Physical characteristics	Results	Results
PS	278	276
ZP	-44.5	-44.7
%EE	95.5	95.04

5.3.5.5. Powder Flow Characterization of Xn-NLCs Powder

S-Xn-NLCs powder were subjected to micromeritics characterization which include angle of repose, bulk density, tapped density, Carr's index, Hausner ratio. The results of all parameters were found to be excellent. The angle of repose was found to be $\Theta = (18^\circ)$, Bulk density was 0.55 ρ , Tapped density was 0.61 ρ , Carr's index was 9.8 and Hausner's ratio was found to be 1.10. The results of scale of flowability show that the flow character of dry powder S-Xn-NLCs were in excellent range.

5.3.5.6. In vitro drug release studies

In-vitro drug release for the optimized NLCs formulation was performed for a period of 24 h using the dialysis membrane method as shown in fig. 5.21 a & b. During the study, L-Xn-NLCs, S-Xn-NLCs powder and pure Xn suspension showed cumulative drug release of $80.09 \pm 0.8\%$, 79.8% and $40 \pm 1.1\%$, respectively. There were no statistically significant ($p > 0.05$) differences in the Xn release patterns for L-Xn-NLCs and S-Xn-NLCs. The *in vitro* drug release study of the Xn-loaded NLCs revealed that the cumulative amount of Xn released from both NLCs formulation

were significantly ($p < 0.05$) higher than the pure Xn suspension in the release medium. It was found that both the NLCs formulation was significantly ($p < 0.05$) higher than that of the pure Xn suspension in the release medium as shown in Fig. 5.20. The resulting study found that incorporation of Xn in NLCs significantly enhanced solubilization. It provided more space for the drugs to remain retained in the lipids. This led to a significant enhancement in drug release from NLCs. About 2~fold enhancement in release of Xn was observed in case of L-Xn-NLCs and S-Xn-NLCs as compared to pure Xn suspension. Finally, the *in vitro* release study graph was plotted and observed by Origin Pro Software. The results of *in vitro* study of Xn release from NLCs were fitted to the Zero order ($R^2 = 0.96$), First order ($R^2 = 0.98$), Hixson Crowell ($R^2 = 0.98$), Higuchi ($R^2 = 0.94$), and Korsmeyer-Peppas models ($R^2 = 0.982$). The highest correlation coefficient (r) value represents the actual mode of release. The *in vitro* release model was best fitted to “Korsmeyer-Peppas Kinetics” model according to given kinetics, resulting the highest correlation coefficient (r). Thus, the results were analyzed using the Korsmeyer-Peppas model, and the release exponent (n) has been found to be 0.44. The release model of Korsmeyer-Peppas model initially showed a burst release followed by slow releasing indicating the “fickian diffusion” (Duong et al., 2019; H. J. Lee et al., 2020).

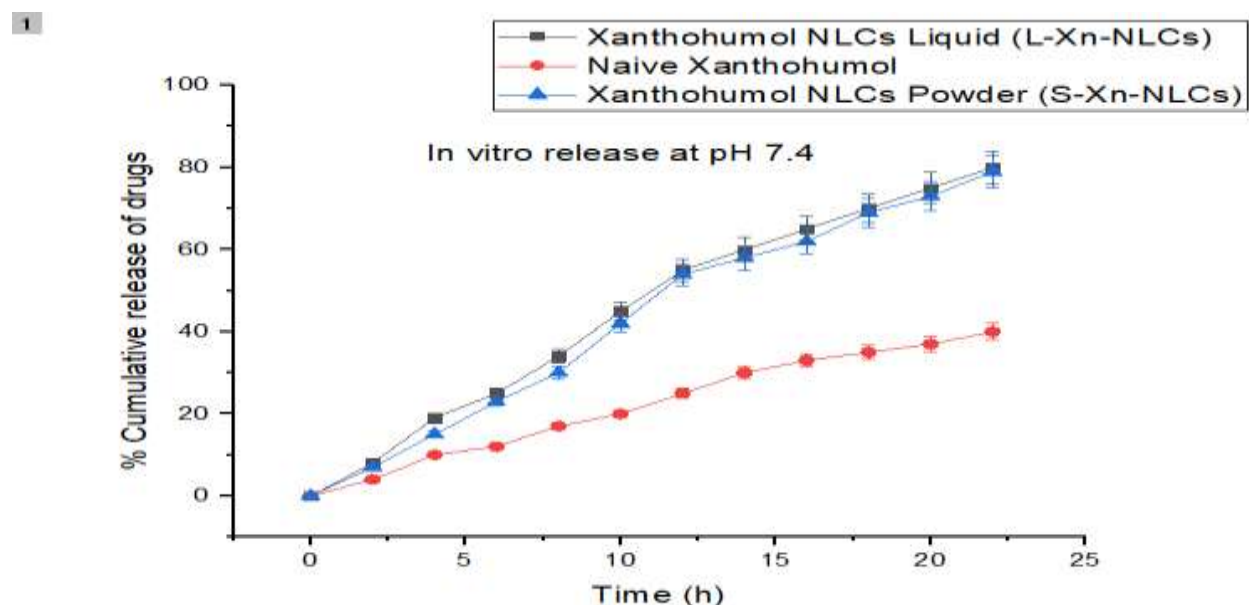


Fig. 5.20 : Graphs of *in-vitro* drug release for L-Xn-NLCs, S-Xn-NLCs and Naive Xn.

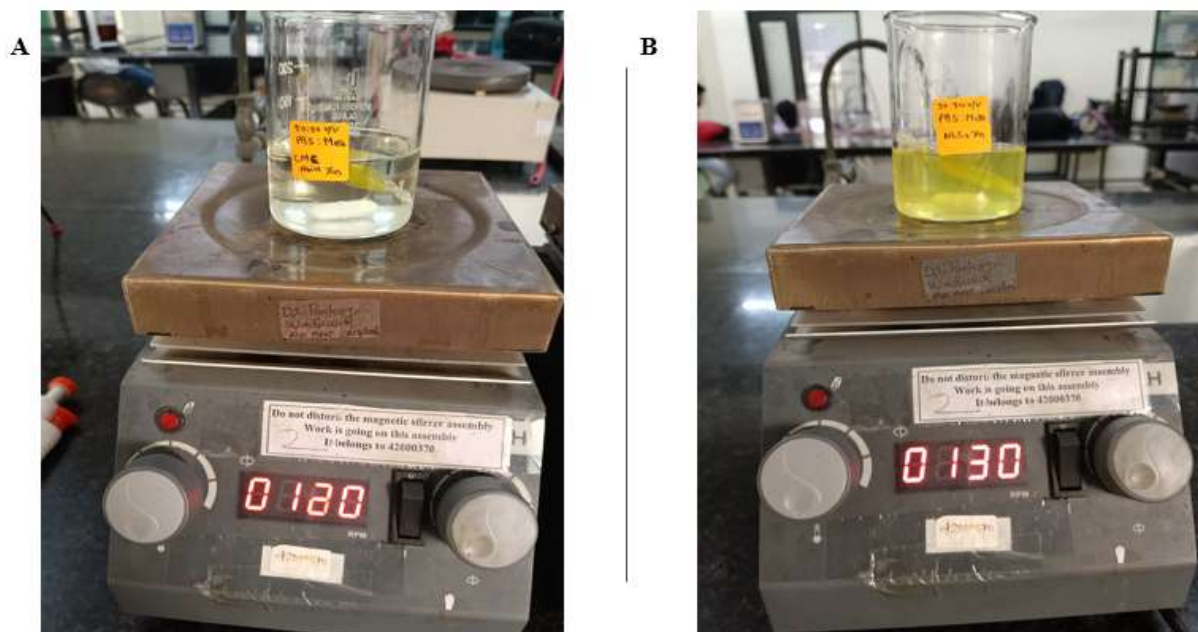


Fig. 5.21 : In vitro drug Released studies: a) Xn; b) NLCs Xn

5.3.5.7. Release kinetics models of NLCs formulation

The results of release kinetics of Xn-NLCs were fitted to the Zero order ($R^2 = 0.96$), First order ($R^2 = 0.98$), Hixson Crowell ($R^2 = 0.98$), Higuchi ($R^2 = 0.94$), and Korsmeyer-Peppas models ($R^2 = 0.982$) as shown in fig. 5.22 a, b, c, d, e. The highest correlation coefficient (r) value represents the actual mode of release as given in Table 5.15. The evaluation of the in vitro release model revealed that it aligns most effectively with the "Korsmeyer-Peppas Kinetics" model. This conclusion was drawn based on the provided kinetics data, which demonstrated the highest correlation coefficient (r), indicating a strong relationship between the model predictions and the observed results. The analysis of the results was conducted using the Korsmeyer-Peppas model, revealing that the release exponent, denoted as " n ," is measured to be 0.44. The release model of Korsmeyer-Peppas model initially showed a burst release followed by slow releasing indicating the "fickian diffusion" (Duong et al., 2019; H. J. Lee et al., 2020).

Table 5.15. Release kinetics models of NLCs formulation

Zero order	0.966
First order	0.98
Higuchi model	0.94

Hixson Crowell model	0.98
Kor's Peppas model	0.982

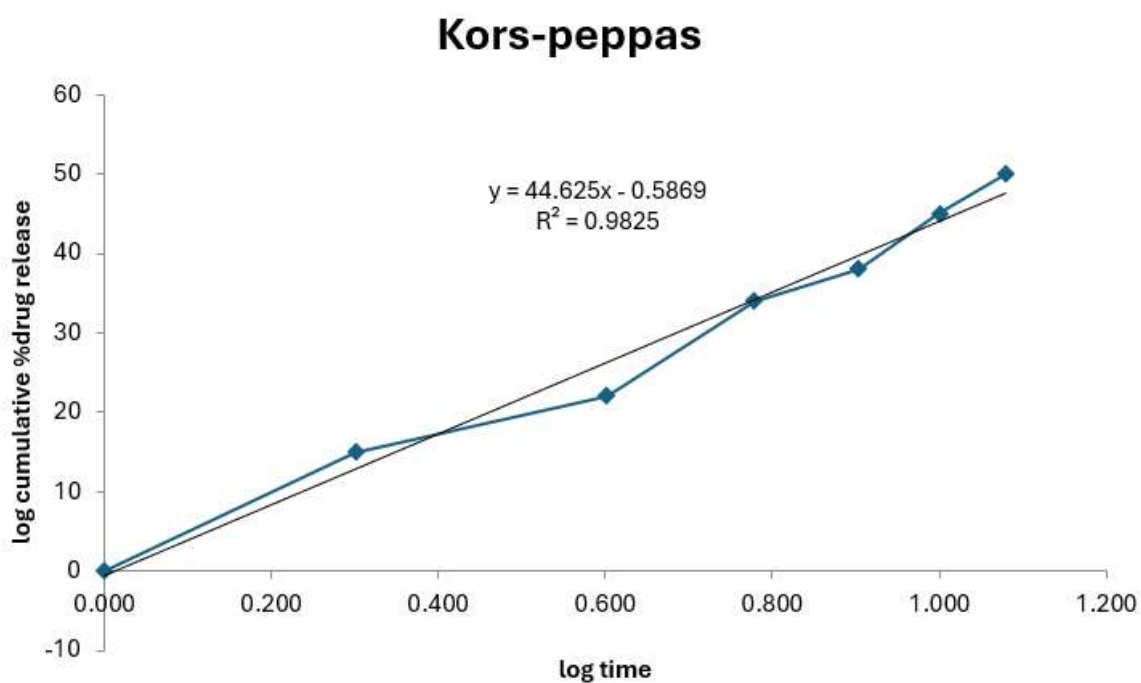


Fig. 5.22a : Release kinetics of NLCs formulation- Korsmeyer Peppas model

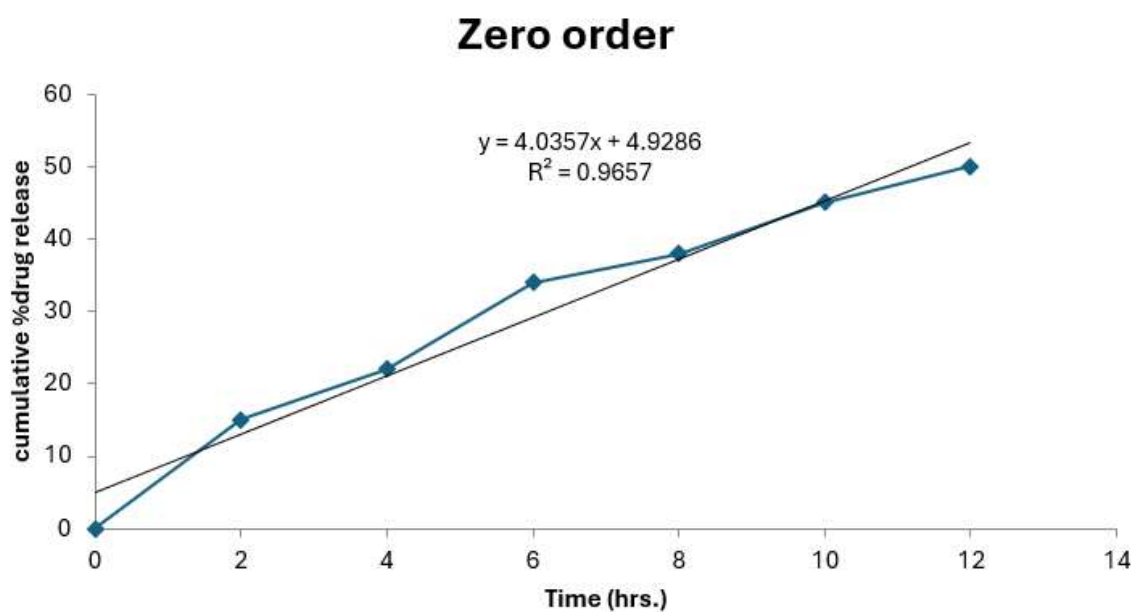


Fig. 5.22b : Release kinetics of NLCs formulation- Zero order model

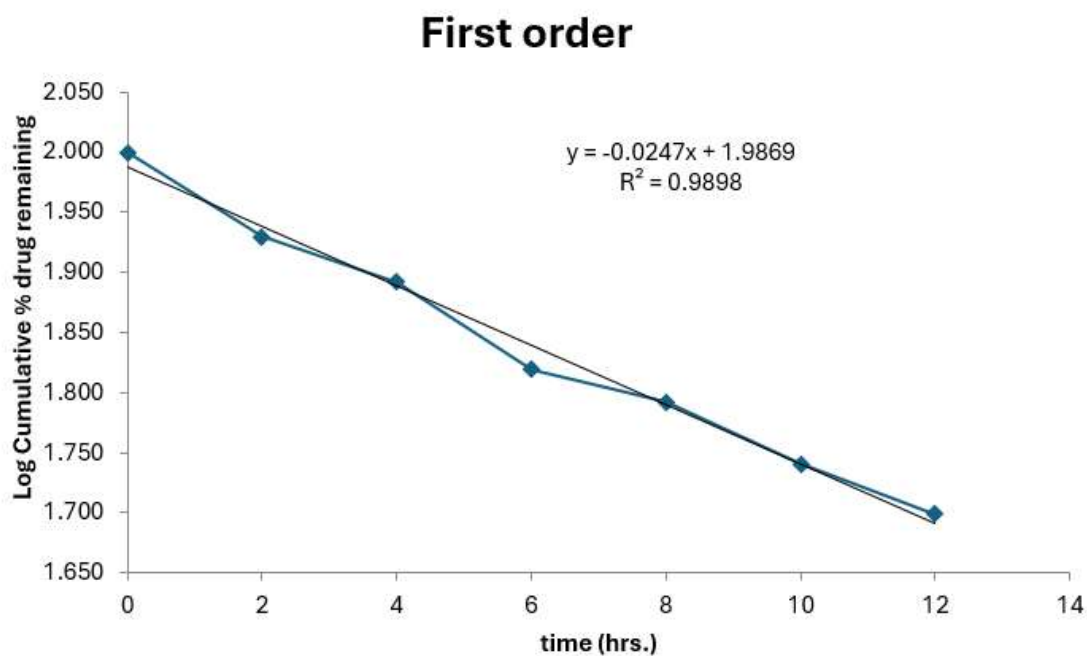


Fig. 5.22c : Release kinetics of NLCs formulation- First order model

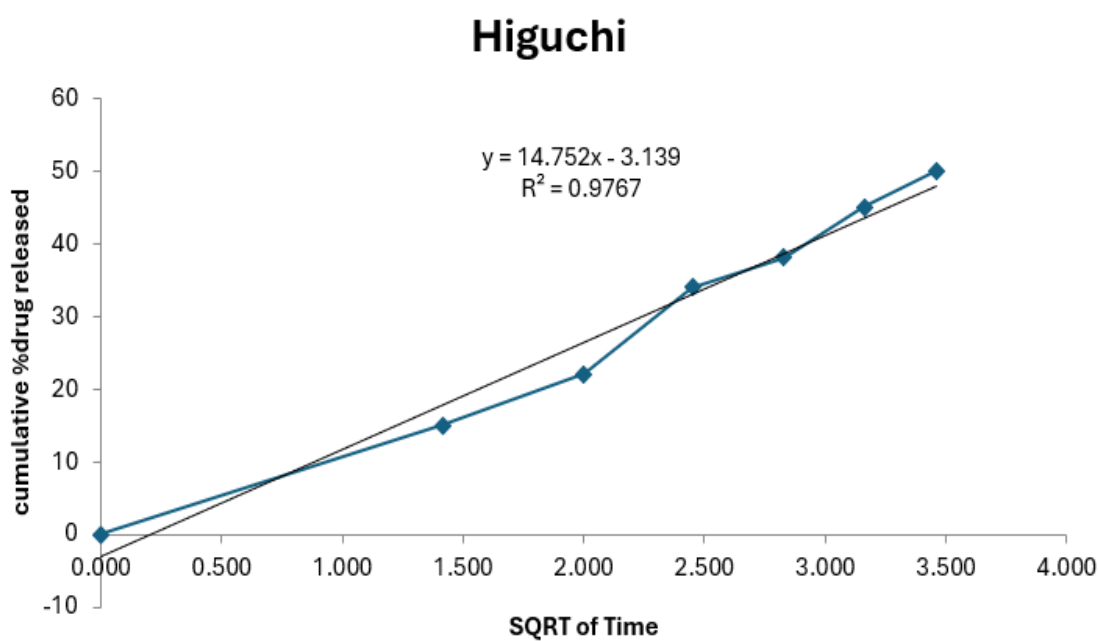


Fig. 5.22d : Release kinetics of NLCs formulation- Higuchi model

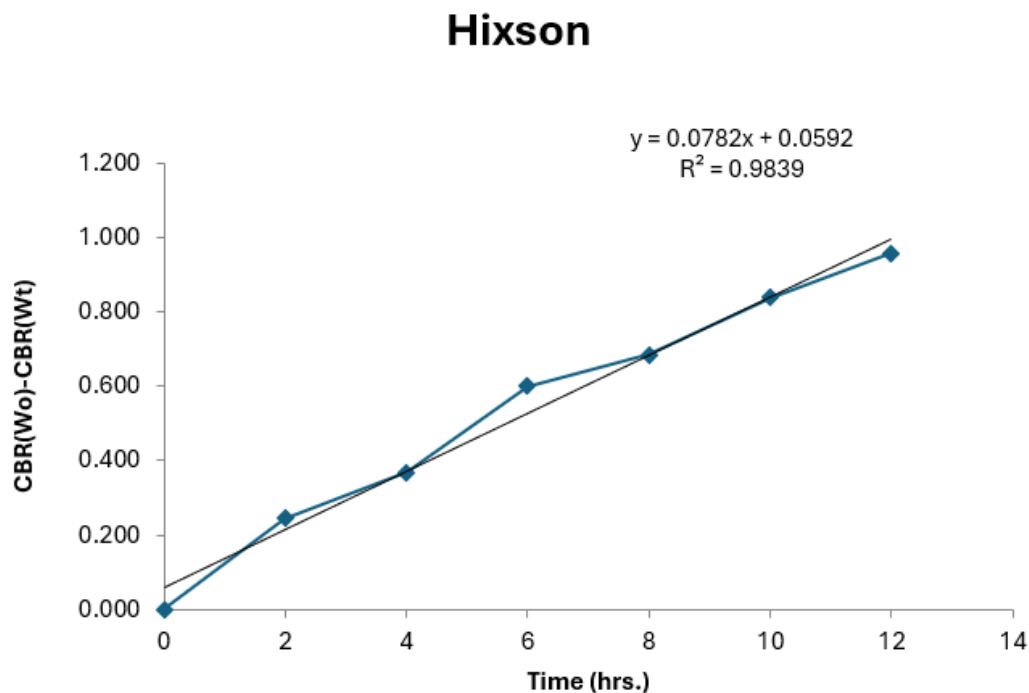
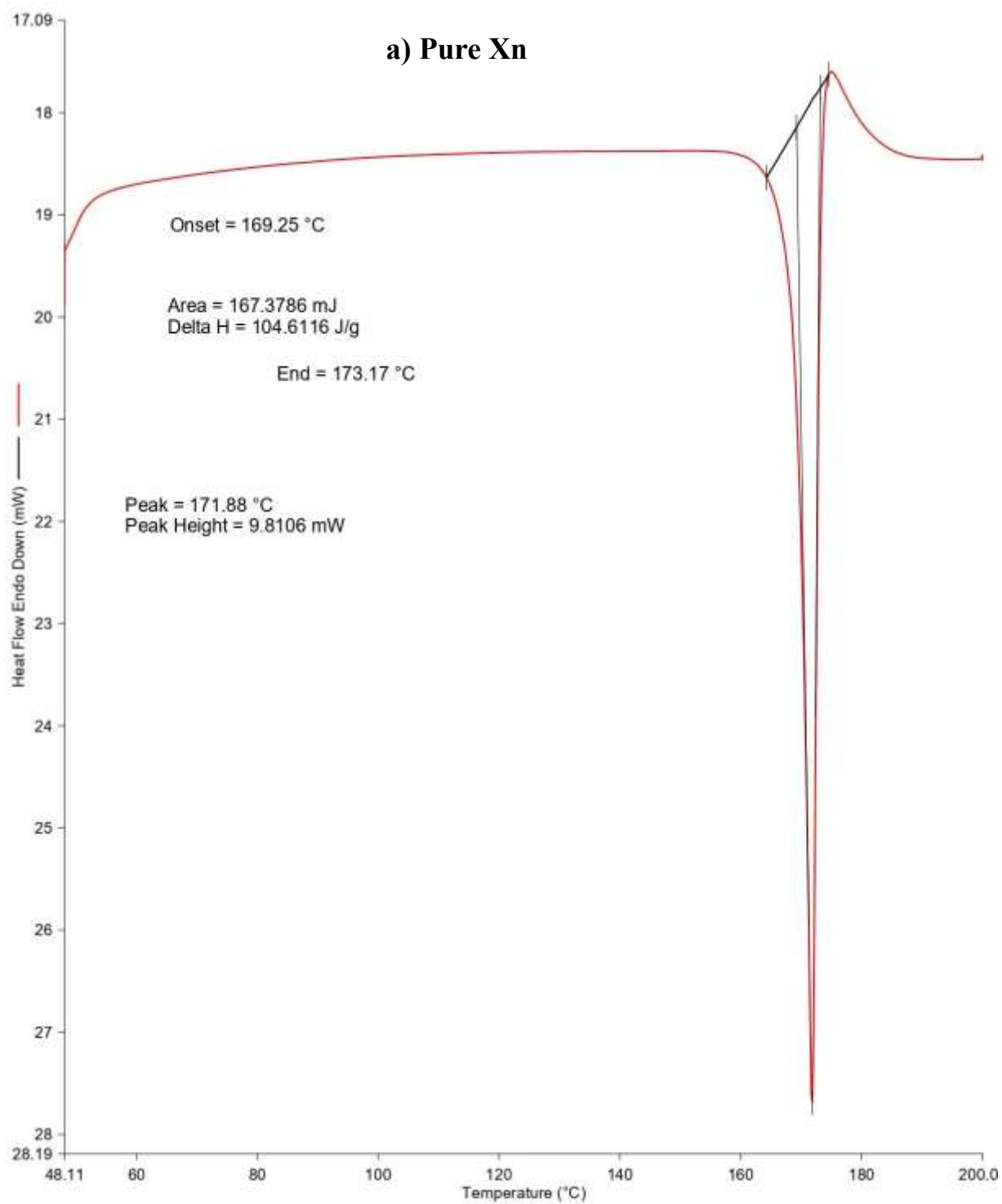


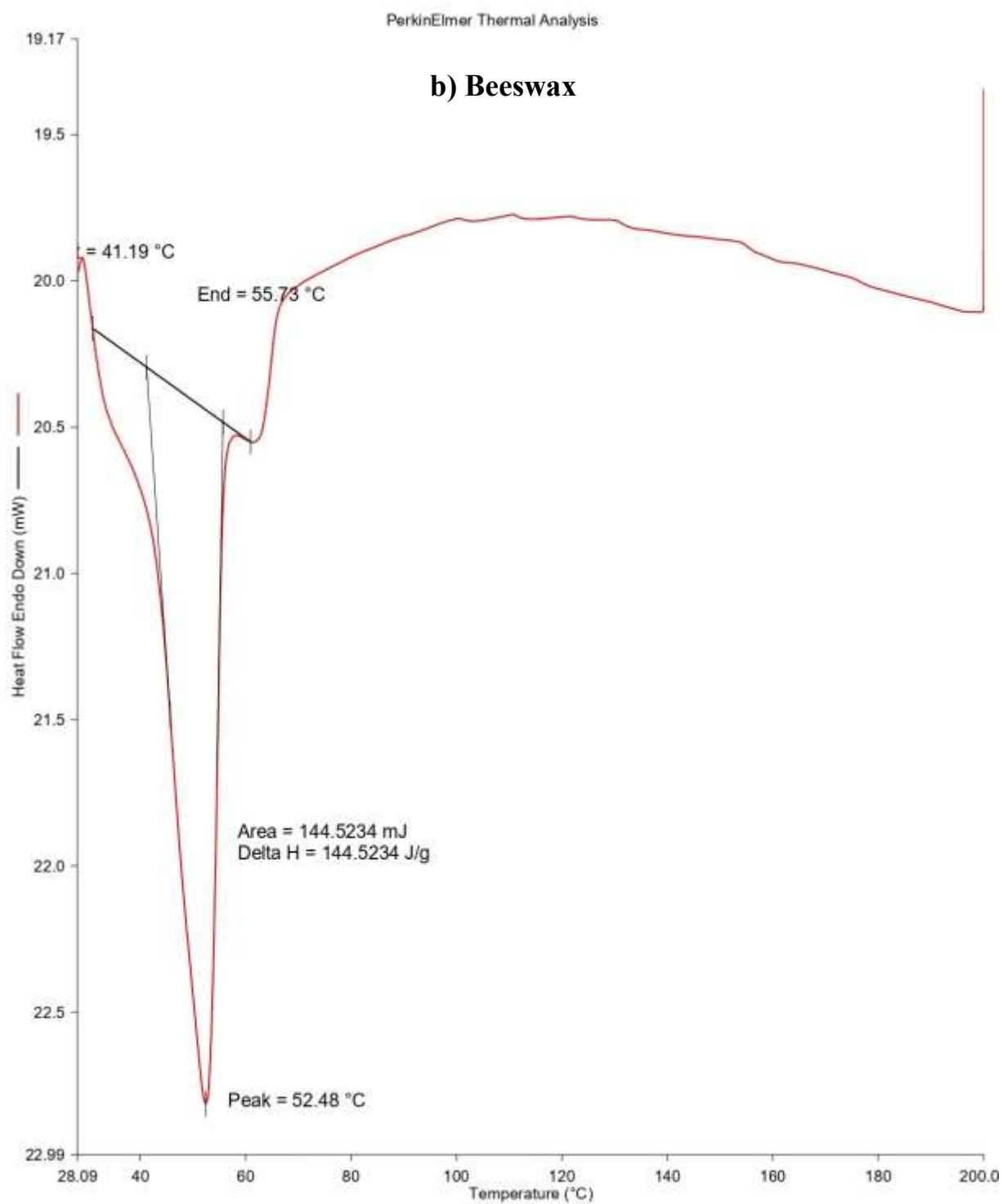
Fig. 5.22e : Release kinetics of NLCs formulation- Hixson Crowell model

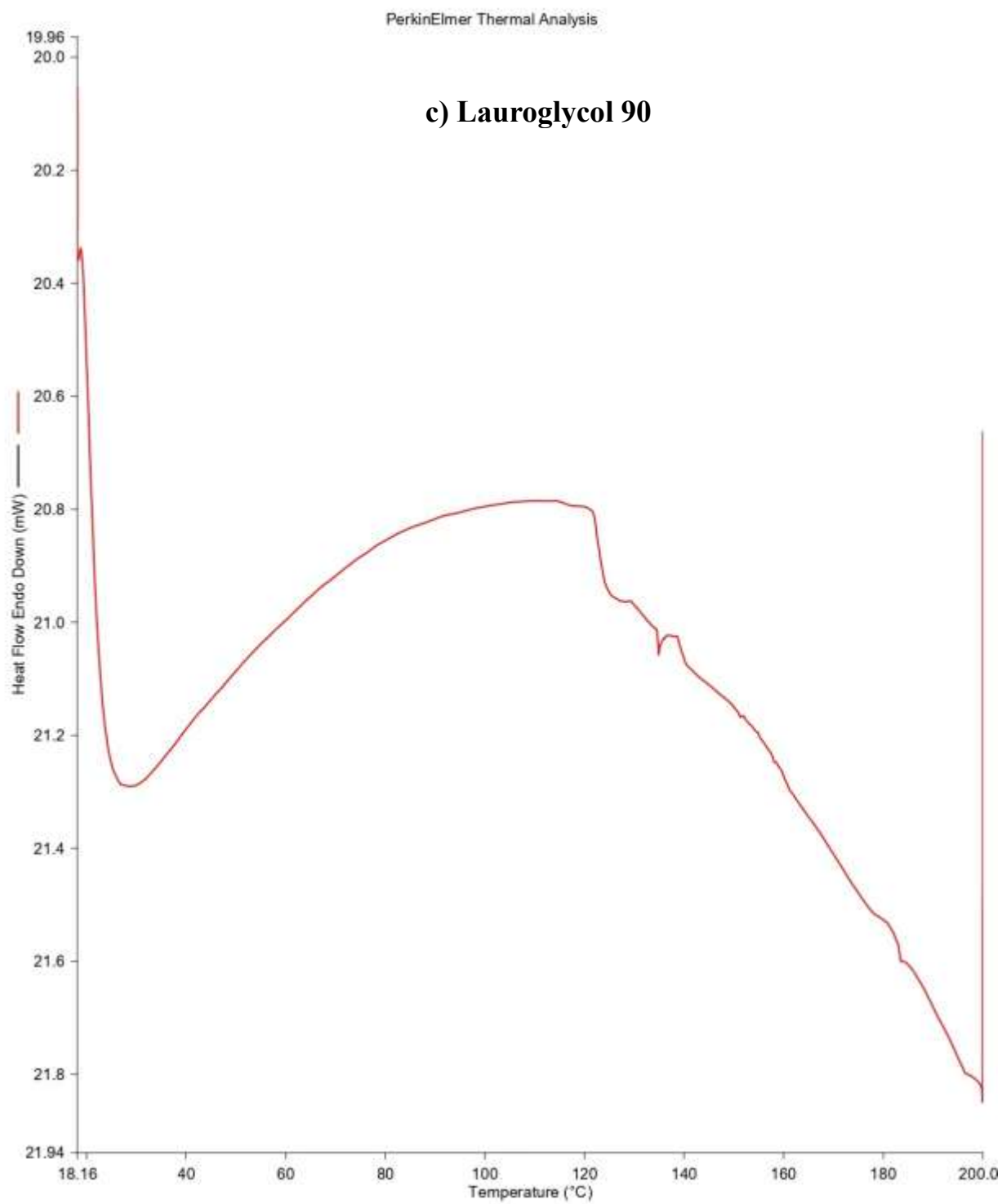
5.3.6. DSC, PXRD, SEM, TEM

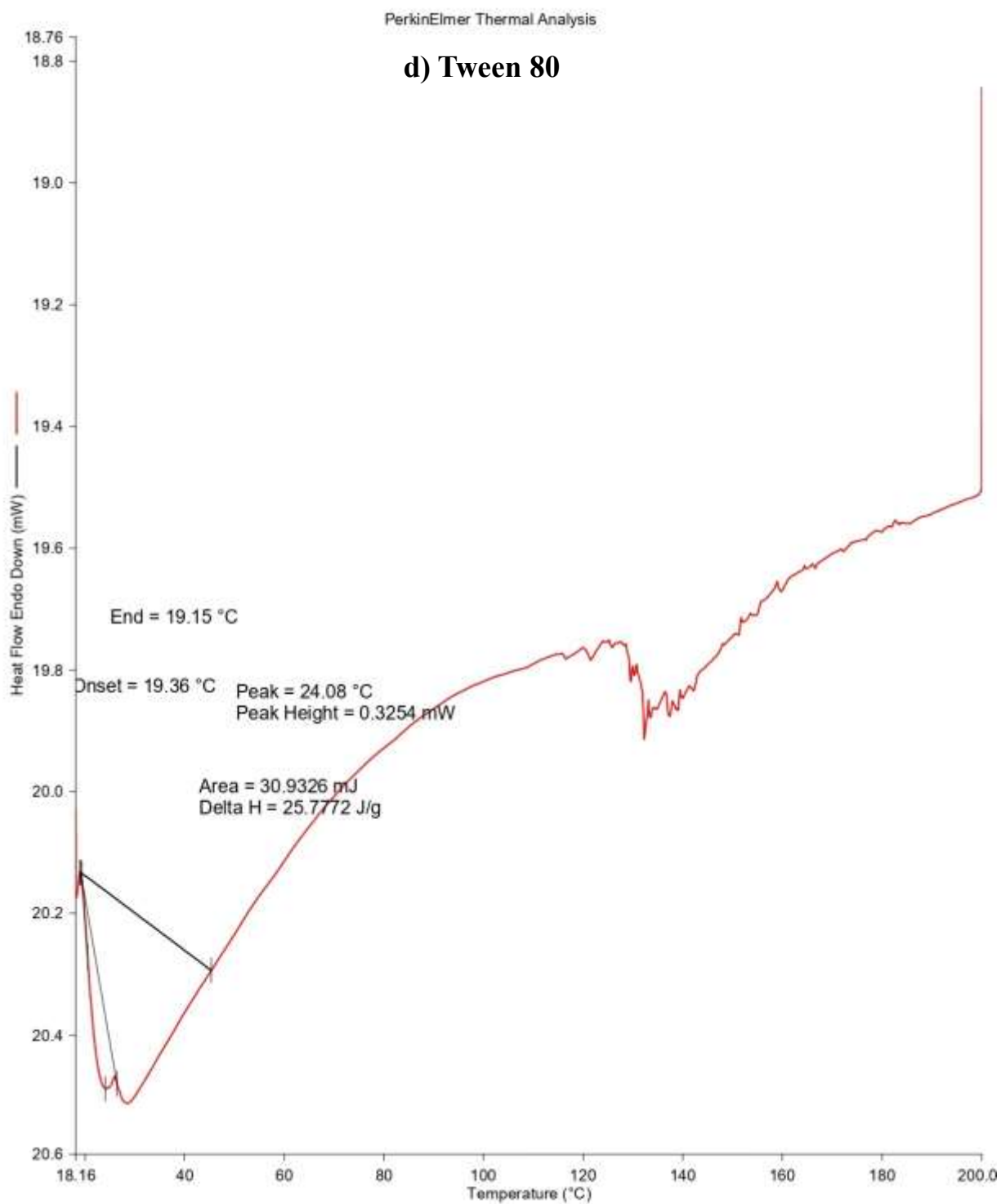
5.3.6.1. DSC

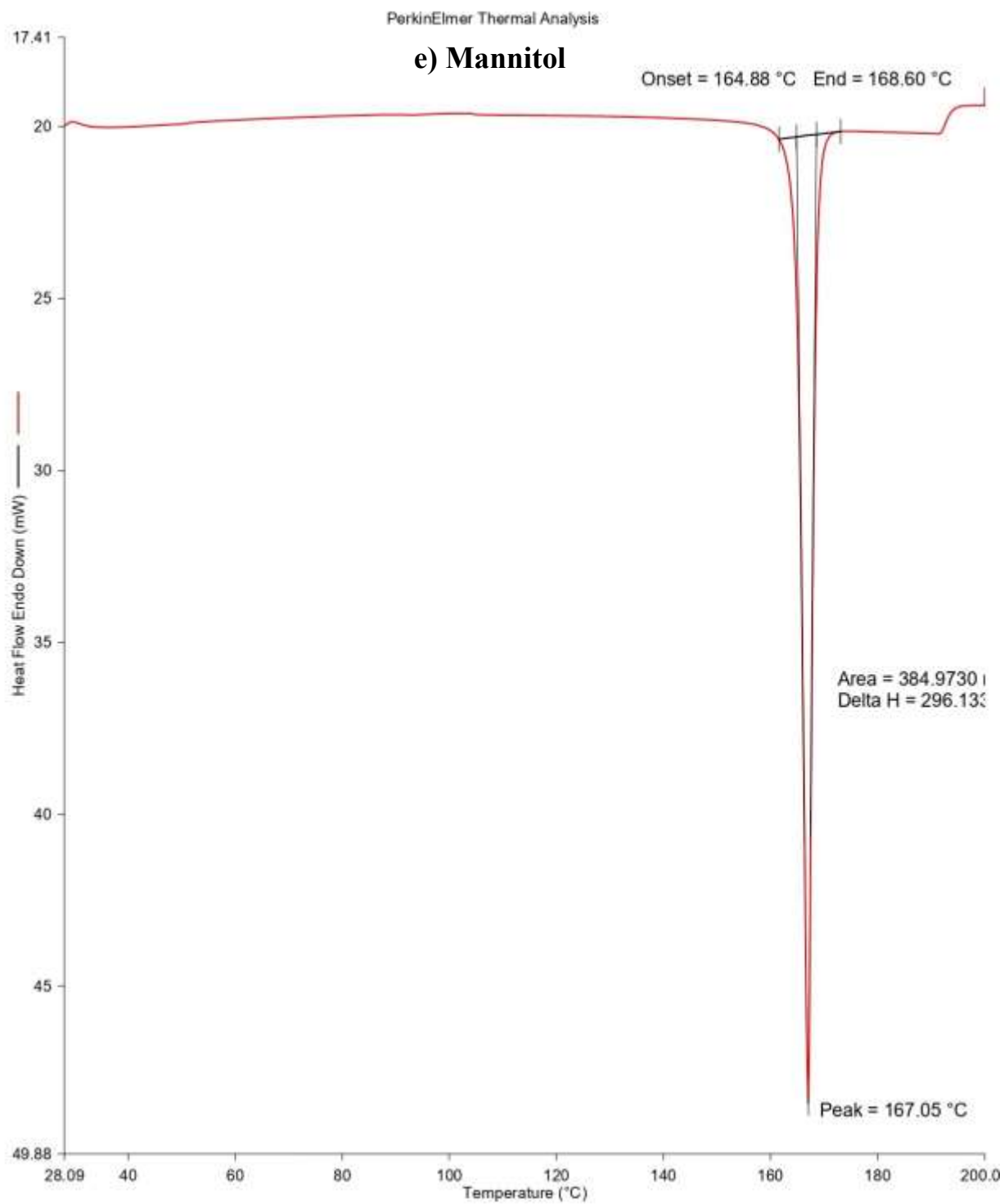
The differential scanning calorimetry (DSC) thermograms for pure Xn, beeswax, Mannitol, lauroglycol-90, Tween 80, L-Xn-NLCs, and S-Xn-NLCs were systematically recorded and analyzed from room temperature to 400°C. The crystalline nature of pure Xn was observed sharp peak at 173.17 °C whereas the thermodynamic peak for Beeswax was observed at 52.48 °C, lauroglycol-90 at 150 °C, Tween 80 at 24.08 °C, Mannitol at 167.05 °C, L-Xn-NLCs at 86.05 °C and S-Xn-NLCs at 166.69 °C as shown in fig. 5.23. The results of the DSC confirmed that the mannitol present in S-Xn-NLCs are crystalline in nature whereas, the peaks related to Xn were completely absent in S-Xn-NLCs that indicated that the Xn lost the crystalline nature during the formulation due to its complete solubility in NLCs.

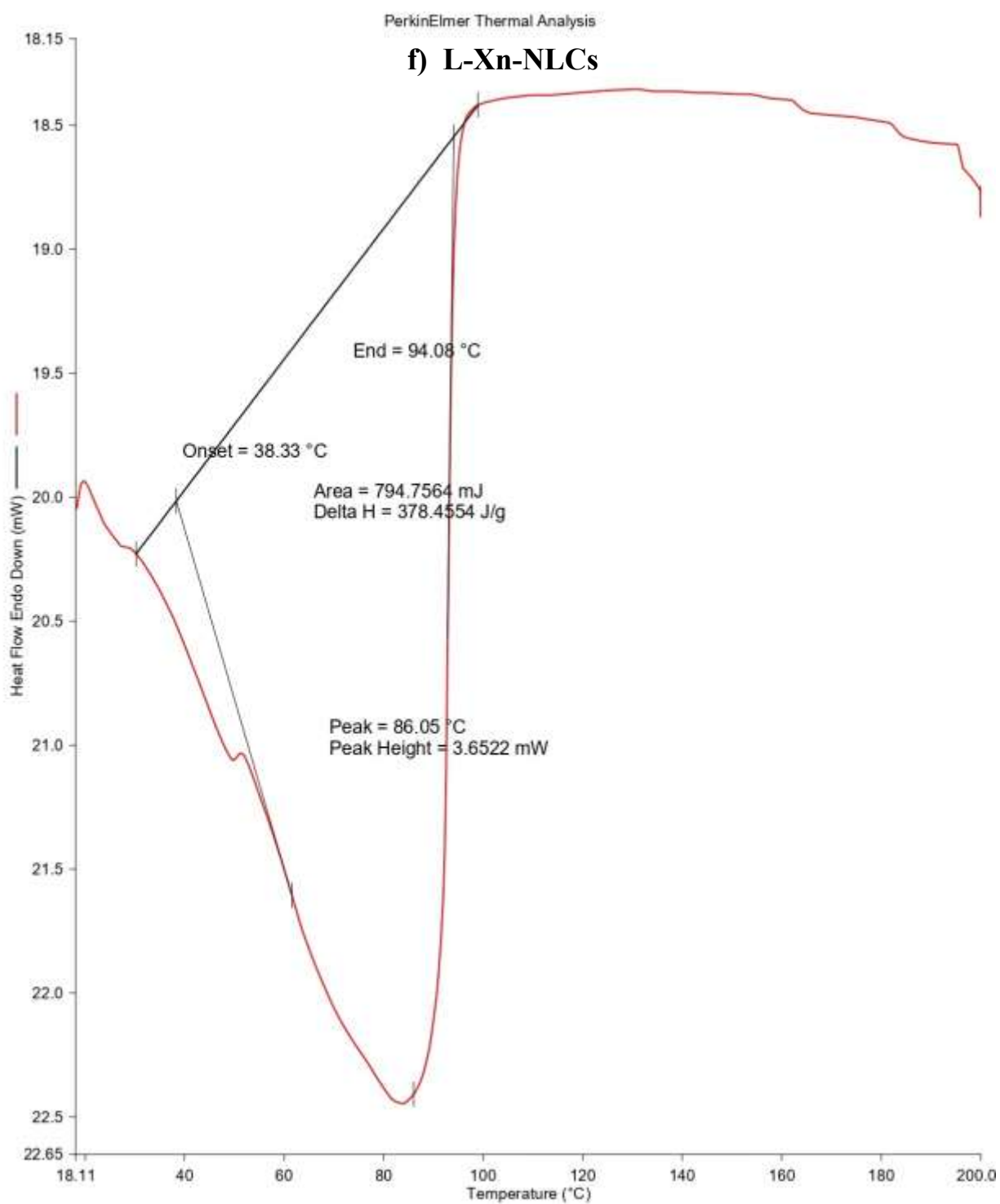












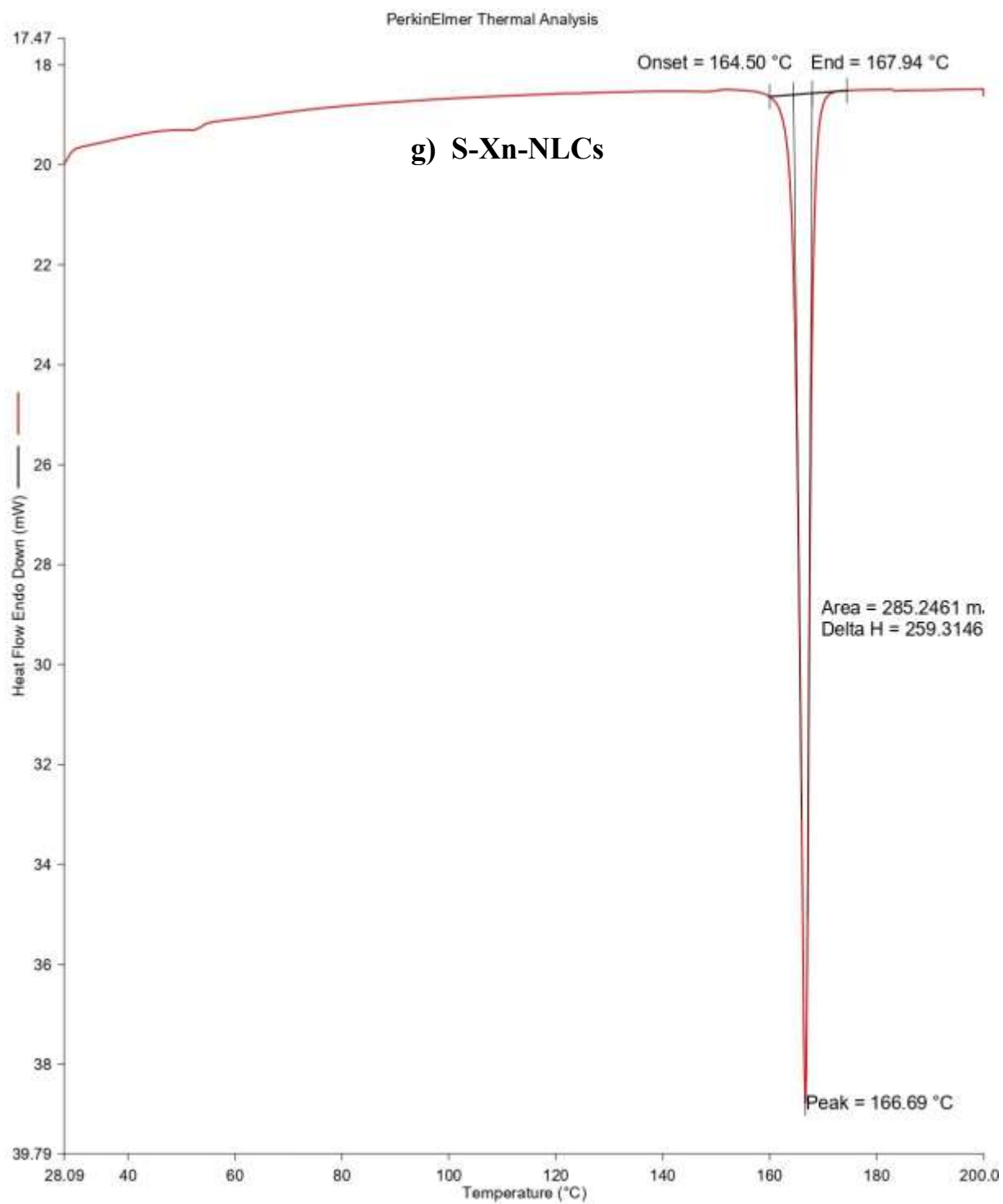
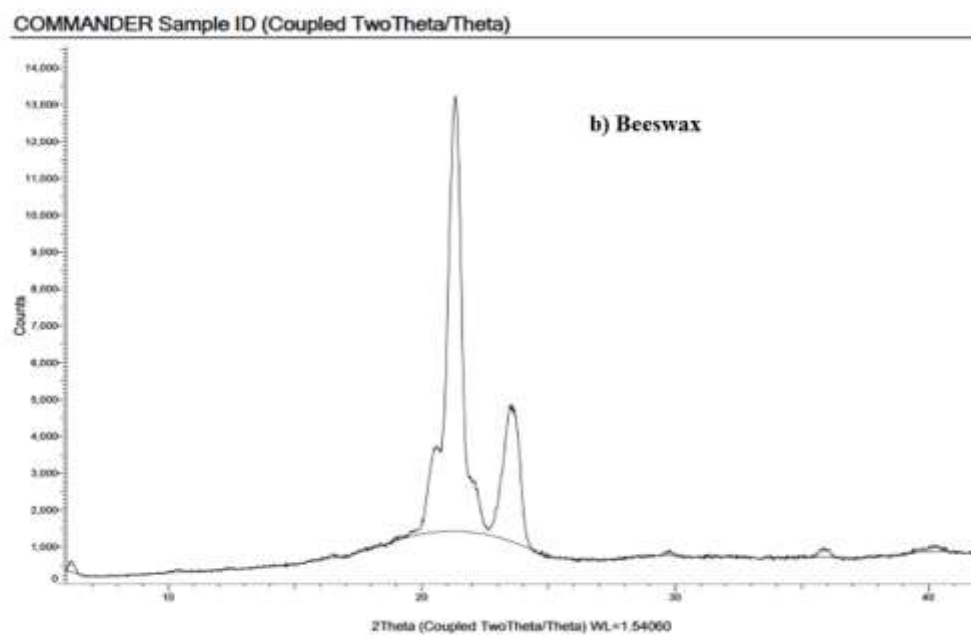
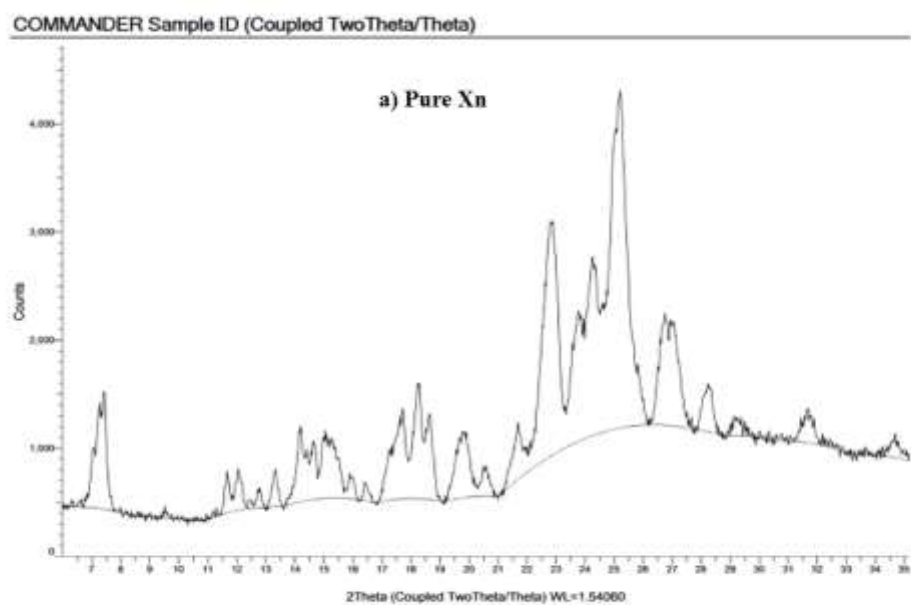


Fig. 5.23 : DSC images of a) Pure Xn, b) Beeswax, c) Lauroglycol-90, d) Tween-80, e) Mannitol, f) L-Xn-NLCs, g) S-Xn-NLCs

5.3.6.2. PXRD

Samples (Xn, beeswax, mannitol, and S-Xn-NLCs) were scanned using PXRD (Bruker Model: D8 Advance) with the monochromatized Cu-K α radiation at the speed of 27.3050s from 25°C with the 2 Θ angle, scanning step size 0.0170 at the voltage of 45 KV and current of 40 mA. Xn, beeswax, mannitol, and S-Xn-NLCs showed sharp peaks at various diffraction angles, exhibiting a typical crystalline pattern. This was understood by observing the diffraction peaks appeared in the diffractogram. For instance, Xn showed sharp peaks at 2 Θ angle at 7.435°, 11.661°, 12.788°, 13.326°, 17.629°, 18.295°, 19.934°, 22.880°, 25.211° with a peak count of 1524, 791, 636, 804, 1292, 1600, 1153, 3100, 4311 respectively. Beeswax showed sharp peaks at 2 Θ angle at 20.985°, 21.369°, 23.546° with a peak count of 6320, 12905, 4882 respectively. Mannitol showed peaks at 2 Θ angle at 10.559°, 14.683°, 18.910°, 20.447°, 21.164°, 23.418°, 28.285°, 29.437° with a peak counts of 3489, 10595, 23991, 5269, 9307, 23367, 4717, 5503 respectively (Patel et al., 2017), whereas S-Xn-NLCs showed sharp peaks at 2 Θ angle at 9.765°, 13.736°, 17.322°, 18.756°, 19.883°, 20.447°, 21.137°, 22.137°, 24.724°, 25.160°, 27.004°, 28.182° with a peak counts of 3837, 5709, 15687, 5016, 8255, 14377, 8906, 2772, 2444, 3219, 2254, 3365 respectively. The results of the XRD confirmed that the mannitol present in S-Xn-NLCs are crystalline in nature whereas, the peaks related to Xn were completely absent in S-Xn-NLCs that indicated that the Xn lost the crystalline nature during the formulation due to its complete solubility in NLCs as shown in Fig. 5.24.



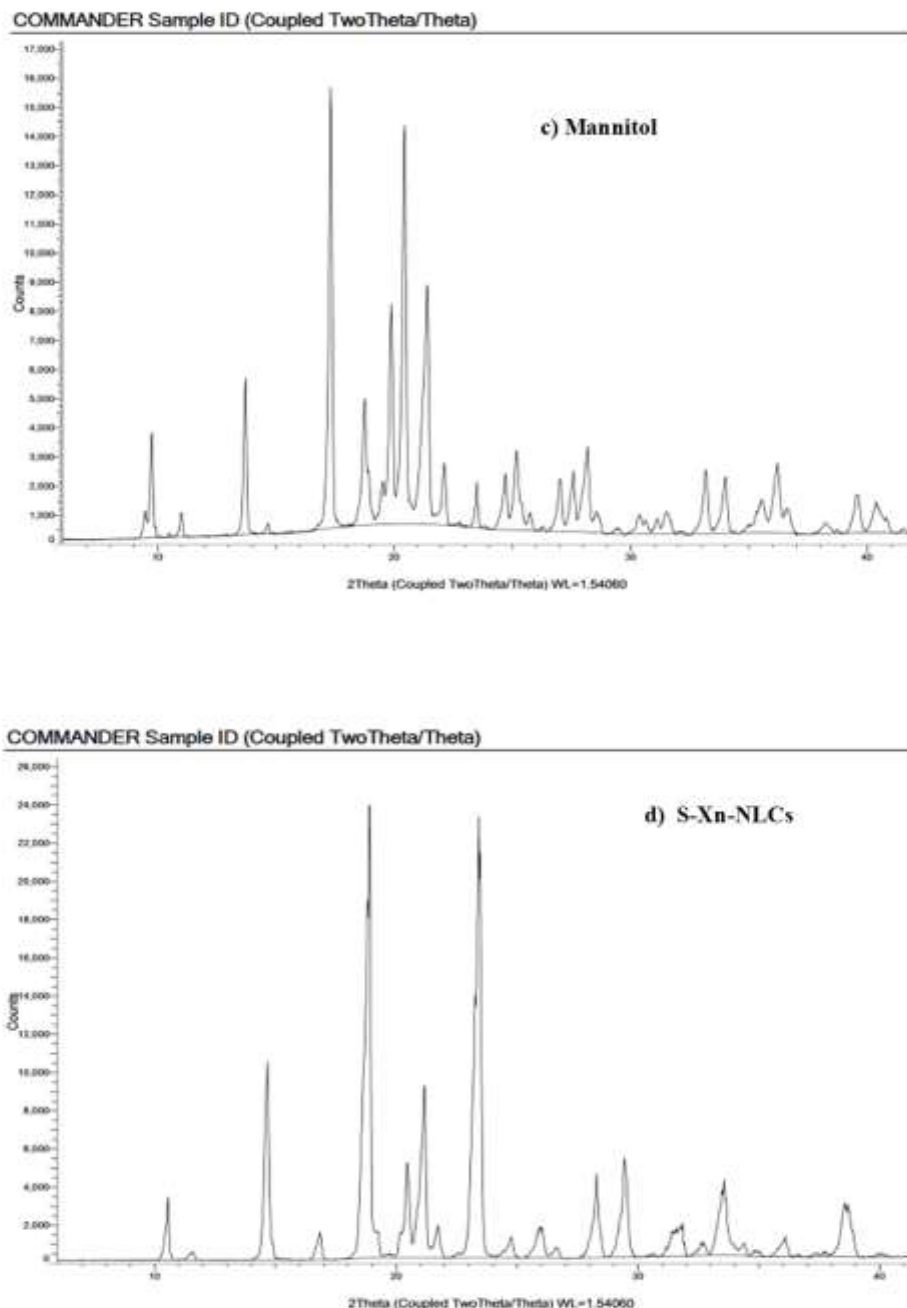


Fig. 5.24 : PXR images of (a) Pure Xn, (b) Beeswax, (c) Mannitol, (d) S-Xn-NLCs

5.3.6.3. Surface morphology (SEM)

The surface morphology of beeswax, Xn, mannitol and S-Xn-NLCs was analyzed using SEM. The images illustrate the surface characteristics of the solid lipid (beeswax) showing waxy in nature,

indicating a lipid nature. Xn showed the small, cylindrical and flat images indicating crystalline nature. Mannitol showed long cylindrical images indicating crystalline nature. S-Xn-NLCs showed a mixed morphology of crystalline indicating the presence of all the components as shown in fig. 5.25. The S-Xn-NLCs were crystalline, cylindrical and waxy, in appearance indicating the presence of all the ingredients used in S-Xn-NLCs formation. However, the dominance of mannitol was found as per image indicating dispersion of Xn-NLCs in the matrix of mannitol (Patel et al., 2017).

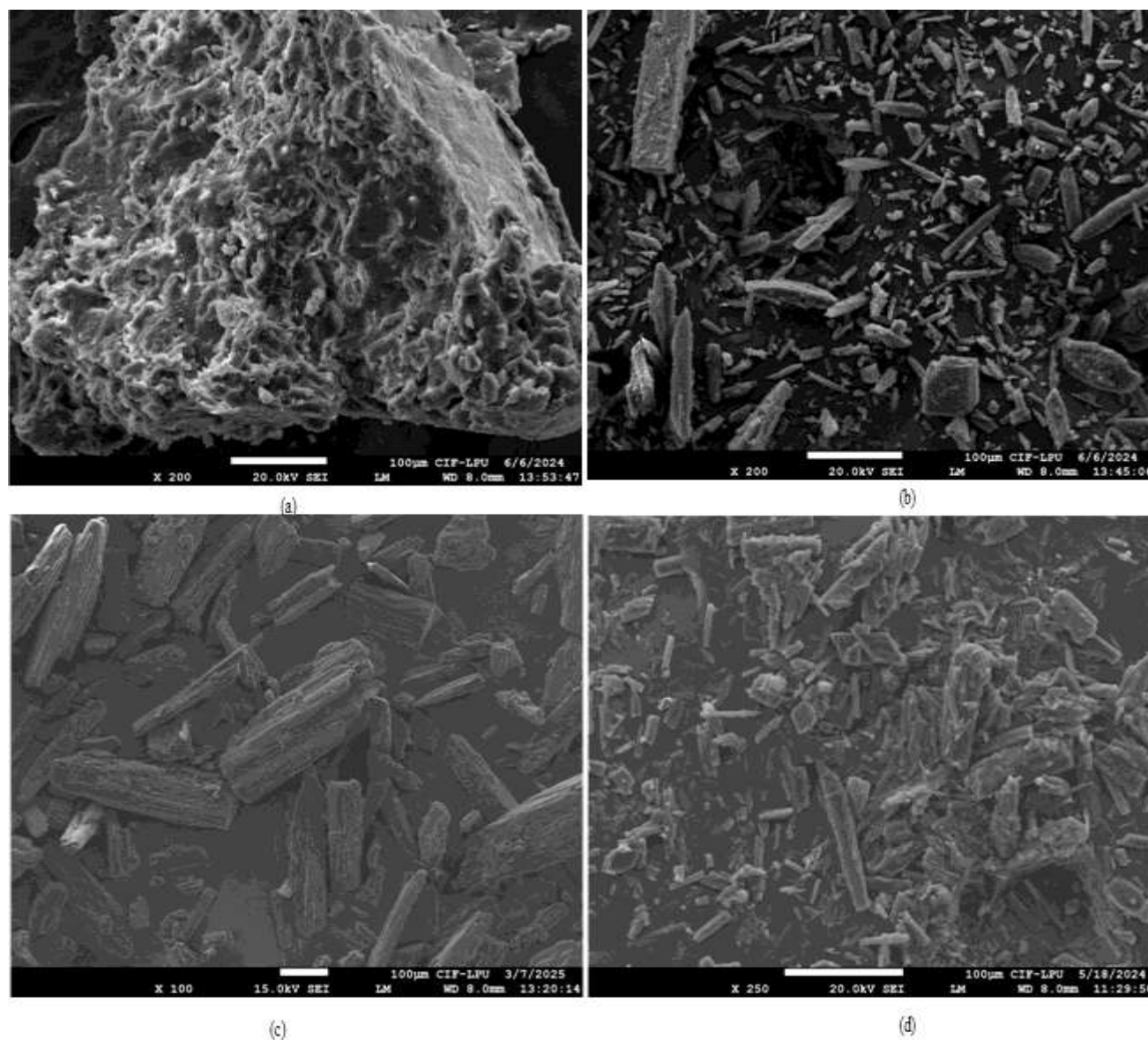


Fig. 5.25 : SEM images of (a) Beeswax, (b) Xn, (c) Mannitol and (d) S-Xn-NLCs

5.3.6.4. TEM analysis

The surface characteristics and morphology of L-Xn-NLCs were analyzed using high-resolution transmission electron microscopy (HRTEM), specifically the Hitachi H-7500 model from Japan, operating at 120 kV and equipped with a CCD camera. In order to perform TEM analysis L-Xn-NLCs were stained 1% aqueous solution of phosphotungstic acid (PTA). PTA is a common negative stain used in TEM analysis. A copper grid coated with pioloform and having a mesh size of 200 microns was used for mounting the sample. The film was examined under a TEM at 40–120 kV after drying for one hour. The results of TEM analysis show the spherical in shape particle size of Xn-NLCs from 200 - 240 nm as shown in fig. 5.26.

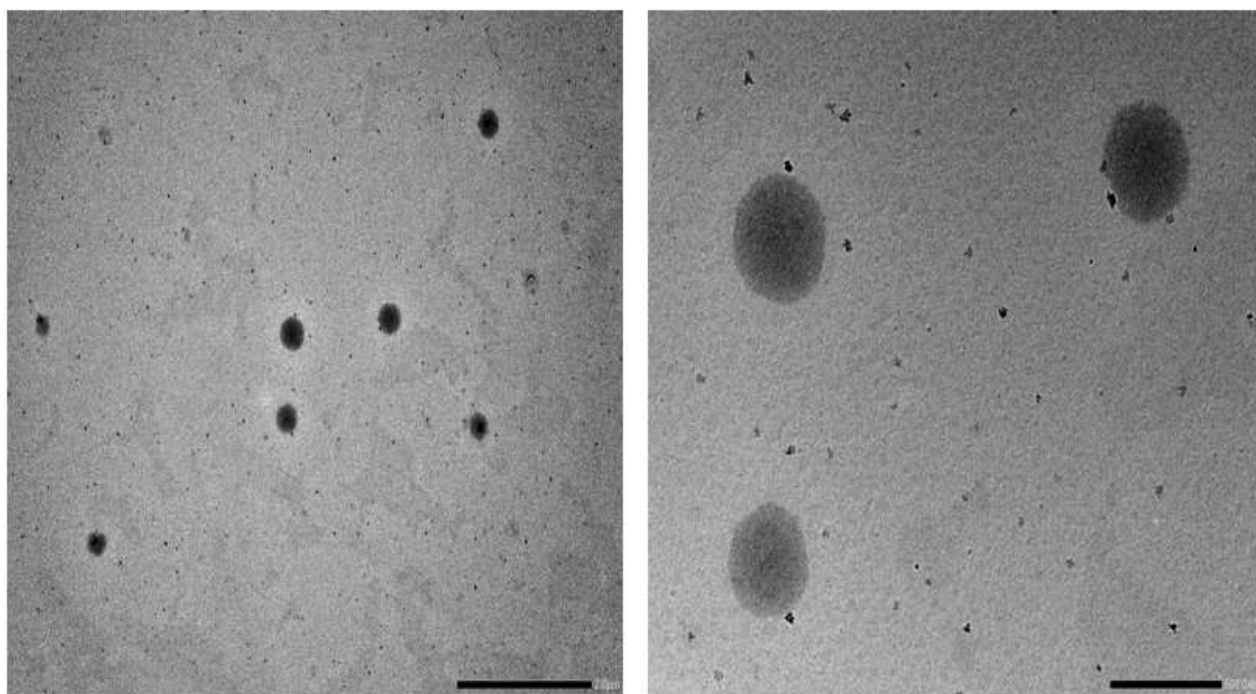


Fig. 5.26 : The results of TEM analysis show the particle size of Xn-NLCs.

5.3.7. In-Vitro A549 cell proliferation assay

5.3.7.1. MTT assay

MTT assay was performed to determine the efficacy of pure Xn and reconstituted S-Xn-NLCs in suppressing A549 cell growth. The percentage inhibition of cell proliferation was assessed at different concentrations ranging 0 - 141 μ M of the pure Xn and S-Xn-NLCs. The untreated cells and DMSO treated cells were used as control groups for comparing the efficacy of pure Xn,

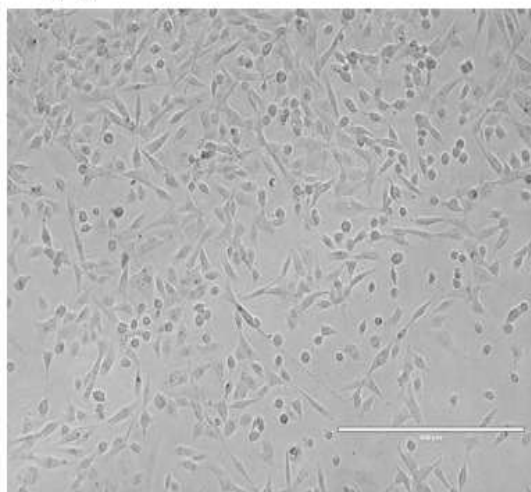
Results and discussion

whereas, in case of S-Xn-NLCs untreated cells and placebo treated cells were taken as control groups. Morphology of A549 cells and confluency after 24h treatment of pure Xn (A) and S-Xn-NLCs (B) is represented in fig. 5.27. The graph shown in fig. 5.28a represents cytotoxicity of pure Xn at various concentrations and the cytotoxic effect of S-Xn-NLCs at different concentrations is shown in fig. 5.28b. Xn showed significant reduction in cell lines proliferation at 28.21 μ M, 56.42 μ M, 84.63 μ M, 112.84 μ M and 141 μ M concentration as compared to all the control groups (** p <0.0001 in case of pure Xn suspension and ** p < 0.0001 in case of S-Xn-NLCs). However, in comparison to pure Xn, S-Xn-NLCs exerted more reduction of proliferation of A549 cells than pure that of Xn. About 1.7~ fold increase in reduction of the proliferation of A549 cells was observed in case of S-Xn-NLCs than pure Xn. The IC₅₀ value of Pure Xn was 140.186 μ M and IC₅₀ of S-Xn-NLCs was 84.63 μ M, which indicated that Xn loaded in S-Xn-NLCs has shown higher cytotoxicity potential as compared to the pure Xn.

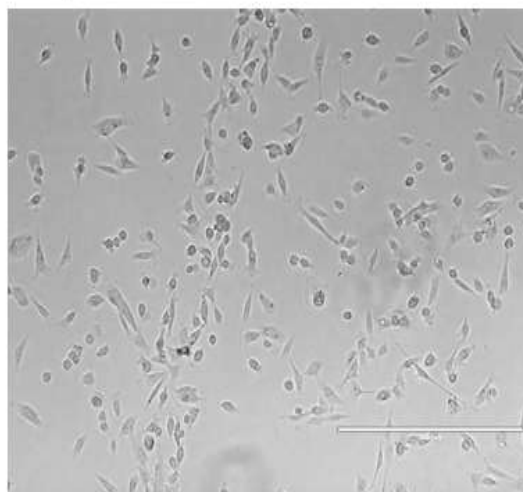
Results and discussion

(A)

MTT assays - A549 cells treated with Pure-Xn



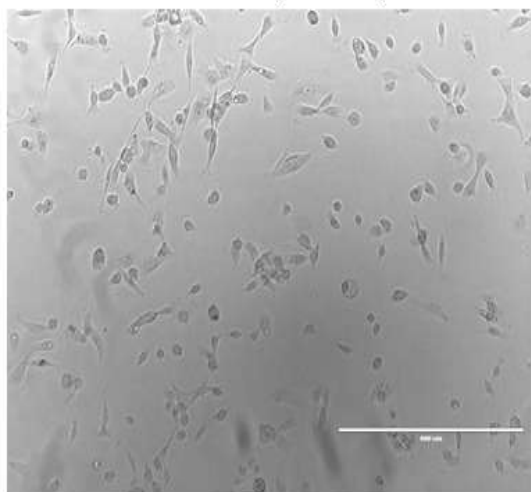
Control (DMSO)



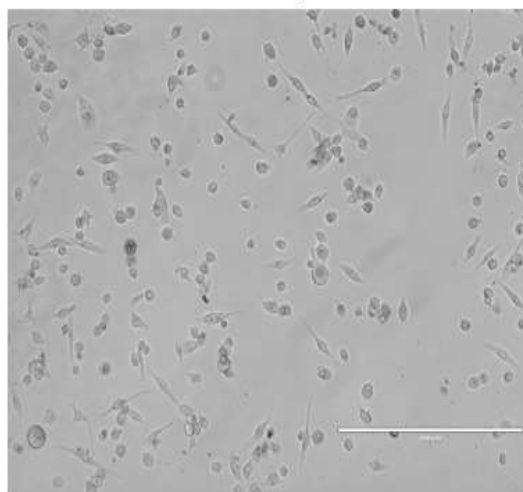
28.21 μ M



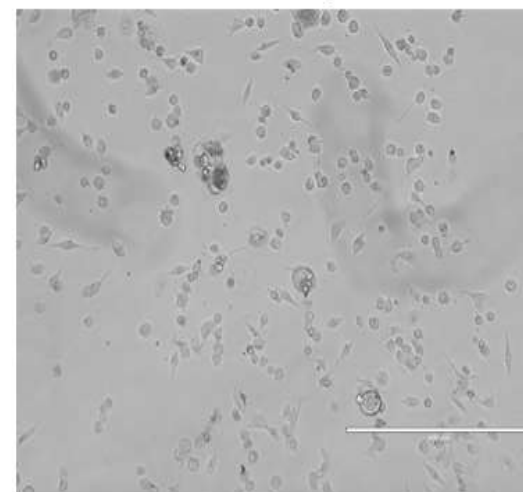
56.42 μ M



84.63 μ M



112.84 μ M



141 μ M

Results and discussion

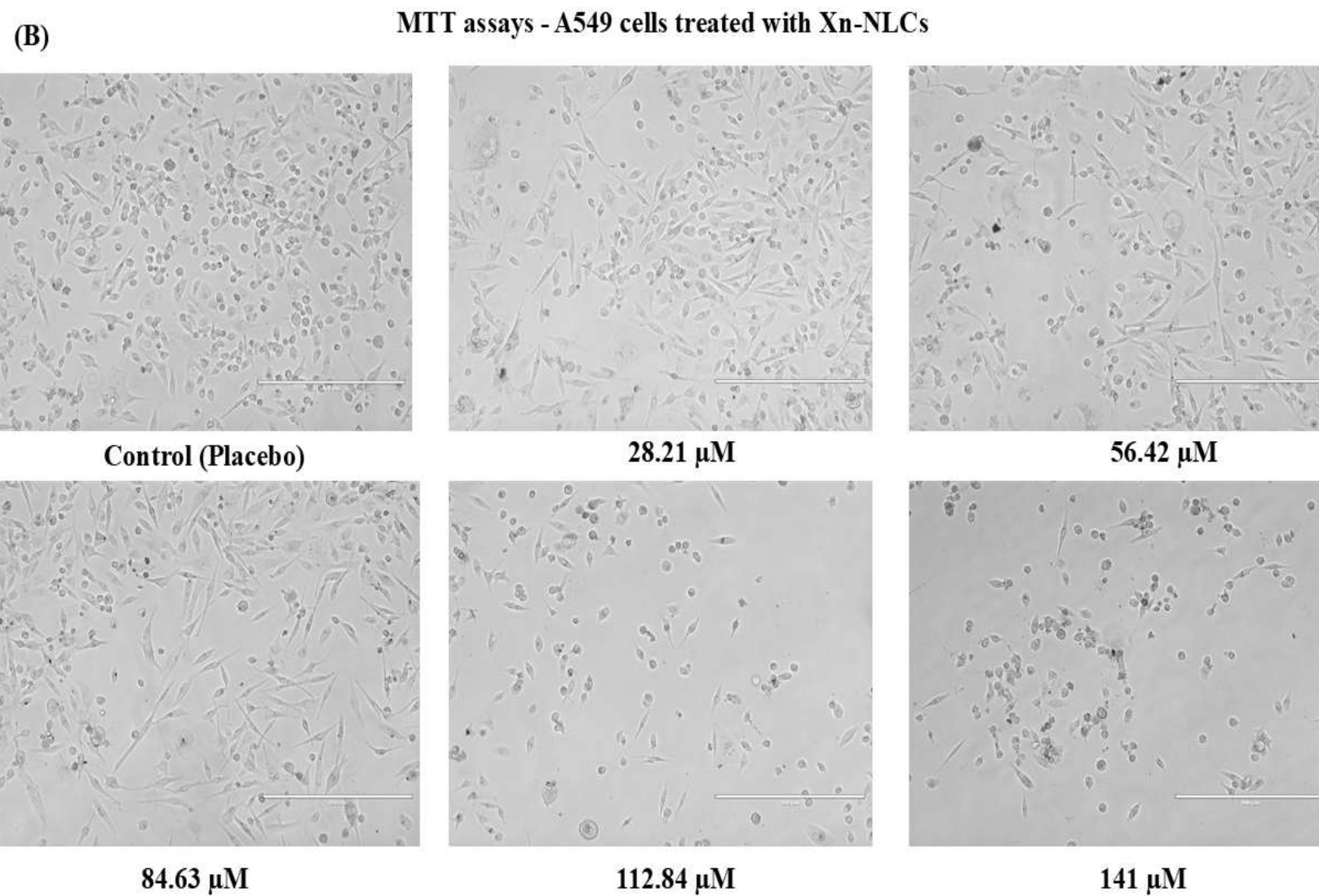
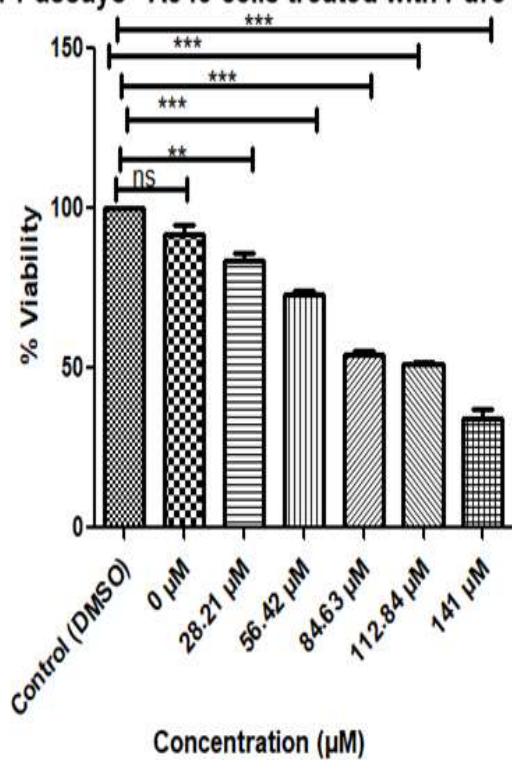


Fig. 5.27 : Morphology of A549 cells and confluency after 24h treatment of pure Xn (A) and S-Xn-NLCs (B). The MTT assays represent images of light microscopy at 20x magnification. Note: Here Xn-NLCs represents pure S-Xn-NLCs.

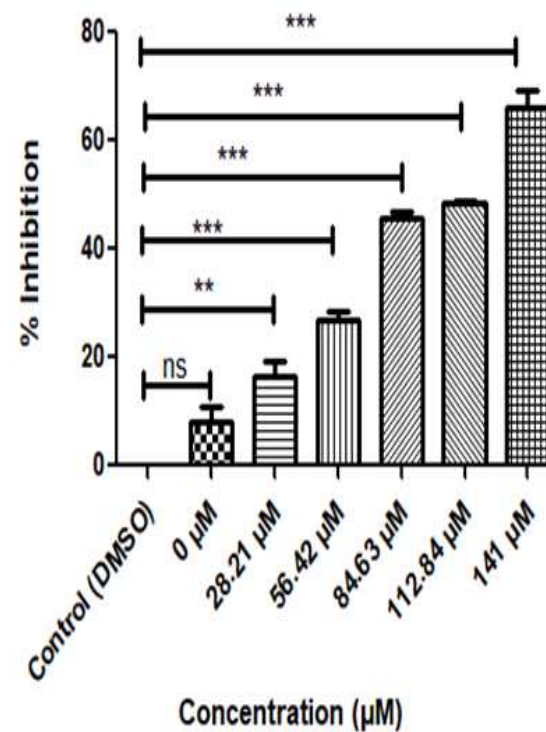
Results and discussion

(A)

MTT assays - A549 cells treated with Pure-Xn



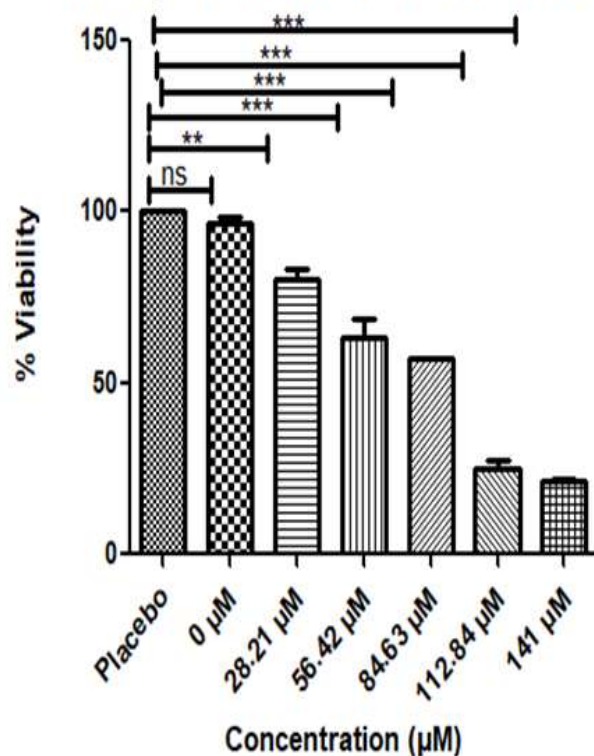
MTT assays - A549 cells treated with Pure-Xn



C

(B)

MTT assays - A549 cells treated with Xn-NLCs



MTT assays - A549 cells treated with Xn-NLCs

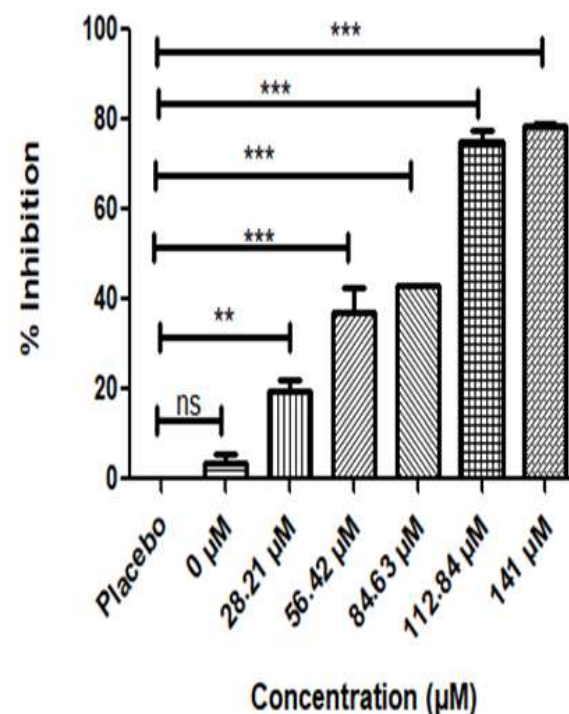


Fig. 5.28 : The statistical analysis of the MTT assay represents the % viability and % inhibition. (A) Pure Xn and (B) S-Xn-NLC, both reduced the proliferation of A549 cells at a concentration of 28.21 µM, 56.42 µM, 84.63 µM, 112.84 µM and 141 µM. S-Xn-NLCs exerted more reduction of cell proliferation than pure Xn. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's multiple comparisons test. The values shown are mean $\pm$ SEM of n = 3 independent experiments, ***p<0.0001, **p<0.01 control vs pure Xn or S-Xn-NLCs. Note: here Xn-NLCs represent S-Xn-NLCs.

Results and discussion

5.3.7.2. Determination of mitochondria membrane potential ($\Delta\Psi_m$)

Mitochondrial function, an essential indicator of cellular health, can be assessed by monitoring changes in the mitochondrial membrane potential ($\Delta\Psi_m$). The retention of rhodamine 123 was used to assess the changes in the mitochondrial membrane potential ($\Delta\Psi_m$) in the A549 cells (after treatment). This cationic dye binds to metabolically active mitochondria. The effect of IC_{50} concentration of Pure-Xn and S-Xn-NLCs on MMP was evaluated in A549 cells using rhodamine-123 dye. In this investigation, the results indicate a decrease in fluorescence intensity at IC_{50} concentration of Pure-Xn and S-Xn-NLCs in comparison to the untreated control cells (100%), indicating loss of MMP as shown in fig. 5.29 and as shown in graph 5.30.

Rhodamine 123 assays staining

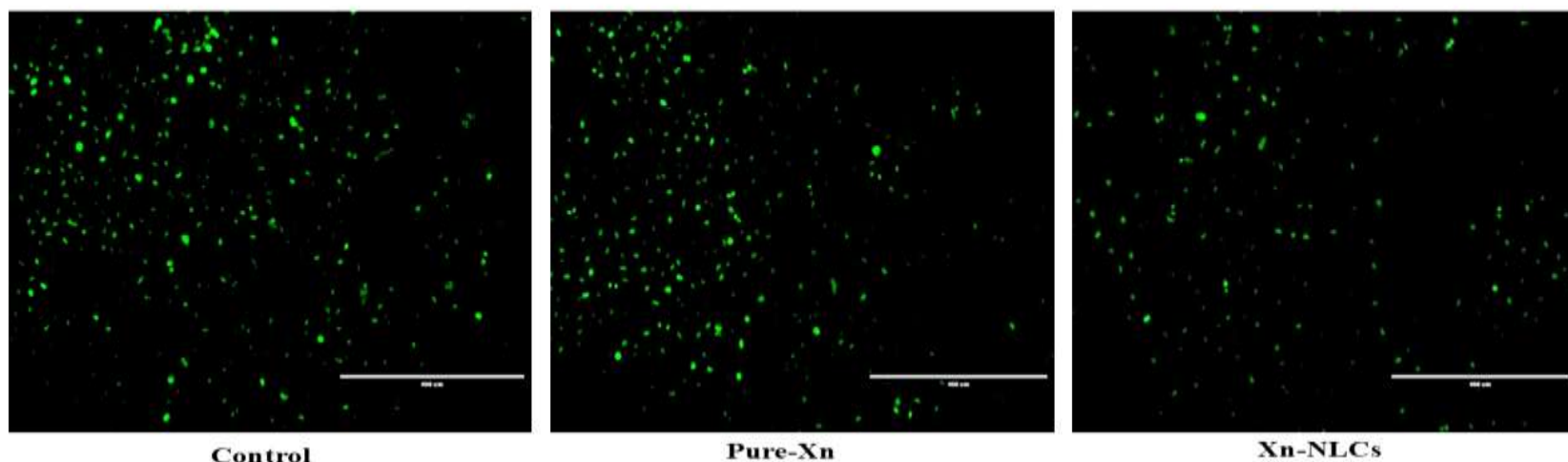


Fig. 5.29 : Measurement of mitochondrial membrane potential using Rhodamine 123 following 24 h treatment of IC_{50} concentration of Pure-Xn and Xn-NLCs on mitochondrial membrane potential (MMP) of A549 cells. Images were taken at 20x magnification (EVOS FL inverted Fluorescence Microscope). Note : here Xn-NLCs represent the S-Xn-NLCs.

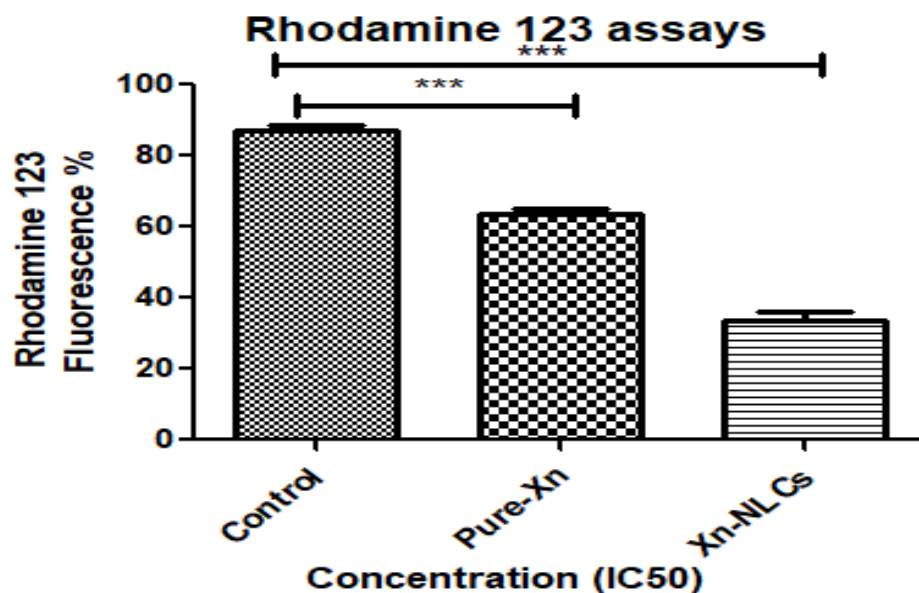


Fig. 5.30 : Effect of Pure-Xn and Xn-NLCs on MMP of A549 cells. The data are shown as the mean $\pm$ SEM of 3 independent experiments, with a *** $p < 0.0001$ significance level. Statistically significant difference from the control ($p < 0.05$) using Dunnett's multiple comparison test. Note : here Xn-NLCs represent the S-Xn-NLCs.

5.3.7.3. DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) Staining - ROS detection

Reactive oxygen species, naturally produced through cellular metabolism, are capable of exerting anti-tumor effects by stimulating various signaling pathways, including cell apoptosis, necrosis, and autophagic cell death. When ROS levels rise, they can trigger signaling pathways mediated by ROS, leading to alterations in the levels of proteins associated with the cell cycle. The DCFH-DA probe, capable of hydrolysis by esterases, permeates the cell membrane to form DCFH. Reactive oxygen species within the cellular environment can subsequently oxidize non-fluorescent DCFH into fluorescent DCF. This transformation enables the measurement of ROS levels via DCF fluorescence intensity. Observations revealed that the control group exhibited no green fluorescence, indicating low ROS levels, whereas the treated groups (Pure Xn and S-Xn-NLCs) demonstrated elevated ROS levels as shown in fig. 5.31 and graph 5.32. Oxidative stress-induced cell death arises from an imbalance between cellular oxidation and resistance to oxidation. It has been further elucidated that this imbalance prompts apoptosis in A549 cells through the ROS-mediated mitochondrial pathway.

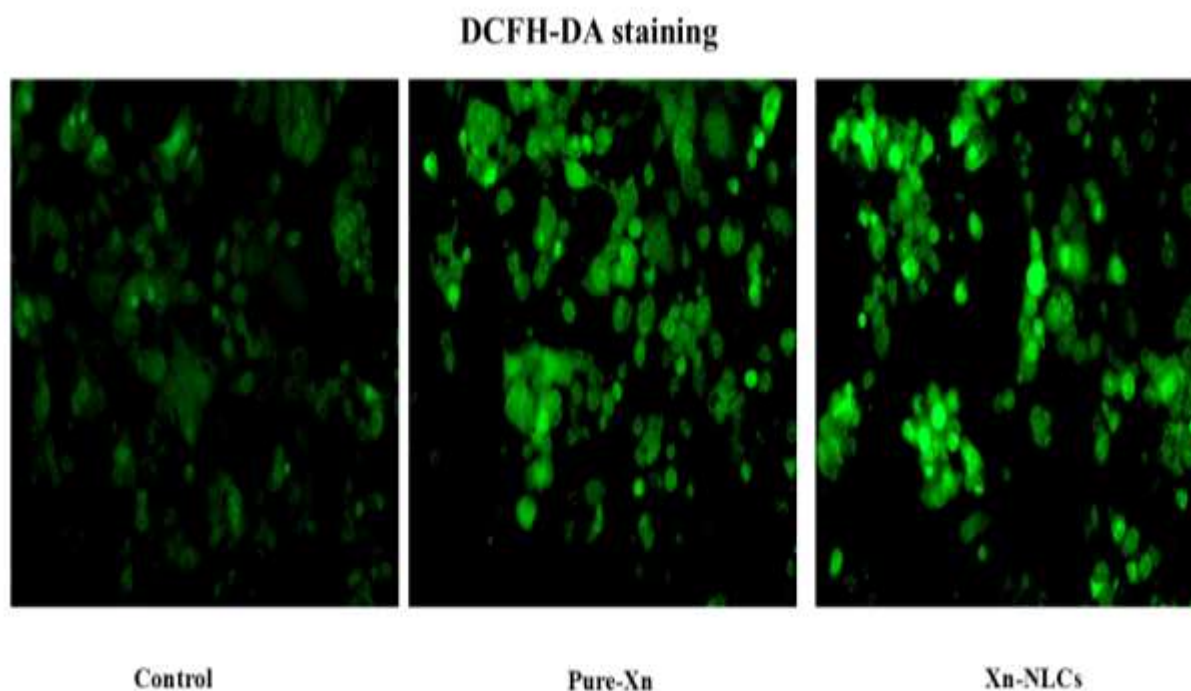


Fig. 5.31 : Fluorescence intensity (DCF) percentage after 24 hours of incubation with Pure-Xn and Xn-NLCs. After straining A549 cells with DCFH-DA, a fluorospectrophotometer was used to measure the ROS levels. Note : here Xn-NLCs represent the S-Xn-NLCs.

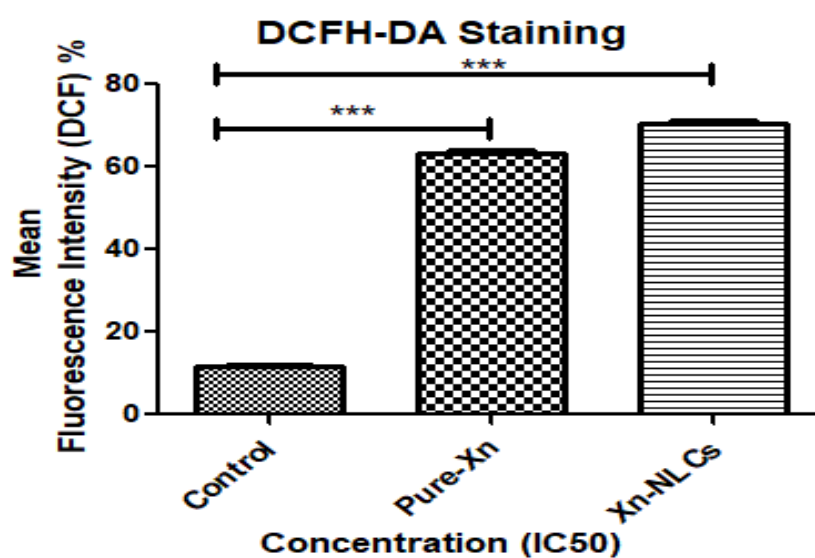
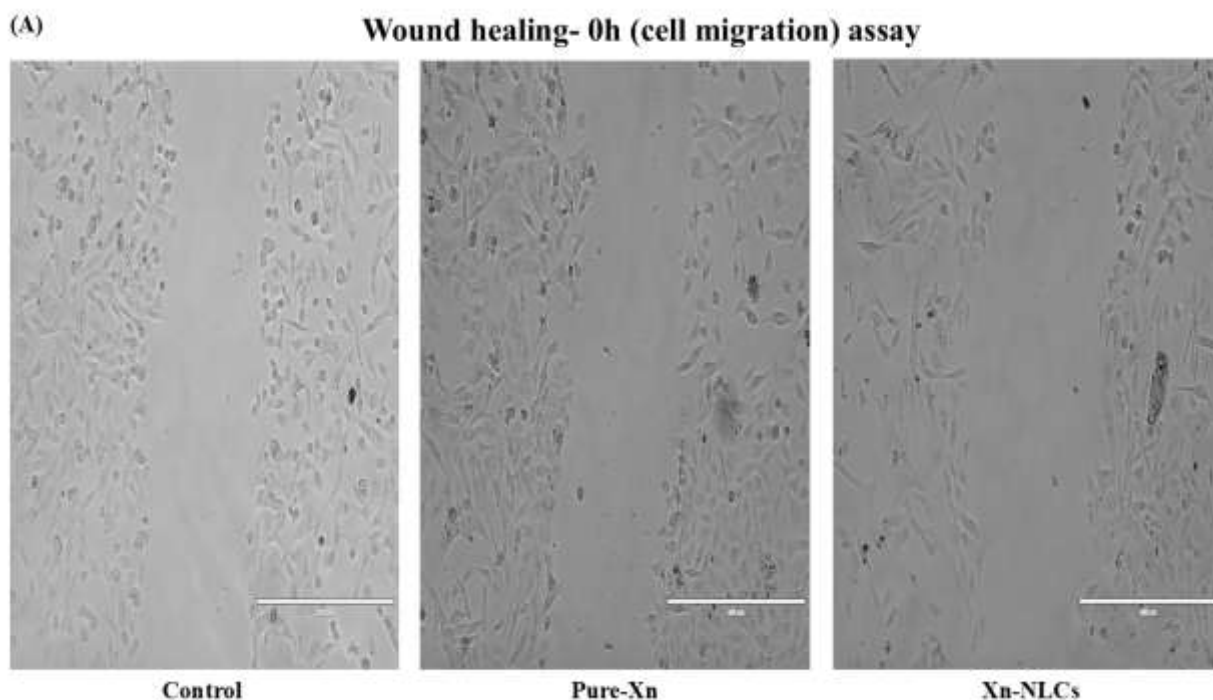


Fig. 5.32 : Mean fluorescence intensity (DCF) percentage after 24 hours of incubation with Pure-Xn and Xn-NLCs. Then, After straining A549 cells with DCFH-DA, a fluorospectrophotometer was used to measure the ROS levels.

Only Xn-NLCs and pure-Xn reduced the amount of A549 by inducing ROS followed by apoptosis vs Control group. The data are shown as the mean $\pm$ SEM of 3 independent experiments, with a *** $p < 0.0001$ significance level (control vs pure-Xn or Xn-NLCs). The analysis was carried out using one-way ANOVA, followed by the Dunnett multi-comparison test. Note : here Xn-NLCs represent the S-Xn-NLCs.

5.3.7.4. Wound healing (cell migration) assay

According to the wound healing assay images, there is no difference in wound closure between the pure Xn (IC_{50}) and control groups; however, A549 cells treated with S-Xn-NLCs showed significant inhibition of wound closure as shown in fig. 5.33. A549 cells treated with IC_{50} concentration of S-Xn-NLCs showed a more than 80% decrease in wound closure as compared to the control group, measured by the percentage wound closure assay. This shows that only S-Xn-NLCs inhibit the migration and invasion of A549 cells than the Pure Xn. The graph is shown as fig. 5.34 representing pure Xn and the S-Xn-NLCs where only S-Xn-NLCs reduced the migration of A549 at IC_{50} as compared to pure Xn (IC_{50}).



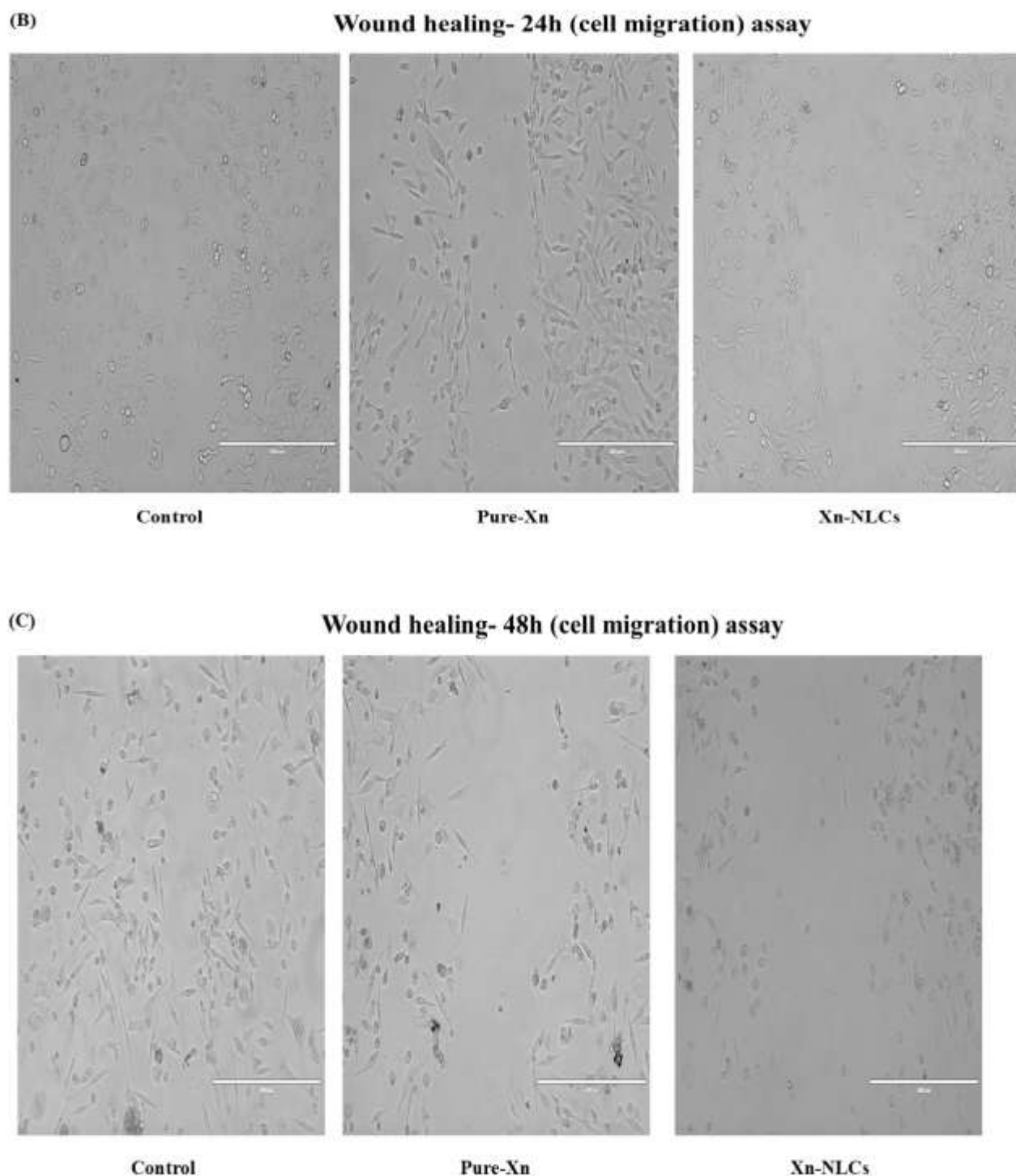


Fig. 5.33 : (A) Representative image from three independent experiments 0 hours. (B) Representative image from three independent experiments 24 hours. (C) Representative image from three independent experiments 48 hours. The cell migration assay was performed after scratching a wound on a monolayer of confluent A549 cells. Then, the cells were cultured for 0, 24, 48 hours with or without pure-Xn and Xn-NLCs. The images were acquired at 0, 24, 48 h of

treatment with a phase contrast microscope at $20 \times$ magnification. Note : here Xn-NLCs represent the S-Xn-NLCs.

Wound healing- 48h (cell migration) assay

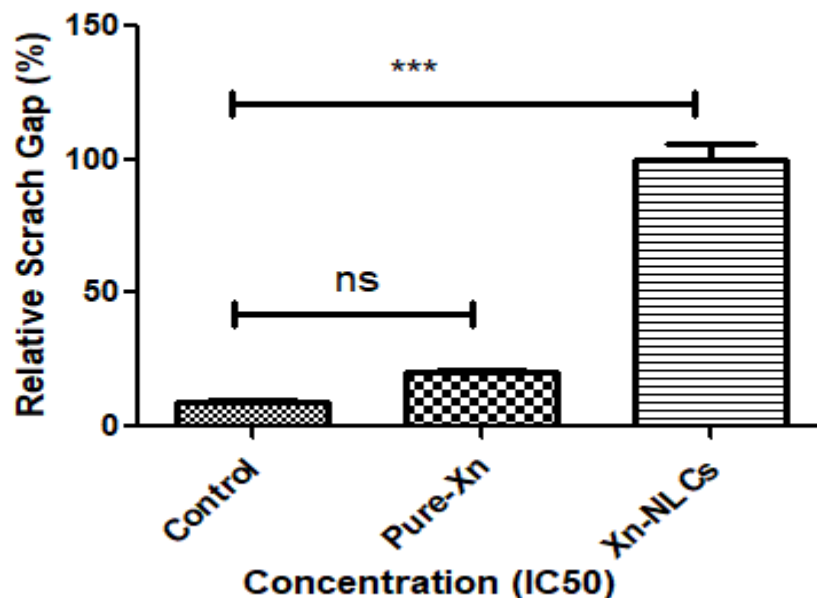


Fig. 5.34 : The percentage of wound closure after 48 hours of incubation with Pure-Xn and Xn-NLCs. The data are shown as the mean $\pm$ SEM of 3 independent experiments, with a *** $p < 0.0001$ significance level, ns = not significant (control vs pure-Xn or Xn-NLCs). The analysis was carried out using one-way ANOVA, followed by the Dunnett multi-comparison test. At IC₅₀, only Xn-NLCs (rather than pure-Xn) reduced the amount of A549 migration. Note : here Xn-NLCs represent the S-Xn-NLCs.

5.3.7.5. Boyden's chamber assay

Boyden's chamber assay represents the anti-migration activity of A549 cells. The cells were treated with Pure-Xn and Xn-NLCs at IC₅₀ concentration for 24hr in a Matrigel chamber. Then, the cells were allowed to migrate from the upper chamber to the lower chamber. The lower chamber were stained with the Crystal violet stain. The invaded cells were observed under a microscope and were counted at 20x magnification. The Xn-NLCs showed a higher reduction in migration activity of A549 cells as compared to the activity of the pure-Xn as shown in fig. 5.35.

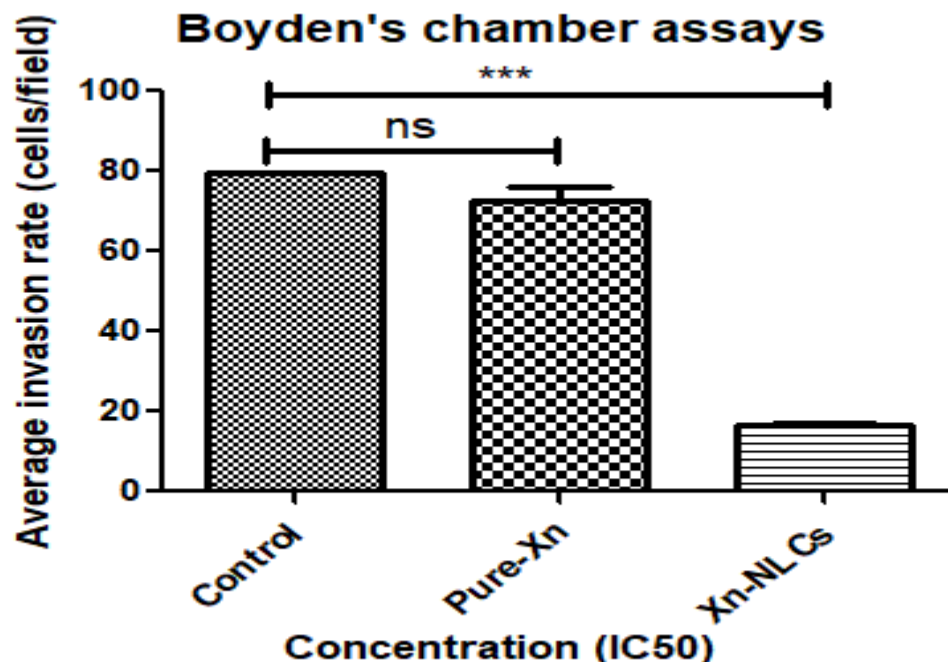


Fig. 5.35 : The anti-migration activities of A549 cells after 24 hours of incubation with Pure-Xn and Xn-NLCs. (B) The data are shown as the mean $\pm$ SEM of 3 independent experiments, with a *** $p < 0.0001$ significance level, ns = not significant (control vs pure-Xn or Xn-NLC). The analysis was carried out using one-way ANOVA, followed by the Dunnett multi-comparison test.

5.3.8. Detection of apoptosis- Flow cytometry analysis

5.3.8.1. Apoptosis by PI and AO/EtBr Staining method

PI and AO/EtBr staining were used to analyze the nuclear morphology in order to determine whether the A549 cell death was caused by apoptosis. In a 6-well plate, A549 cells were seeded (2×10^5 cells/well) and cultured in a CO₂ incubator for overnight. After incubation, cells were treated with the IC₅₀ concentration of pure-Xn and S-Xn-NLCs for 24 hr. Following a 24-hour period, the cells were gently washed once with PBS before being stained with PI (10 μ g/mL) for five minutes in darkness. The AO/EtBr staining technique is performed for visualizing nuclear alteration and differentiating between viable, apoptotic (early or late phases), and necrotic cells. Cells were trypsinized after incubation, and each 25 μ L cell suspension was placed on a glass slide. It was subsequently mixed with (1 μ L) of dual fluorescent staining solution, which contained 10 μ g/mL AO and 10 μ g/mL EB, and covered with a coverslip. The morphology of apoptotic cells

was examined and photographed using an EVOS FL inverted fluorescence microscope. Acridine orange (AO) stains both live and dead cells, while ethidium bromide targets only dead cells that has loss the membrane integrity. Live cells display a uniform green color, whereas early apoptotic cells exhibit green dots within their nuclei. Late apoptotic cells stain orange and demonstrate condensed or fragmented nuclei. In contrast, necrotic cells also stain orange, maintaining a nuclear morphology similar to healthy cells, but lacking condensed chromatin.

5.3.8.2. Cell cycle analysis - G0-G1, S, and G2/M phases

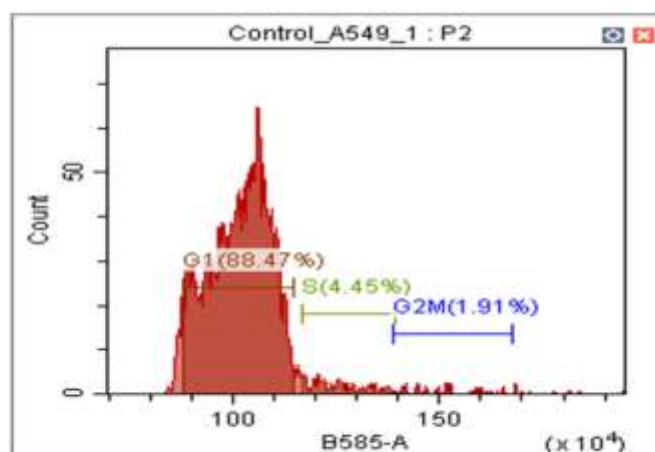
The cytotoxicity of S-Xn-NLCs and Pure Xn on A549 cells is related to cell cycle distribution. A549 cells were treated to IC₅₀ concentrations (μ M) of pure-Xn and S-Xn-NLCs for 24 hours, followed by PI staining. Flow cytometry was used to quantify cell populations in each phase of DNA content. The results of the cell cycle phases are depicted in Fig. 5.36 and Graph 5.37a and as shown in Table 5.16. The cell cycle analysis were determined by FACS CytoFlex-Beckman Coulter. The cell preparation method followed the protocol established by Davis et al. (1990). Data analysis was conducted using the DIVA software, through which the percentage of cells in the G0-G1, S, and G2/M phases was calculated. According to the results of cell cycle analysis, S-Xn-NLCs caused A549 cells to be arrested during the G2/M phase of the cell cycle. The experimental results show that S-Xn-NLCs treatment significantly accelerated the cell cycle progression Fig. 5.37(c). The percentage of cells entering into the G2/M phases is (57.02%) significantly higher than control Fig. 5.37(a) & 5.37(b). In the control group, the percentage of cells in the G2/M phase was 6.37%, whereas in the pure-Xn group, this percentage increased markedly to 31.65%. The mean PI (percentage of the S+G2/M phases), an index reflecting the proliferation characteristic of cells, was also evaluated. The PI of the Xn-NLCs group (57.02 %) was significantly higher than those of the control group (6.37 %) and pure-Xn group (31.65 %) (Table 5.16). The cell cycle analysis study showed that S-Xn-NLCs induced G2/M arrest significantly higher than the pure Xn.

Table 5.16 : Cell cycle distribution of A549 cells

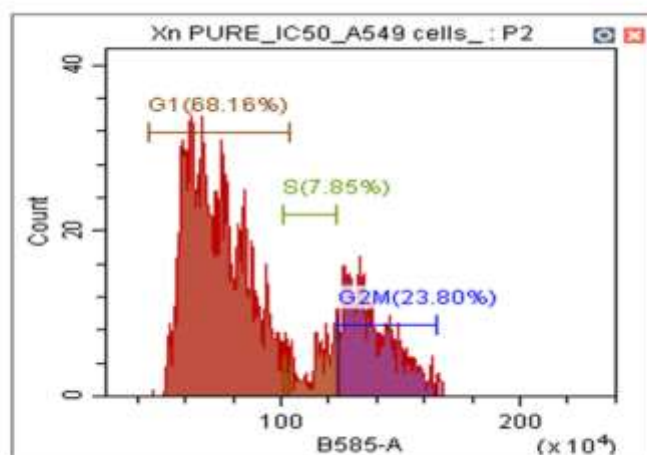
Cell cycle distribution of A549 cells				
Treatment	Phase			PI
	G0/G1	S	G2/M	
Control	88.47	4.45	1.91	6.37

Results and discussion

Pure-Xn	68.16	7.85	23.80	31.65
Xn-NLCs	39.87	10.29	46.73	57.02



a) Control



b) Pure-Xn

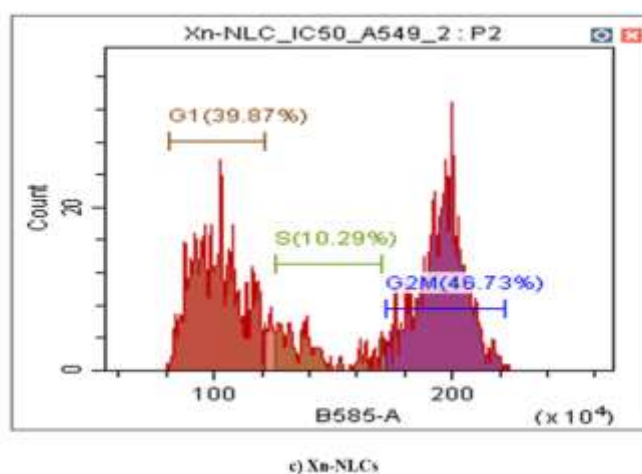


Fig. 5.36 : Flow cytometry analysis: Cell cycle distribution of A549 cells. The percentages of cells in G1, S, and G2/M phase of cell cycle. Cells were treated with IC₅₀ concentration (μ M) of pure-Xn and Xn-NLCs for 24 hours. (a) Control (without any treatment). (b) pure-Xn treatment. (c) Xn-NLCs treatment. The x-axes represent DNA content and y-axes represent cell count, respectively. Note : here Xn-NLCs represent the S-Xn-NLCs.

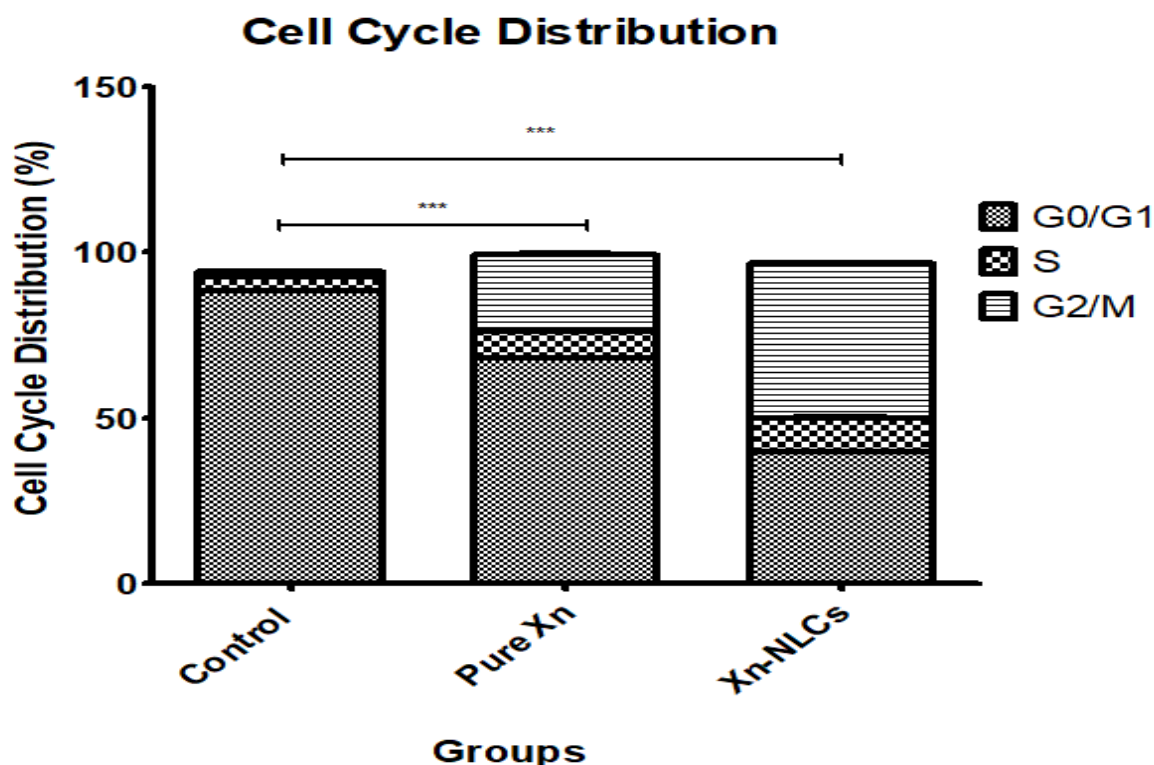


Fig. 5.37a : The statistical data of the cell cycle phase distributions of A549 cells. The cells were then harvested and stained with propidium iodide. Cells were treated with IC₅₀ concentration (μ M) of pure-Xn and Xn-NLCs for 24 hours. The analysis was carried out using two-way ANOVA, followed by the Bonferroni post-test. The data are shown as

the mean $\pm$ SEM of 3 independent experiments, with a *** p <0.0001 significance level.

Note : here Xn-NLCs represent the S-Xn-NLCs.

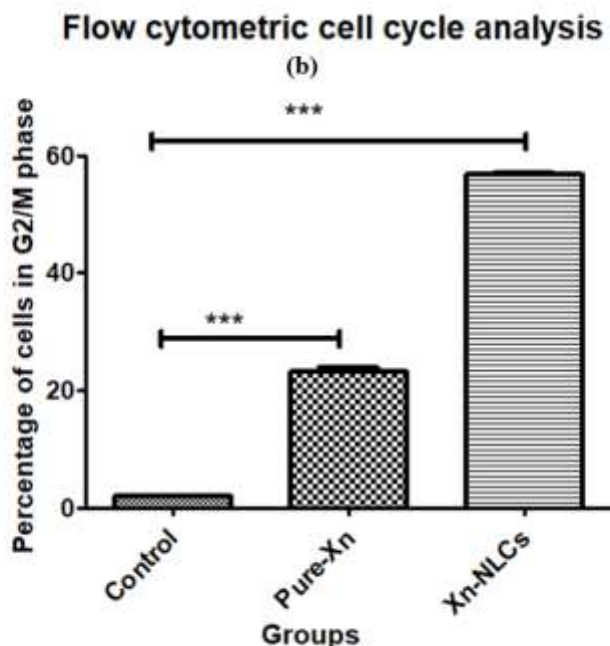


Fig. 5.37b : Cell cycle analysis by flow cytometry. The cells were then harvested and stained with PI. Histograms represent the percentage of cells in G2/M phase. The data are shown as the mean $\pm$ SEM of 3 independent experiments, with a *** p <0.0001 significance level. The analysis was carried out using one-way ANOVA, followed by the Dunnett multi-comparison test.

5.3.9. Gene expression - by Reverse transcriptase-quantitative polymerase chain reaction (q-RT-PCR)

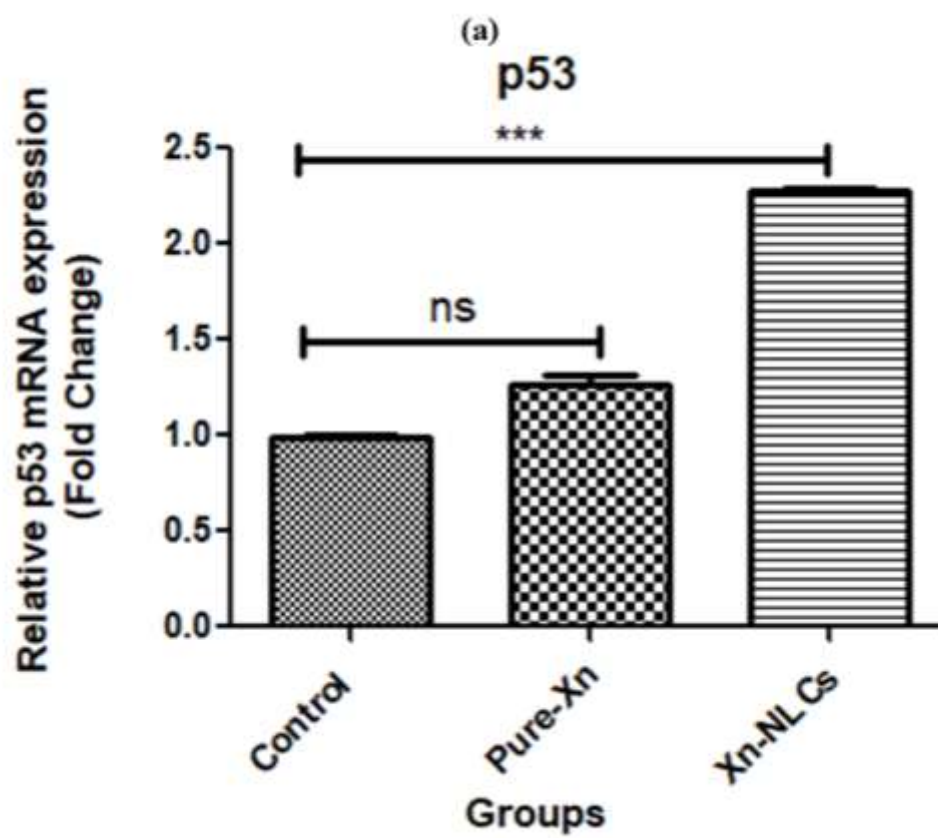
5.3.9.1. Gene expression - Real-Time qPCR

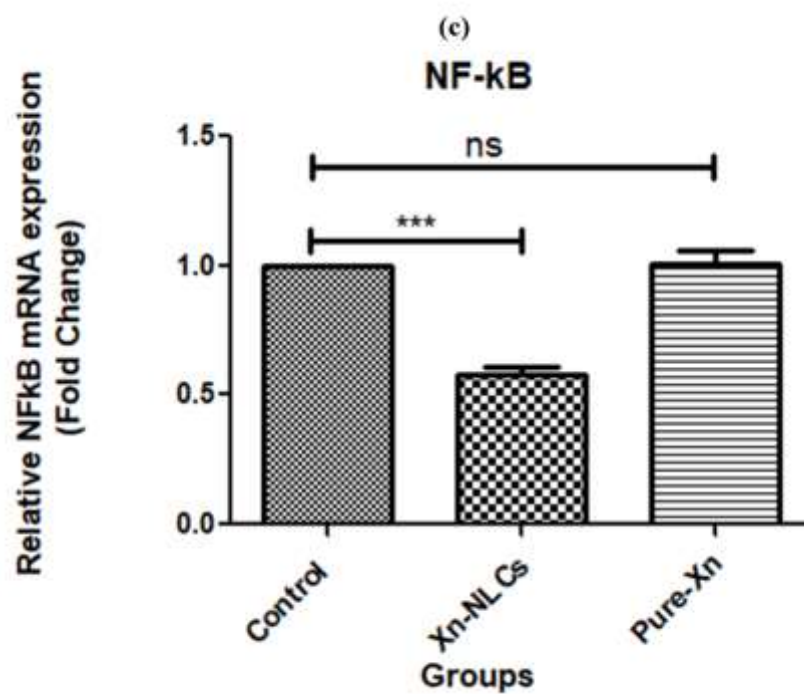
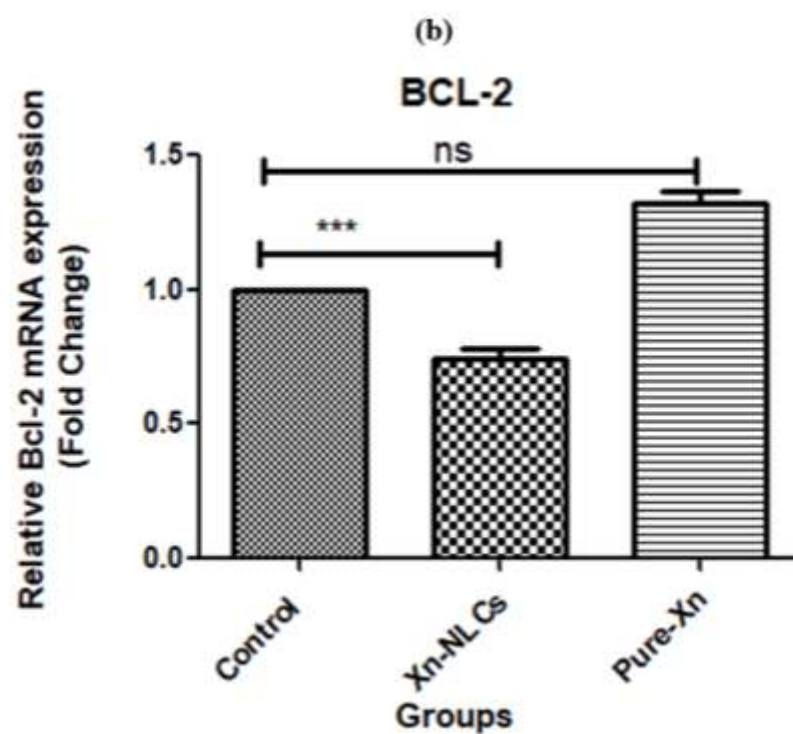
5.3.9.1.1. Relative m-RNA expression of p53, BCL-2, NF-kB, Caspases -3

Major apoptotic genes were identified in order to determine a potential mechanism of action for the growth inhibitory effects of pure-Xn and S-Xn-NLCs on the A549 cell line. After 24 hours of exposure to IC₅₀ concentrations, we found that S-Xn-NLCs had potentially stronger impacts than pure-Xn on A549 cancer cell lines. The pro-apoptosis p53 and caspase-3 were significantly upregulated, and the anti-apoptosis BCL-2 was downregulated. The tumor suppressor protein p53 induces apoptosis, or programmed cell death, by acting as a transcription factor to activate genes that promote cell death or by interacting with mitochondria and BCL-2 family proteins. In cancers,

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BCL-2 gene suppresses the programmed cell death and encourages cell survival, which supports drug-resistant in cancer cells. In order to induce apoptosis, it is essential to modify the expression of proteins (p53 and caspases upregulation, BCL-2 downregulation). Caspases-3 activation is an important step in the apoptosis process which lead to activation of cascade activity that can cause several proteins in cancer cells to cleave and become inactive. According to the q-RT-PCR results, S-Xn-NLCs showed increased in mRNA expressions of p53 ($\sim 1.29 \pm 0.1$ -fold change) and caspase-3 ($\sim 6.46 \pm 0.05$ -fold change) and decreased in mRNA expressions of BCL-2 ($\sim 0.22 \pm 0.1$ -fold) as shown in graph fig 48. NF- κ B (Nuclear Factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of genes involved in inflammation, immunity, and cell survival. The mRNA expression of the cell survival signaling protein NF- κ B in A549 cells was investigated, revealing substantial differences in NF- κ B expression between the untreated control A549 cells and those treated with S-Xn-NLCs. As indicated from results of q-RT-PCR, S-Xn-NLCs showed decreased mRNA expression of NF- κ B (by $\sim 0.53 \pm 0.1$ -fold), whereas pure-Xn doesn't showed any significant difference in mRNA expression with control untreated A549 cells as shown in graph fig. 5.38. Thus, the downregulation of NF- κ B by the action of S-XN-NLCs on A549 cells shows that the mechanism of inhibition phosphorylation of STAT protein in Janus kinases (JAKs) pathways which activate nuclear transcription factor NF- κ B which further activate c-myc in cancer cells.





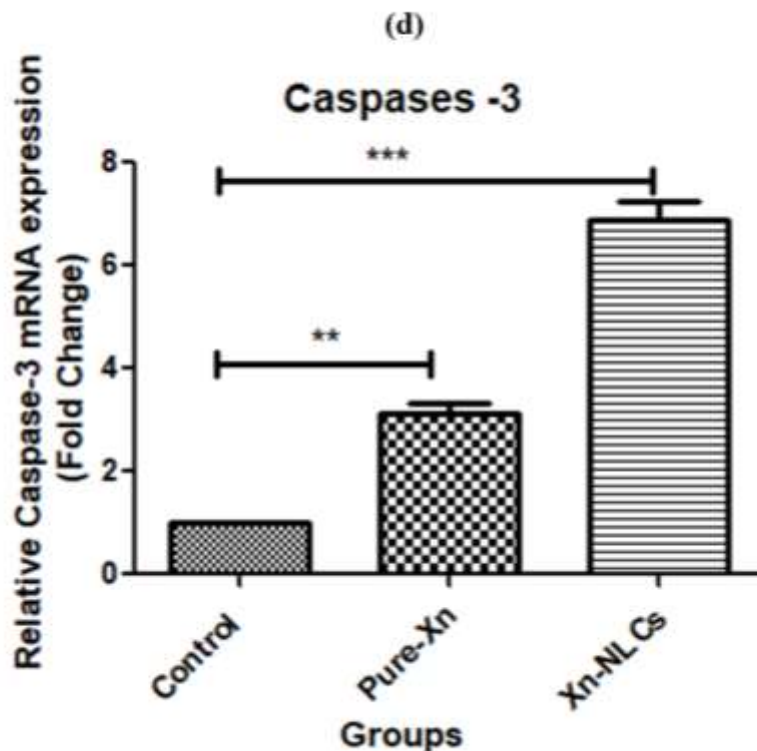
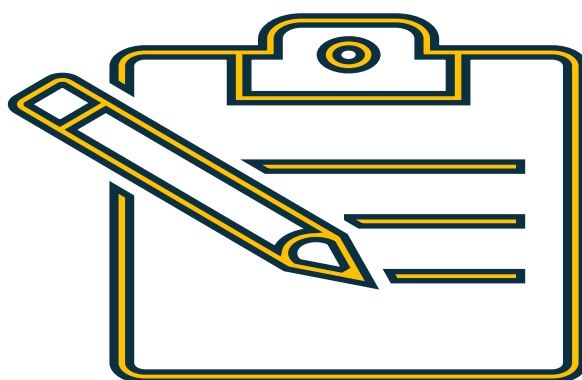


Fig. 5.38 : RT-PCR values for the apoptotic markers a) p53, b) caspase-3, c) NF-kB, and d) BCL-2 in A549 cells. Cells were treated with IC_{50} concentration (μM) of pure-Xn and Xn-NLCs for 24 hours. The data are shown as the mean $\pm$ SEM of 3 independent experiments, with a *** $p < 0.0001$ significance level. The analysis was carried out using one-way ANOVA, followed by the Dunnett multi-comparison test.

Note : here Xn-NLCs represent the S-Xn-NLCs.



Chapter 6



Conclusion and Future Perspective...

CHAPTER 6

Conclusion and Future Perspective

The current research was initiated based on the hypothesis **“Intranasal administration of xanthohumol loaded nanostructure lipid carriers as a novel treatment strategy for lung cancer ”**. Prior to formulation development of Xn. The Xn drugs was subjected to HPLC examination, and characterization of active Pharmaceuticals ingredients (API) such as physical characterization, melting point, determination of Log P value, DSC, PXRD, FTIR and solubility studied in different solid lipids, liquid lipids and surfactant. An isocratic analytical method was used for the method development using analytical column C18, 5 μ m, 250 $\times$ 4.6mm, which had a detection wavelength (λ) of 370 nm. The mobile phase consisted of a combination of HPLC grade Methanol and HPLC grade water buffer. The ratio of mobile phase consists of 95:5 v/v, with a flow rate of 1.0 mL/min. These were the chromatographic conditions and the mobile phase. In analytical procedures, it was established that the retention time for Xn is approximately 4.5 minutes within a total run duration of 10 minutes. This indicates the specific timeframe during which Xn remains in the system before elution, which is crucial for accurate identification and analysis. The suggested approach's accuracy was confirmed using recovery studies, and the result was 96.3%. Tests of repeatability on the standard and sample solutions show that the precision of the procedure is within reasonable limitations. The precision determination resulted in a relative standard deviation (RSD) of under 2%, accompanied by limits of detection (LOD) and quantification (LOQ) values of 0.85 μ g/mL and 2.6 μ g/mL, respectively. The fast, simple, accurate, precise, and linear stability indicating HPLC method developed. The uniqueness of the research work is lesser rt value with this new method development no data of Xn till now have been reported yet with lesser rt value. Under various chromatographic settings, the approach exhibits sufficient robustness to replicate precise and accurate results. The ICH guidelines were followed during the validation process.

Further, the study deciphered the successful formulation of Xn-loaded solid NLCs using a ternary phase diagram and BBD as optimization tools. The formulation of NLCs was accomplished using “hot high-pressure homogenization” followed by “ultrasonication”. Xn was incorporated into molten beeswax, followed by the addition of lauroglycol-90 and then lipid phase was dispersed in

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aqueous solution of tween-80 into the heated mixture stirred at 2000 rpm for 30 minutes to form a homogeneous emulsion and ultrasonication at 37°C for 10 minutes. Then, the optimized NLCs were converted into free-flowing powder (S-Xn-NLCs) by rotary evaporation, followed by drying them in a tray dryer along with mannitol. Further characterization of L-Xn-NLCs and S-Xn-NLCs was done. The optimized formulation of L-Xn-NLCs and S-Xn-NLCs showed PS, ZP, and EE%, L-Xn-NLCs show a PS of 101.60 ± 1.47 nm, PDI of 0.77 ± 0.029 , and ZP of -25.6 mV, indicating particle stability, EE% of $97.02 \pm 1.05\%$, thus indicating that NLCs exhibit superior drug encapsulation effectiveness and the reconstituted S-Xn-NLCs showed PS of 278 nm, PDI of 0.22, ZP of -44.3 mV, and EE% of 95.5 % indicating the formulation of stable NLCs. The results of DSC, PXRD, and SEM showed that L-Xn-NLCs had completely adsorbed on the mannitol surface. *In vitro* drug release demonstrated significantly higher drug release from both liquid and solid NLCs. The resulting study found that incorporation of Xn in NLCs significantly enhanced solubilization. It provided more space for the drugs to remain retained in the lipids. This led to a significant enhancement in drug release from NLCs. About a 2-fold enhancement in the release of Xn was observed in the case of L-Xn-NLCs and S-Xn-NLCs as compared to pure Xn suspension. The *in vitro* release model was best fitted to the “Korsmeyer-Peppas Kinetics” model according to the given kinetics, resulting in the highest correlation coefficient (r).

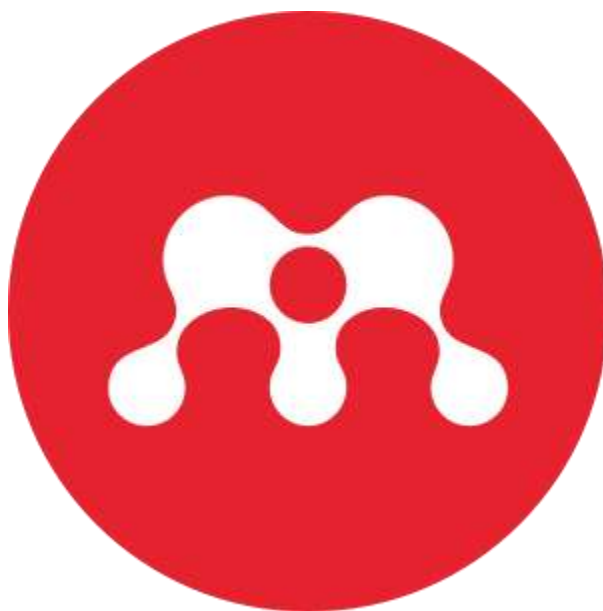
Further, the cytotoxicity studies performed on A549 cells indicated significantly higher toxicity of Xn-loaded S-Xn-NLCs as compared to their pure form. About 1.7~fold increase in reduction of the proliferation of A549 cell lines was observed in case of S-Xn-NLCs than pure Xn. The cytotoxic efficacy of developed S-Xn-NLCs was further confirmed by Rhodamine 123 staining and further by cell migration assays and DCFH-DA Staining. According to the percentage wound closure assay, S-Xn-NLCs showed a more than 80% reduction in wound closure at lower IC₅₀ concentrations as compared to the control group and pure Xn. This indicates that S-Xn-NLCs are the only ones that prevent A549 cells from migrating and invading more than pure Xn. DCF fluorescence intensity was used to determine ROS levels. It was found that the ROS level was higher in the treated groups of Pure Xn and S-Xn-NLCs than in the control group, which showed no green fluorescence. Using q-RT-PCR, the study explored the expression of key apoptotic genes to potentially elucidate the mechanism by which pure-Xn and S-Xn-NLCs exert their growth-inhibiting effects on the A549 cell line. The study found that pro-apoptotic genes p53 and caspase-3, were dramatically increased while anti-apoptosis genes BCL-2 were downregulated and NF-κB

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were also downregulated by the action of S-Xn-NLCs. Thus, the downregulation of NF- κ B by the action of S-XN-NLCs on A549 cells shows that the mechanism of inhibition phosphorylation of STAT protein in Janus kinases (JAKs) pathways which activate nuclear transcription factor NF- κ B which further activate c-myc in cancer cells. Flow cytometry analysis were used to determine the distribution of cells across the different phases of the cell cycle, specifically identifying the percentages of cells in the G0-G1, S, and G2/M stages. Cell cycle analysis showed that S-Xn-NLCs caused A549 cells to be arrested in the G2/M phase of the cell cycle. The obtained results indicated a promising effect of developed NLCs *in vitro* against LC cells, which can be further correlated with future pre-clinical studies that could be done on suitable animal models.



Chapter 7



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Chapter 8



Annexures ...



**Centre for
Research Degree Programmes**

LPU/CRDP/PHD/EC/20231207/002577
Dated: 22 Sep 2023

Shubham Singh

VID: 12224392

Programme Name: Doctor of Philosophy (Molecular Biology and Genetic Engineering)

Subject: Letter of Candidacy for Ph.D.

Dear Candidate,

We are very pleased to inform you that the Department Doctoral Board has approved your candidacy for the Ph.D. Programme on 22 Sep 2023 by accepting your research proposal entitled: "Formulation and Optimization of Xanthohumol loaded Nanostructured lipid carriers for the treatment of Lung cancer"

As a Ph.D. candidate you are required to abide by the conditions, rules and regulations laid down for Ph.D. Programme of the University, and amendments, if any, made from time to time.

We wish you the very best!!

In case you have any query related to your programme, please contact Centre for Research Degree Programmes.

Head

Centre for Research Degree Programmes

Note:-This is a computer generated certificate and no signature is required. Please use the reference number generated on this certificate for future conversations.

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Review paper published :



Pathology - Research and Practice

Volume 256, April 2024, 155222



Review

Emerging role of tumor suppressing microRNAs as therapeutics in managing non-small cell lung cancer

Shubham Singh^a, Sangeeta Saxena^b, Himani Sharma^c, Keshav Raj Paudel^d,
Amlan Chakraborty^{e,f}, Ronan MacLoughlin^{g,h,i}, Brian G. Oliver^{j,k}, Gaurav Gupta^{l,m},
Poonam Negiⁿ, Sachin Kumar Singh^{c,o}  , Kamal Dua^{o,p}  

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Opening avenue for the targeted treatment of lung cancer using xanthohumol loaded nanostructured lipid carriers

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Section

Research paper published :



Journal of Chromatography B

Volume 1252, 1 February 2025, 124437



Method development and validation on RP-HPLC method for estimation of xanthohumol in nanostructured lipid carriers drug delivery systems

Shubham Singh ^a, Himani Sharma ^b, Vijay Kumar ^a, Gaurav Gupta ^{c, d}, Samir Patel ^e,
Archita Patel ^e, Kamal Dua ^{f, g}, Sachin Kumar Singh ^{b, g}

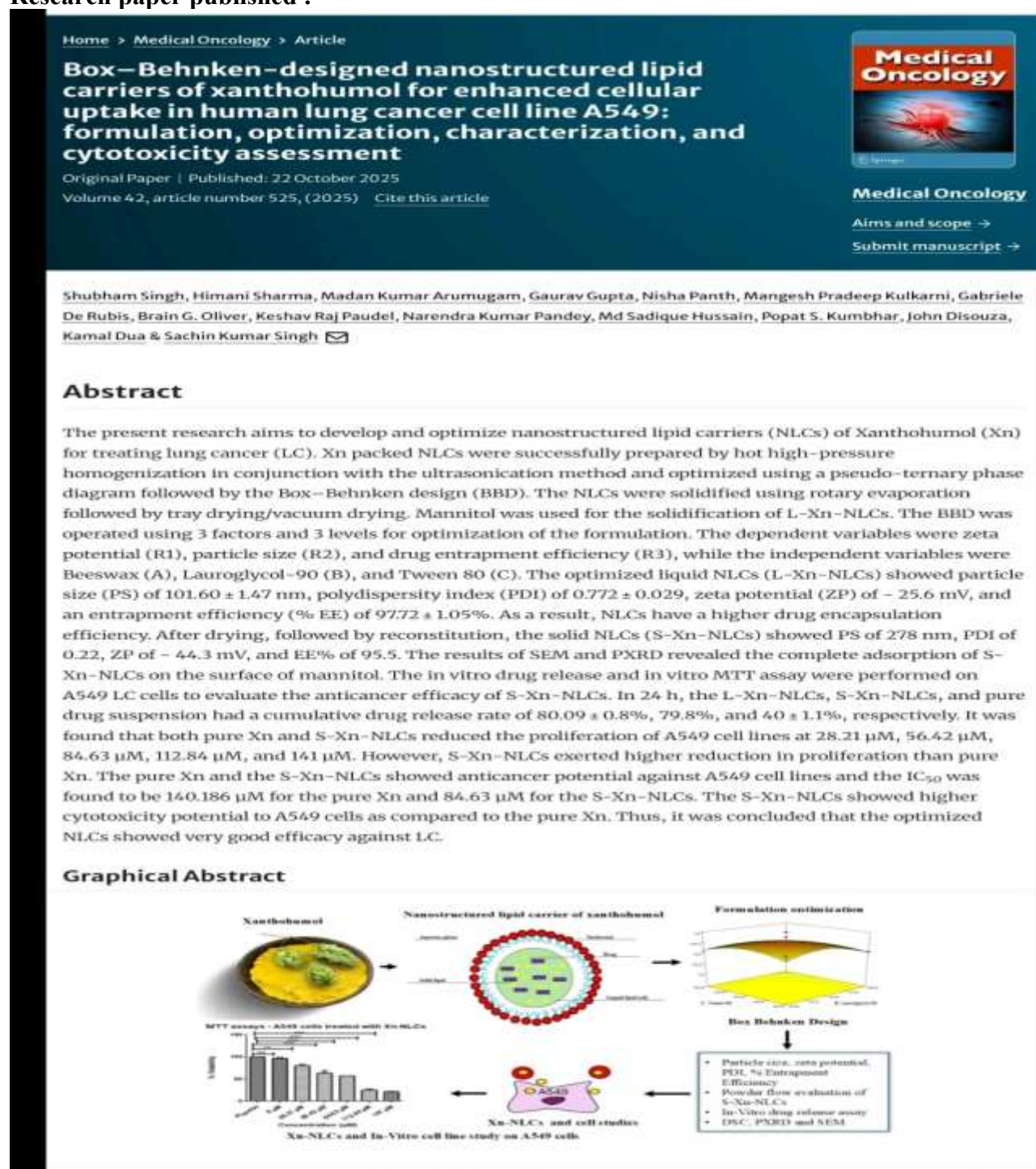
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Certificate 1: 4th International conference of Pharmacy (ICP-2024)

Title : Intranasal Administration of Xanthohumol Loaded Nanostructure Lipid Carriers as a Novel Treatment Strategy for Lung Cancer: Hypothesis









Certificate 2: ICRAHS (International Conference on Recent Advances in Health Sciences)

Title : Tumor suppressing microRNAs inhibit the proliferation and migration of A549 non-small lung cancer cells by targeting various signalling pathways: as therapeutics in managing Non- small lung cancer





Certificate 3: SHE Science Conference (National Conference on Sustainable Health & Empowerment in Science)

Title : Xanthohumol loaded nanostructured lipid carriers for targeted lung cancer treatment : A Box Behnken approach



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Certificate of Presentation

This is to certify that Dr./Mr./Ms. SHUBHAM SINGH presented a poster/oral presentation ✓
 on Xanthohumol loaded nanostructured lipid carriers for targeted lung in the National Conference
Cancer treatment : A Box- Behnken design approach
SHE-SCIENCE 2025 (Sustainable Health & Empowerment in Science) held on August 26th, 2025 organized by School of
 Pharmaceutical Sciences, in collaboration with Society of Pharmaceutical Education & Research (SPER), Noida at Lovely
 Professional University, Punjab.

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