
**CHARACTERIZATION OF DESIGNED
NANOPARTICULATE DRUG DELIVERY SYSTEM OF
DASATINIB FOR CANCER THERAPY**

**Thesis submitted to
THE KLE ACADEMY OF HIGHER EDUCATION AND
RESEARCH, (BELAGAVI)
(KLE DEEMED UNIVERSITY)**

[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide
Govt. of India Notification No.F.9-19/2000-U.3 (A)]

(Accredited 'A⁺' Grade by NAAC) (3rd Cycle)

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For the award of the degree of

Doctor of Philosophy

In the Faculty of Pharmacy

By

Hemanth G M.Pharm.

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List of abbreviations

SI – International system of Units

API – Active Pharmaceutical Ingredient

nm- nanometer

PVA- Poly Vinyl Alcohol

DTB – Dasatinib

DTBNps – Dasatinib loaded Eudragit L 100 Nanoparticles

US – United States

TKI – Tyrosine Kinase Inhibitor

TPP -Tripoly Phosphate

(+) - Positive

(-) – Negative

IHC – Immunohistochemistry

CML- Chronic Myeloid Leukemia

TNBC – Triple Negative Breast Cancer

mV – milli volts

% - Percentage

EE – Entrapment Efficiency

MQC – Medium Quality Control

HQC – High Quality Control

LLQC – Lower Limit Quality Control

LQC – Lower Quality Control

LC-MS – Liquid Chromatography Mass Spectroscopy

UV – Ultra Violet

FT-IR – Fourier Transformation Infrared Spectroscopy

SEM – Scanning Electron Microscopy

AFM – Atomic Force Microscopy

C_{max} – Maximum Plasma Concentration

AUC – Area under curve

DMSO – Dimethyl Sufoxide

DMEM – Dulbecco's Modified Eagle Medium

DMBA - 7,12-dimethylbenz[a]-anthracene

P-XRD – Powder X-Ray Diffraction

DSC – Differential Scanning Calorimeter

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ABSTRACT

Background: Breast cancer a prevalent malignancy among female worldwide also a leading cause of death globally. Breast cancer is developed due to abnormal and unchecked growth of breast cancer epithelial cells driven by combination of multiple factors. Chemotherapy a primary treatment is often limited due to its drawbacks includes systemic toxicity, gastrointestinal disturbances, neuropathy and others. Further drug resistance remains as a major challenge contributing to failure of treatment and relapse of disease. In recent years nanotechnology is gaining its importance to enhance chemotherapy effectiveness by enhancing drug solubility, tumor targeting capabilities and thereby reducing side effects.

Objective: The goal of the project was to develop nanoparticulate drug delivery system of Dasatinib (DTB) loaded Eudragit (EL 100) nanoparticles utilizing full factorial design to address the drawbacks of conventional system.

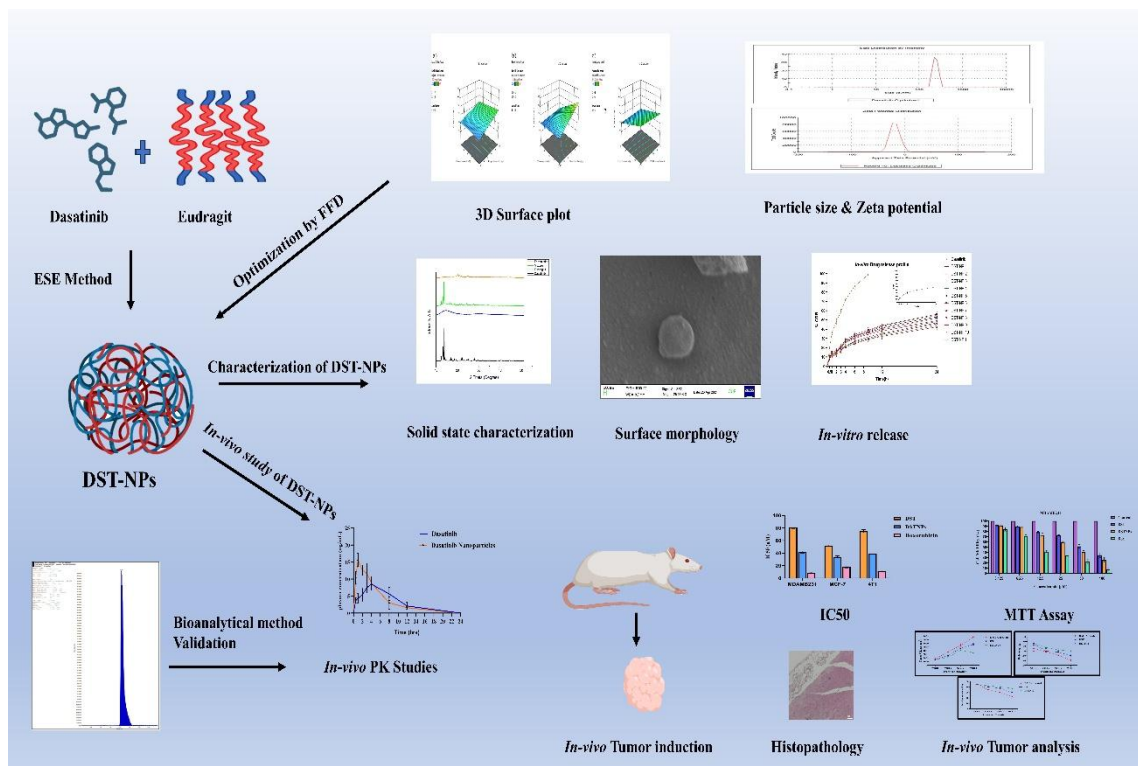
Results and discussion: Eudragit L 100 loaded DTB nanoparticles (DTBNps) were formulated using emulsion solvent evaporation technique by varying formulation parameters. The optimized formulation DTBNp5 exhibited a particle size of 202.1 ± 5.7 nm, a zeta potential of -18 ± 1.01 mV, and an entrapment efficiency of $93.11 \pm 0.2\%$. The FT-IR, DSC and XRD confirmed the amorphization of DTB from crystalline to amorphous in polymer matrix. The *in-vitro* release shown a sustained release behaviour over 24 hours. A novel method for detection of concentration of DTB in rat plasma was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The accuracy and precision for MQC, HQC, LLQC, and LQC were 106.09 %, 107.33%, 99.83%, and 94.15%, respectively. Pharmacokinetic studies *in-vivo* revealed higher C_{max}

(*** $p < 0.001$) and AUC_{0-t} (***) $p < 0.001$) for DTBNps compared to pure DTB, indicating improved bioavailability. Tissue distribution studies showed enhanced targeting of DTBNps, with higher concentrations in the liver ($*p < 0.05$) and spleen ($*p < 0.05$). *In vivo* efficacy in a DMBA-induced mammary carcinoma model demonstrated that DTBNps significantly reduced tumor volume, maintained stable body weight, and improved survival rates compared to pure DTB. Hematologic analysis indicated a favorable blood profile with DTBNps, and histopathological examinations confirmed the restoration of normal mammary gland and liver architecture. MTT assays showed higher cytotoxicity of DTBNps against MCF-7, MDA-MB231, and 4T1 cell lines, with lower IC₅₀ values than pure DST. Stability studies indicated that DTBNps maintained their properties over three months at various storage conditions.

Conclusion: The smaller size of nanoparticulate drug delivery system was found to be effective for improving pharmacokinetic parameters and safety due to efficient biodistribution. In this regard the developed nanoformulation can be considered as a promising strategy for treatment of cancer.

Keywords: Dasatinib, Nanoparticle Drug Delivery System, Breast Cancer, Factorial design, Eudragit L 100

GRAPHICAL ABSTRACT



1. INTRODUCTION

1.1 BACKGROUND

Breast Cancer

Breast cancer is defined as a condition in which aberrant breast cells develop out of control and shape into tumors. If not treated, the tumors can spread all through the body and become lethal. Breast cancer cells develop in milk ducts and/or in milk-producing lobules in breast. The early stage is not life threatening, when spread into adjacent breast tissue it forms tumors that cause knots or thickening.

Metastasis can be life-threatening and lethal. Treatment varies depending on the individual typically includes a combination of surgery, radiation, and chemotherapy. In 2022, there were 2.3 million people detected with breast cancer and 670 000 death. Breast cancer exists worldwide in females at any age after adolescence but with increasing rates in afterward life.

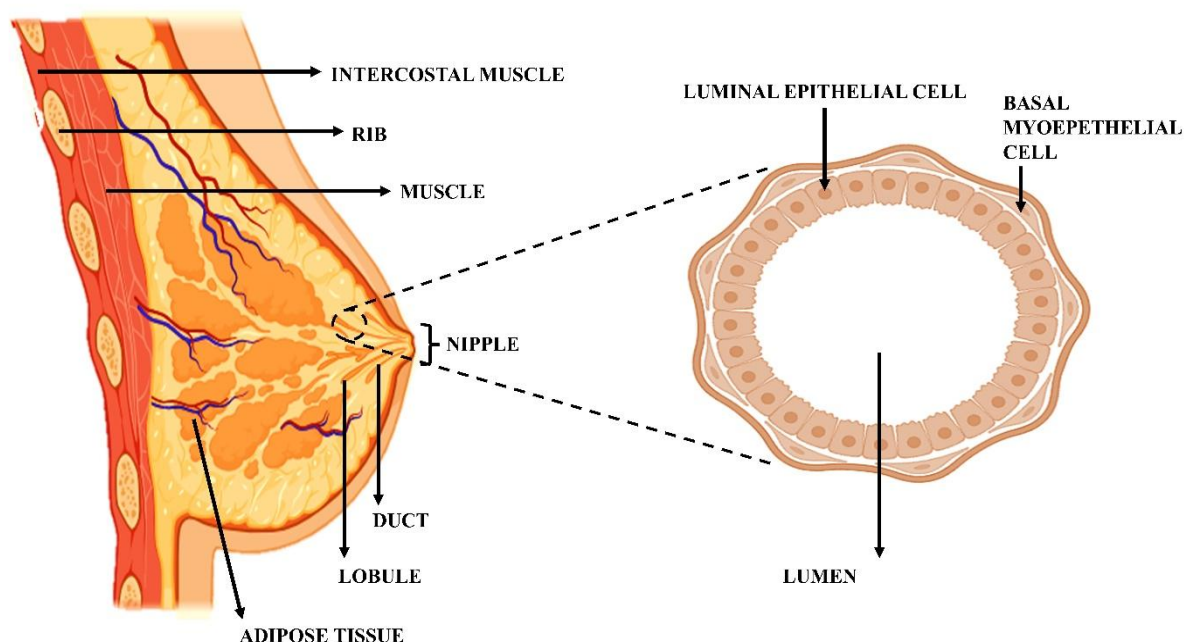


Figure 1: Anatomy of female breast.

Females are mostly affected by breast cancer. Roughly 99% of breast cancers happen in females and 0.5–1% of breast cancers happen in male. Risk factors of breast cancer include aging, weight, excessive alcohol consumption, family history of cancer, regenerative history, tobacco utilize and postmenopausal hormone treatment. Around half of breast cancer cases diagnosed in females had no identifiable breast cancer chances.

Certain acquired highly penetrable quality transformations incredibly contribute to breast cancer chances, the foremost dominant being changes within the qualities BRCA1, BRCA2 and PALB-2. Females found to have transformations in these major qualities may consider chance decrease strategies such as surgical expulsion of both breasts or chemoprevention techniques.¹

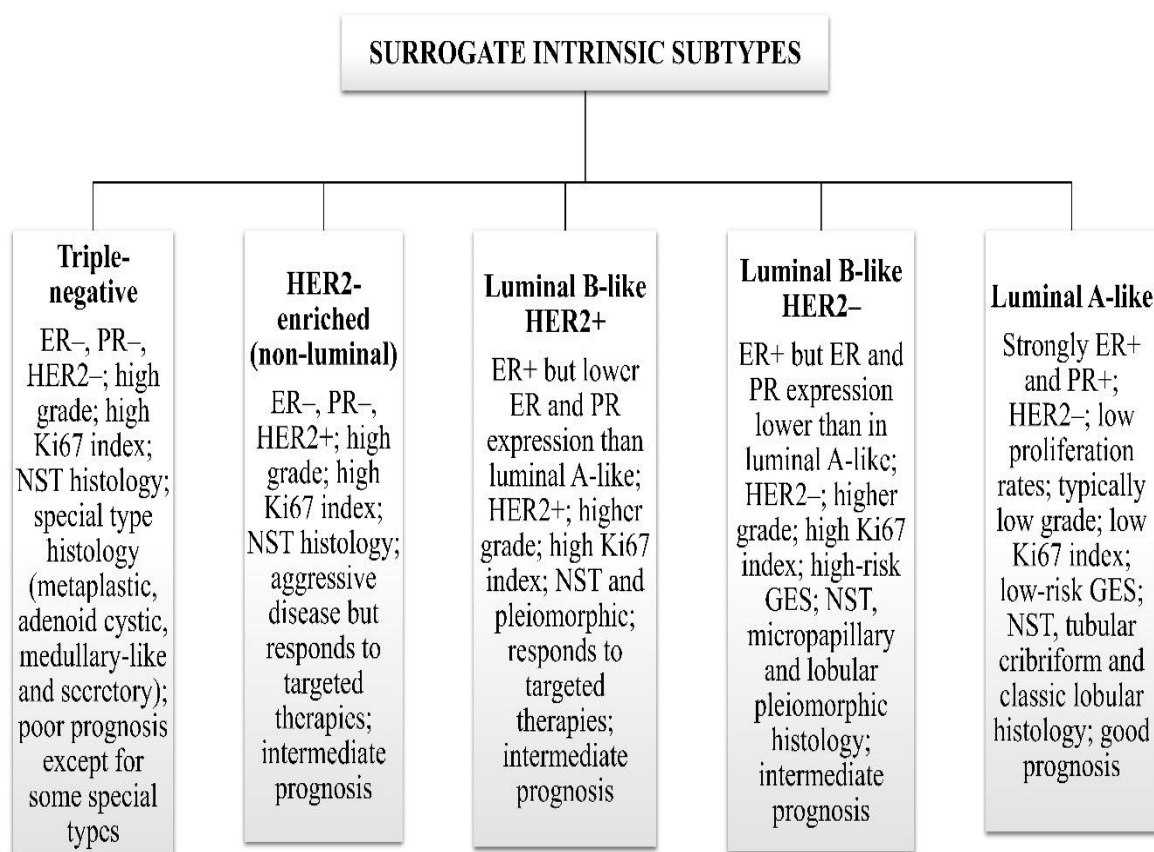


Figure 2: Surrogate intrinsic subtypes of breast cancer based on IHC markers.

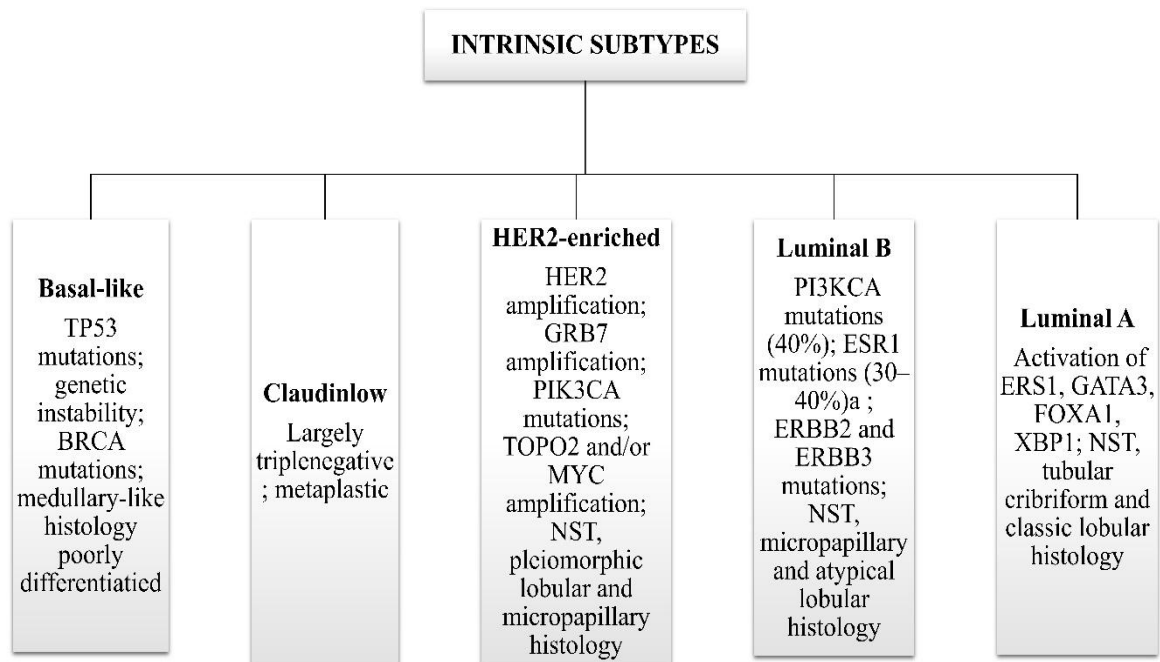


Figure 3: Intrinsic subtypes of breast cancer based on gene expression.

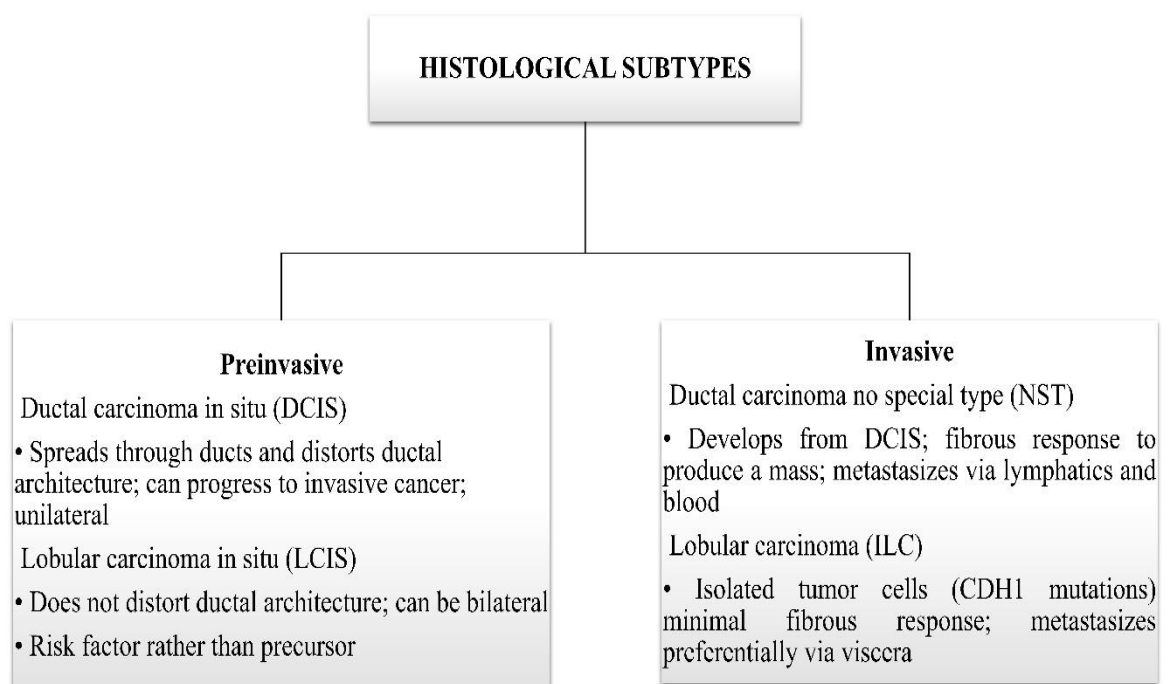


Figure 4: Histological subtypes of breast cancer based on microscopical appearance.

The larger part of breast cancers are adenocarcinomas, with 85% of adenocarcinoma cases emerging from the breast ducts and 15% from the lobular epithelium. The ductal pathology span from ductal carcinoma in situ, to invasive carcinomas which have spread past the basement membrane into adjoining breast parenchyma.²

Metastasis is vital in breast cancer treatment since it can be play dual role as a therapeutic target and a detectable marker. Targeted therapeutics for breast cancer incorporate substances or drugs that stop cancer development by interfering with the purpose of certain molecules for tumor cell growth and survival. Focused on treatment for metastatic breast cancer is choosed by the existence or non-existence of hormone receptors, HER2, cancer reappearance, metastasis rate, and metastasis location. In specific, personalized treatment for patients can upgrade the restorative viability and minimize chemotherapy-induced harmfulness in breast cancer. In any case, the recurrence of metastasis of breast cancer still exists.³

Nanoparticles

The nanoparticle system includes an extraordinary potential to improve the performance of drugs through enhancing pharmacodynamics, enhanced stability and solubility, thereby limiting its toxicity.⁴

The term "nano" originate from the ancient Greek word "nanos," meaning tiny, and was later adopted into Latin as "nanus," signifying something very small. Nano-technology refers to design, characterization, production, & application of materials, devices, & systems by controlling their shape & size at nano-meter scale. According to the SI, nanotechnology operates at scale of one billionth of a meter, with 1 nanometer (nm) equalling 10^{-9} meters. At this microscopic scale, the behaviour of molecules and atoms

changes significantly, functioning as cohesive units with distinct properties and improved transport characteristics, which offer a wide range of benefits.

Nanoparticles [NPs] are defined as particulate dispersions or drug carrier that may be or may not be biodegradable. The drug is dissolved entrapped, encapsulated to a nanoparticle matrix. The term nanoparticle is a combination of nanospheres and nanocapsules. Drug is surrounded by a polymer membrane called nanocapsule, nanospheres are matrix systems in which the drug is physically and uniformly dispersed.⁵⁻⁶

Need for development of nanoparticles

The primary goal in designing the nanoparticles for delivery system is to control particle size, surface properties and release of API to achieve site specific action of drug at the rationale rate. This approach also helps enhance the stability of the drugs, ensuring they remain effective and intact during transport to their intended location.⁷⁻⁸

Advantages of nanoparticles

By using nanoparticles as drug delivery systems offers several key advantages.

a) Surface Properties and particle size: Nanoparticles can be easily modified in terms of their surface characteristics and size, enabling passive & active targeting of the drug after administration, which enhances delivery precision.

b) Sustained and Controlled Drug Release: Nanoparticles can control the release of drug both during transport and at site of action. By modifying the distribution of the drug across organs & regulating its clearance, they increase therapeutic efficacy while minimizing side effects.

- c) **Targeted Drug Delivery:** Attaching specific ligands to the surface of nanoparticles allows for site-specific targeting, ensuring the drug reaches its intended location more effectively.
- d) **Modifiable Release and Degradation:** The release & degradation characteristics of nanoparticles can be adjusted easily by selecting divergent matrix components. Additionally, nanoparticles can have high drug loading capacity, enabling incorporation of drugs without requiring chemical reactions, which helps preserve the drug's activity.
- e) **Administration Routes:** Nanoparticle-based systems can be adapted for various routes of administration, including nasal, oral, intraocular, and parenteral, offering flexibility in drug delivery methods.⁹

Disadvantages of nanoparticles

- a) The smaller size & larger surface area leads to particle aggregations, which make the physical handling of nano-particles complex in liquid & dry forms.
- b) The particles which are smaller in size & large surface area readily result in the limited loading of drug & burst release. The problem needs to be solved before nanoparticles can be clinically used or been made available commercially.¹⁰⁻¹¹

Preparation of nanoparticles.

The choice of an appropriate method for preparing nano-particles depends on the physicochemical properties of both the polymer and the drug to be incorporated. Nanoparticles can be made from a wide variety of materials, including proteins, polysaccharides and synthetic polymers. The selection of matrix materials is influenced by several factors, including:

- ❖ **Antigenicity of final product:** Whether the product triggers an immune response.
- ❖ **Biocompatibility and toxicity:** The material must be safe and compatible with the body without causing adverse effects.
- ❖ **Degree of biodegradability:** The material should degrade in a way that is safe and efficient over time.
- ❖ **Desired drug release profile:** How quickly and in what manner the drug is released from the nanoparticles.
- ❖ **Intrinsic properties of drug:** Factors like the drug's aqueous solubility & stability need to be considered.
- ❖ **Required nanoparticle size:** The size of the particles should be tailored for the intended use.
- ❖ **Surface characteristics:** These include the charge and permeability of the particles, which affect their interaction with the body and target cells.

Nano-particles are typically formulated using one of the following methods:

- ❖ **Dispersion of preformed polymers:** This involves, dispersing pre-existing polymer to form nano-particles.
- ❖ **Polymerization of monomers:** This method creates nanoparticles by polymerizing individual monomer units.
- ❖ **Ionic gelation or coacervation of hydrophilic polymers:** This process involves using ionic interactions or phase separation to form nanoparticles from hydrophilic polymers.¹²

Preparation Techniques

- **Solvent Evaporation:** Solvent evaporation was the initial method developed for formulation of nano-particles. In this method primarily nano-emulsion is prepared. Polymer is dissolved in organic solvent {dichloromethane, chloroform, or ethyl acetate}. Drug is dispersed solution then the mixture emulsified in an aqueous phase containing surfactant {poly-sorbates, poloxamers, sodium dodecyl sulfates, polyvinyl alcohols, gelatin,} make an o/w emulsion by utilizing mechanical stirring, sonication, micro-fluidization [high pressure, homogenization, through narrow channels]. After the formation of the emulsion the organic solvent evaporated by increase in the temperature & reduced pressure with continuous stirring.¹³⁻¹⁵

- **Spontaneous Emulsification or Solvent Diffusion Method**

This method represents an improvised version of the solvent evaporation technique, wherein water miscible solvent along with a small amount of water miscible organic solvent is utilized as an oil phase. The spontaneous diffusion of solvents an interphasial turbulence is created between both phase lead to formation of small particles. An increase in the concentration of the water-miscible solvent results in a decrease in particle size, allowing for better control over nanoparticle characteristics. Both solvent evaporation & diffusion methods can be adopted for hydrophilic & hydrophobic drugs. For hydrophilic drugs, a multiple (w/o/w) emulsion must be created, where the drug is dissolved in internal aqueous phase. This ensures efficient drug encapsulation and controlled release within the nanoparticle formulation.¹⁶

- **Salting Out**

This is one of the most commonly adopted methods for nanoparticles preparation involving the formation of an (o/w) emulsion. The process begins with the incorporation of a saturated aqueous solution of PVA into an acetone solution containing the polymer under magnetic stirring. Unlike nano-precipitation technique where polymeric solution (acetone) is completely miscible with external aqueous medium, this technique the miscibility of both phases is restricted by the saturation of the external phase with PVA. Nanoparticle formation occurs when water is gradually added to the external phase, allowing the diffusion of acetone from the internal phase into the aqueous phase. This controlled diffusion induces polymer precipitation, leading to formation of nanoparticles. The salting-out technique is particularly suitable for drugs and polymers that are soluble in polar solvents such as acetone/ethanol, making it a versatile method for encapsulating various pharmaceutical compounds.¹⁷

- **Emulsion-Diffusion Method**

This is another widely utilized method for nanoparticle preparation, offering several advantages such as high encapsulation efficiency, reproducibility, ease of scale-up, and a narrow size distribution. The encapsulating polymer is dissolved in partially water miscible solvent (such as propylene carbonate, benzyl alcohol) and saturated with water to ensure the initial thermo-dynamic equilibrium of both of liquids. Subsequently, the polymer water saturated solvent phase is emulsified in an aqueous solution containing stabilizer, leading to solvent diffusion to the external phase & the formation of nano- spheres nano- capsules depending upon the oil to polymer ratio. Finally, the solvent is eliminated by evaporation/filtration according to the boiling point. This method is highly efficient because it does not

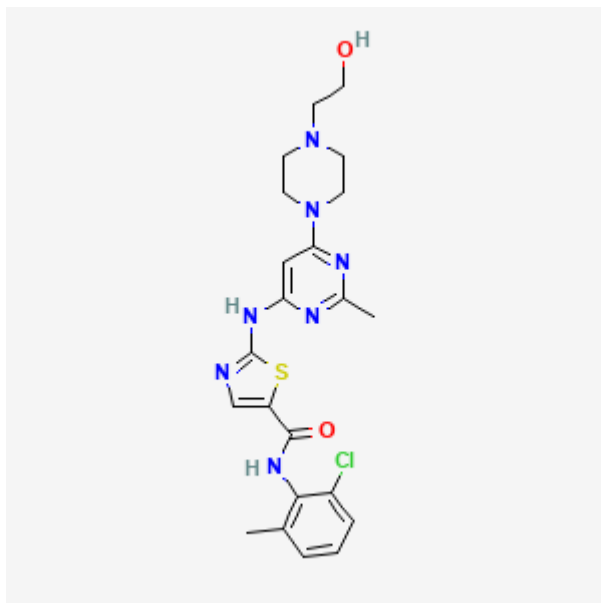
require homogenization and provides consistent batch-to-batch reproducibility, making it a reliable and scalable approach for nanoparticle synthesis.¹⁸

- **Polymerization method**

In this technique, monomers are polymerized in an aqueous solution to form nanoparticles. The drug is incorporated either by dissolving it in the polymerization medium or by adsorbing it onto the nanoparticles after polymerization is complete. Once the nanoparticle suspension is formed, it is purified to remove stabilizers & surfactants used during the polymerization process. This purification is typically done through ultra-centrifugation, followed by resuspending the nanoparticles in an isotonic medium that is free of surfactants. This method has been used to create nano-particles from materials such as PBCA/PACA.¹⁹⁻²¹

- **Coacervation or Ionic gelation Method**

Nanoparticle preparation can be achieved using biodegradable hydrophilic polymers such as chitosan, gelatin, and sodium alginate. One method for preparing hydrophilic chitosan nanoparticles is through ionic gelation. In this technique, the positively charged amino groups of chitosan interact with the negatively charged TPP, leading to the formation of coacervates. These coacervates then aggregate into nano-particles, typically within the nano-meter size range. This process takes advantage of the ionic interaction between the two components to form stable nanoparticles.²²

DASATINIB**Figure 5: Structure of Dasatinib (DTB)**

IUPAC name: *N*-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)piperazin-1-yl]-2-methylpyrimidin-4-yl]amino]-1,3-thiazole-5-carboxamide

Molecular formula: $C_{22}H_{26}ClN_7O_2S$

Molecular weight: 488.0 g/mol

Log P: 3.6

Solubility: Soluble in DMSO.

Half Life: 4-5 hours.

Bioavailability: 14-34%

Dose: 70mg twice daily or 100mg once daily

Tyrosine kinase inhibitors (TKIs) are a category of small molecule drugs that inhibit intracellular signals which power proliferation in malignant cells by particularly inhibit

the kinase function of individual intracellular pathways associated in receptor-mediated growth signalling.

DTB is short-acting dual ABL/SRC tyrosine kinase inhibitor (TKI) administered orally. It strongly inhibits BCR-ABL and SRC family kinases (SRC, LCK, YES, FYN), but also c-KIT, PDGFR-a and PDGFR-b, and ephrin receptor kinase. DTB is a viable treatment for chronic myeloid leukemia (CML) and Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ ALL). These diseases are characterized by a constitutively active tyrosine kinase; BCRABL. DTB inhibit the BCR-ABL with greater efficiency compared to other BCR-ABL inhibitors and is active in CML resistant or intolerant to imatinib. DTB is endorsed for the treatment of all phase CML and for the treatment of Ph+ ALL, resistant or intolerant to prior imatinib treatment.²³⁻²⁴

1.2 REVIEW OF LITERATURE

Ahmed A. H. *et al.*,2022- Sorafenib (SFB) is an anticancer medication that has limited solubility in water and decreased bioavailability. The nanoformulation of SFB could improved its dissolution rate and solubility. The SFB nanoparticles were created using the solvent evaporation method at a concentration of 50 μ M. To do this, 1. 3 mg of SEB was mixed with 10 mL of methanol at room temperature to form solution I. The aqueous phase, or solution II, contained 40 mL of the water-soluble polymer PEG-4000 in a 1:1 drug-to-polymer ratio, along with 10 mL of glycerol as a stabilizer. Solution I was then combined with solution II using a syringe and stirred at 600 rpm to help evaporate the methanol.²⁵

Bahman F *et al.*,2021- Breast cancers rank as the most common type of tumor among women in the U. S. , and in 2021, it is estimated that 280,000 new breast cancer cases will be identified . The condition is impacting approximately 1 in 8 women in the U. S. throughout their lives. The deaths caused by breast cancer may be linked to metastasis by 80–90%. Triple negative breast cancer (TNBC) is a particularly aggressive form of breast cancer, accounting for approximately 15% of cases. It does not exhibit HER2 amplification, is not reliant on hormones, and lacks both estrogen and progesterone receptors, resulting in no effectiveness against conventional treatments. Presently, the mainstay of treatment primarily involves chemotherapy, yet resistance and recurrences frequently occur. DTB, a second-generation tyrosine kinase inhibitor, demonstrates potential for treating TNBC. To improve its efficacy, DTB was encapsulated within poly(styrene-co-maleic acid) SMA micelles, resulting in the creation of SMA–DTB nanoparticles. These nanoparticles displayed enhanced anti-tumor activity against TNBC cell lines and yielded better outcomes in an animal model compared to the drug in its

unencapsulated form. This method underscores the capability of nanoformulations to shield drugs from degradation and proposes a novel approach for treating TNBC.²⁶

Lin Qiu, *et al.*,2019- Ursolic acid nanoparticles was prepared by the emulsion solvent evaporation method, UA is a type of pentacyclic triterpenoid. It is commonly found in nature, for instance in the leaves and fruit of bearberry from the Ericaceae family, the leaves of Paulownia tomentosa Steud from the Scrophulariaceae family, and the leaves of Ligustrum lucidum Ait from the Oleaceae family. UANs have a higher oral bioavailability, being 2.68 times greater than raw UA. They also showed better antioxidant activity against cells, with their EC₅₀ decreasing by 37.5 times compared to raw UA. The levels of trichloromethane and ethanol were kept within safe limits. Overall, UANs may offer advantages for oral bioavailability.⁴

Karimi N *et al.*,2020- Nanoparticle combination chemotherapy using polymer-drug grafts aims to improved traditional chemotherapy. In this study, all-trans retinoic acid (ATRA) was attached to poly β -amino ester to create nanoparticles that carry docetaxel (DTX). The size of these nanoparticles was optimized using response surface methodology. Their size and stability were tested with a Zetasizer, while their shape was examined using transmission electron microscopy. Release studies showed that ATRA and DTX were released faster at lower pH levels. The nanoparticles demonstrated better anti-cancer effects compared to free drugs. Overall, ATRA-g-PBAE nanoparticles are a good option for controlled drug release in chemotherapy.²⁷

Enrique Niza *et al.*,(2019): DAS has shown activity in human epidermal growth factor receptor 2 (HER2) positive tumors, so conjugation of this compound with the anti-HER2 antibody trastuzumab (TAB) with the use of nanocarriers could improve its efficacy. TAB-targeted DAS-loaded nanoparticles were generated by nanotechnology. The

guided nanocarriers enhanced in vitro cytotoxicity of DAS against HER2 human breast cancer cell lines. Cellular mechanistic, release studies and nanoparticles stability were undertaken to provide evidences for positioning DAS-loaded TAB-targeted nanoparticles as a potential strategy for further development in HER2-overexpressing breast cancer therapy.²⁸

Enrique Niza *et al.*, (2019): Cellular mechanistic release studies and nanoparticles stability were undertaken to provide evidences for positioning Dasatinib loaded Trastuzumab targeted nanoparticles as a potential strategy for further development in HER2 overexpressing breast cancer therapy.²⁹

Mehandole A *et al.*, (2024): The study aimed to enhance the oral delivery and efficacy of DTB in treating triple-negative breast cancer (TNBC) by developing mucoadhesive lecithin-chitosan hybrid nanoparticles (DTB-L/CS-NPs). The key outcomes of the research are as follows: Nanoparticle Optimization. In conclusion, the study demonstrates that the developed hybrid nanoparticles significantly improve the oral bioavailability and therapeutic efficacy of DTB for treating triple-negative breast cancer, positioning them as promising carriers for poorly water-soluble drugs.³⁰

Sankha Bhattacharya., (2020): The main objective of this research was to develop chitosan based polymeric nanoparticles of Imatinib (IMT-PNPs) for colorectal cancer targeting. The ionic gelation technique and central composite design was implemented to prepare IMT-PNPs. The key outcomes of the research are as follows: Nanoparticle Optimization. In conclusion, the study demonstrates that the developed nanoparticles significantly improve the bioavailability and therapeutic efficacy of Imatinib for treating colon cancer, positioning them as promising carriers for poorly water-soluble drugs.³¹

Mohammed Elmowafy *et al.*,(2021): The aim of study was to develop luteolin loaded hybrid polylactic acid/Eudragit L100 nanoparticles. By nanoprecipitation, different formulations were prepared, optimized and characterized in terms of physicochemical properties, stability and *in vivo* performance. The findings of this study revealed that hybrid polylactic acid/Eudragit L100 nanoparticles are responsible for improved pharmacological efficacy of orally administered luteolin, thereby indicating the benefits of using such system to overcome the problems facing luteolin oral delivery.³²

Qing *et al.*,(2017): aimed in improving tumor targetability and drug efficacy and decrease drug resistance of DTB. Two major functional polymers polyethylene glycol – MMP2 sensitive peptide – phosphoethanolamine (PEG5K –PP-PE) and folic acid – polyethylene glycol – phosphoethanolamine (FA-PEG2K-PE). In-vivo bio-distribution studies, the MMP2 and FR dual targeting strategy could significantly prolong the systemic circulation, decrease the non specific distribution in non-tumor and increase the tumor accumulation of the polymeric accumulation of the polymeric micelles in melanoma xenograft mouse 5 model. The MMP2 sensitive FR targeted micelles might have great potential as a tumor targeted platform for delivery of molecular targeted therapies.³³

1.3 NEED FOR STUDY

Breast cancer is type of cancer that begins in the cells of the breast, often in the milk ducts or lobules. It occurs when ab-normal cells in the breast grow uncontrollably, forming a tumor. The tumor can be either benign (non-cancerous) or malignant (cancerous), with the latter having the potential to spread to other parts of body through lymphatic system or bloodstream. Treatments include surgery, radiation, chemotherapy, hormone therapy & targeted therapies, depending on the type and stage of the cancer.³⁴

DTB is a potent oral TKI against Sarcoma family kinases, BCR- ABL, platelet derived growth factor receptor & C-kit has demonstrated multiple effects on solid tumors & has been approved for use in patients with CML refractory or intolerant to imatinib & in patients with PhACC ++ leukemia.³⁵ However, DTB treatment has been reported to cause serious hematologic & non-hematologic adverse effects due to its interaction with non disease related processes & cells, which often leads to dose reduction or treatment discontinuation in clinic. Peripheral edema & pleural effusion are the common non-hematologic side effects occurred during DTB treatment, which is likely caused by endothelial hyperpermeability.³⁶ DTB undergoes first pass metabolism by the cytochrome P450 enzyme 3A4 and terminal half life between 1.5 to 5 hours after oral administration.³⁷

Some of the important technological advances of nanocarrier drug delivery systems prove better shelf life and can be administered by oral, nasal, parenteral etc. The optimal size and characteristics of nanoparticles increase the circulation time and also enhanced permeability and retention effect.³⁸ DTB, when administered in its traditional form, often suffers from poor bioavailability due to its low solubility. Nanoparticles can enhance the solubility of DTB, allowing for better absorption and more effective delivery to the site of

action. By localizing the delivery of DTB to the tumor site, nanoparticles help minimize exposure to healthy tissues, which can reduce the side effects commonly associated with chemotherapy and targeted therapies. Nanoparticles can protect DTB from degradation, improving its stability during storage and transport in the body. This can contribute to the overall effectiveness of the drug, especially in complex biological environments.

Hence the development of DTB nanocarrier based drug delivery system could potentially improve therapeutic action thereby minimizing the systemic toxicity.

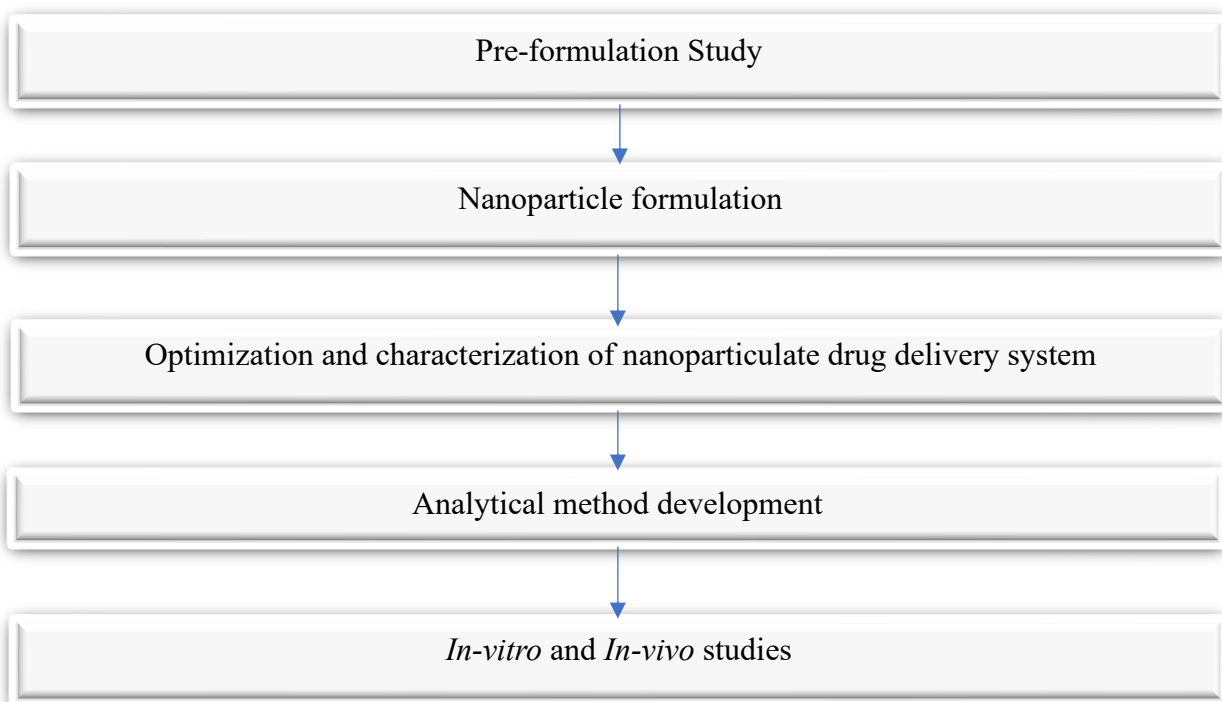
1.4 AIM AND OBJECTIVE

Aim: To Formulate and Evaluate Nanoparticle Drug Delivery System of DTB.

Objectives

- To formulate and optimize nanoparticulate drug delivery system of DTB using Factorial Design.
- To characterize the formulated nanoparticulate drug delivery system.
- To carry out the *in-vivo* study of formulated nanoparticulate drug delivery system.

STUDY PLAN



2. MATERIALS AND METHODS

Table 1: List of Equipment

Sl. No	Instrument Name	Model and Make
1.	Electronic Balance	Shimadzu, BL-220
2.	Magnetic Stirrer	Remi Instruments Ltd.
3.	Homogenizer	T18 digital Ultra Turrax®, IKA®, Ultra Instruments, Bengaluru, India
4.	Centrifuge	PR 24, Remi World, Mumbai, India
5.	Probe Sonicator	Labmann
6.	Bath Sonicator	2.5L GT-Sonic Ultrasonic bath sonicator
7.	FT-IR	Jasco, 460 Plus, Jasco Inc., United States
8.	DSC	Shimadzu DSC 600
9.	P-XRD	Bruker D8 ADVANCE X-ray Diffractometer, United States
10.	SEM	Zeiss Gemini 300
11.	AFM	Innova SPM
12.	Freeze dryer	Alpha Christ
13.	Nanosizer	Malvern Mastersizer -v3.62 (Malvern Instruments Ltd., UK
14.	UV-Spectroscopy	Shimadzu UV-Vis Spectrophotometer 1900i, Shimadzu Corporation, Japan
15.	LC-MS	Sciex, API-4000, Shimadzu

Table 2: List of Chemicals

Sl. No	Chemical	Supplier
1.	DTB	MSN Laboratories Pvt Ltd. Hyderabad
2.	Eudragit L 100	Evonik, Mumbai
3.	Poloxamer – 407	Yarrow chemie, Mumbai
4.	DMSO	Sigma Aldrich, Mumbai
5.	DMEM	Sigma Aldrich, Mumbai
6.	Fetal Bovine Serum	Sigma Aldrich, Mumbai
7.	DMBA	TCA, Hyderabad
8.	Methanol	Thermo Fishcer Scientific, Mumbai
9.	Acetonitrile	Thermo Fishcer Scientific, Mumbai
10.	Mannitol	Thermo Fishcer Scientific, Mumbai
11.	Sodium Hydroxide	Thermo Fishcer Scientific, Mumbai
12.	Potassium Dihydrogen Phosphate	Thermo Fishcer Scientific, Mumbai
13.	Orthophosphoric acid	Thermo Fishcer Scientific, Mumbai
14.	MTT	Sigma Aldrich, Mumbai

2.1 Analytical Methods for the identification of DTB

2.1.1 UV Spectroscopic method

DTB exhibits solubility in DMSO. Hence DMSO was used as solvent throughout the study.

Around 10 mg of DTB was used to scan in the range of 200 – 400 nm wavelength by UV visible spectrophotometer 1900-i.

Around 10mg of DTB was weighed accurately and transferred in phosphate buffer 1.2 pH & 6.8 pH in a 100mL volumetric flask. From the aliquots of 1, 3, 5, 7, 9 mL were taken to produce concentrations of 10, 30, 50, 70, 90 µg/mL. The absorbance was measured using Shimadzu double beam UV-visible spectrophotometer 1900-i at 324 nm. The linear regression data obtained from the calibration curve showed a linear relationship over the concentration range of 10 to 100µg/mL. The experiments were performed in triplicates.³⁹

2.1.2 FT-IR Spectroscopy

FT-IR of DTB, the sample was prepared by finely grinding in a glass mortar and pestle with potassium bromide to minimize IR scattering on the particle surface, The prepared sample was placed in the sample holder and scanned in the range of 4000 – 1000 cm⁻¹ using JASCO Plus FTIR.

2.1.3 Thermal Analysis

Analysis was performed on pure DTB using SHIMADZU DSC -60. Sample weighing 5 mg placed on aluminum pans and sealed. The data were collected under nitrogen gas between 25 °C and 300 °C at scan rate of 10 °C/min.⁴⁰

2.2 Development and validation of LC-MS method for DTB

2.2.1 Instrumentation

The liquid chromatography and mass spectrometry analysis was performed using an AB Sciex 4000 instrument, equipped with a Chromasol Garnet C18 polar analytical column (50×4.6 mm, 4 μ m, 120 Å). The column oven was maintained at $45 \pm 2^\circ\text{C}$, while the autosampler was kept at $10 \pm 2^\circ\text{C}$. The mobile phase consisted of a 60:40 v/v mixture of water and methanol, delivered at a flow rate of 0.8 mL/min, with an injection volume of 2 μ L.

2.2.2 Method development

The liquid chromatography procedure was carried out over a four-minute duration. The mass spectrometer operated in positive electrospray ionization molecular structure on (+ESI) mode, employing multiple reaction monitoring (MRM) to detect the mass-to-charge ratio transitions for DTB (m/z 488.1 $\rightarrow$ 401.0) and Verapamil (internal standard, m/z 455.6 $\rightarrow$ 165.3). This setup ensured precise and efficient separation and detection of the analytes.⁴¹

2.3 Compatibility using FT-IR Spectroscopy

FT-IR of DTB, EL were obtained to examine for possible interactions between drug and excipients. The samples were prepared by finely grinding in a glass mortar and pestle with potassium bromide to minimize IR scattering on the particle surface, The prepared sample was placed in the sample holder and scanned in the range of 4000 – 1000 cm^{-1} using JASCO Plus FTIR.⁴⁰

2.4 Experimental Design

To maximise the synthesis of DTB nanoparticles (NPs), a factorial design was used. The influence of the sonication power, surfactant concentration, and polymer concentration on the physicochemical characteristics of the produced nanoparticles was investigated. The effect of the three independent variables, Eudragit® L100 concentration (X1), Poloxamer 407 concentration (X2), and sonication power (X3) on the dependent variables, P.S, Z.P, and EE of the formulated NPs, was examined using a full 2^3 factorial design with a total of 11 experiments. Throughout the course of the trial, DTB levels remained steady. The low and high levels, which were denoted by the signs (-1) and (+1), were explored for each factor over a range of two levels. The levels and parameters under investigation were chosen after consulting the literature and results of earlier experiments carried out at our lab. The full factorial design, responses, design summary, significant values and experimental trial are given. Using Design Expert 13.0.5 software, the response parameter data were statistically evaluated.⁴²

Table 3: Variables utilized in 2^3 factorial design for the DTBNps.

Coded Level	-1	+1
Independent Variables		
X1 – Drug: Polymer Ratio (mg)	50	100
X2 – Stabilizer Concentration (%)	1	2
X3 – Sonication Power (mV)	30	50
Dependent Variables		
Y1 – Particle Size (nm)	Minimize	
Y2 – Zeta Potential (mV)	In range	
Y3 – Entrapment Efficiency (%)	Maximize	

Table 4: Formulation Table

	Independent Variables		
	Drug: Polymer ratio (mg) (Dasatinib:Eudragit L)	Surfactant concentration (%) (Poloxamer 407)	Sonication Power mV
DTBNp1	1:1	1	30
DTBNp2	1:1	2	30
DTBNp3	1:2	1	30
DTBNp4	1:2	2	30
DTBNp5	1:1	1	50
DTBNp6	1:1	2	50
DTBNp7	1:2	1	50
DTBNp8	1:2	2	50

2.5 Preparation of DTBNps

NPs were formulated using Single – emulsion (o/w) – solvent evaporation method following the parameters indicated in the experimental design. Briefly weighed amount of DTB and Eudragit L were weighed and then added to organic phase [DMSO, DCM, Ethanol]. The organic phase added dropwise [0.5ml/min] to a beaker filled with deionized water with certain proportions of poloxamer- 407 under probe sonication (Labmann) conditions. The resultant solution was further homogenised (IKA T18 Ultra Turrax) at 6000 rpm for 10 minutes to achieve the nanoemulsion. The particles were separated by centrifugation at 10000 rpm for 15 minutes [Remi, PR 24 Mumbai,

India] along with washing thrice with water. The nanoformulation was freeze dried into powder using vacuum freeze dryer using mannitol (2%) as cyroprotectants [Alpha Christ] and stored for further studies as presented in the figure 6.⁴³⁻⁴⁵

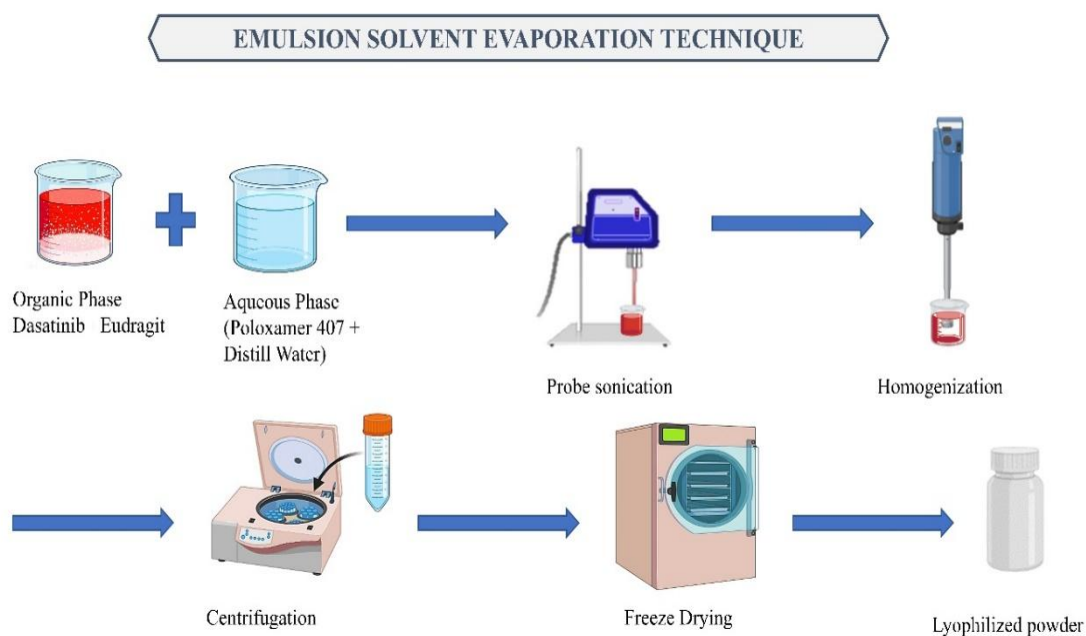


Figure 6: Emulsion solvent evaporation method of preparation for formulation of DTBNPs.

2.6 Physicochemical Characterization studies

2.6.1 Particle Size Determination

Particle size is associated with pharmacokinetics. The Particle size of prepared DTB-NPs were determined using Dynamic light scattering method at 25°C using a quartz cell at ambient temperature. Malvern Zetasizer (Nano-ZS90, Malvern Instruments and UK). The scan were performed in triplicates.

2.6.2 Zeta Potential Determination

The zeta potential is the measure of the electric charge at the surface of the particles indicating the physical stability of colloidal systems. In this study, Malvern Zetasizer (Nano-ZS90, Malvern Instruments and UK) and used to measure the zeta potential values by determining the particle electrophoretic mobility at 25°C. The measurements were performed triplicate in ultra-purified water adjusted to a standardized conductivity.⁴⁶

2.6.3 Entrapment Efficiency

Entrapment efficiency was estimated using an indirect technique. Briefly, DTBNps were dissolved in DMSO and stirred for 1 h, followed by sonication using an ultra-sonic cleaner (GT Sonic Ultra Sonic Cleaner) for 10 min. The solution was then filtered using a 0.25 µm PDF syringe filter and subjected to centrifugation at 15,000 rpm for 15 min (Remi, PR 24 Microprocessor Research Centrifuge, India). The supernatant, containing the free drug, was analyzed by UV spectroscopy at 324 nm. Measurements were performed in triplicates.⁴⁴ The entrapment efficiency was calculated using the following formula:

$$\text{Entrapment Efficiency \%} = \frac{\text{Free Drug in supernatant} - \text{Total drug}}{\text{Total Drug}} \times 100$$

2.7 Surface Morphology of DTBNPs.

The surface texture of the optimized nanoparticles was observed by Scanning Electron Microscopy & Atomic Force Microscopy.

2.7.1 SEM

The DTBNPs were carefully casted on glass slides and degassed under vacuum to eliminate the contaminants. A thin layer of a gold-palladium alloy was sputter-coated

onto the nanoparticles to enhance conductivity and improve image quality After drying, the nanoparticle's surface morphology was captured at a suitable magnification using Zeiss Gemini 300 SEM.⁴⁷

2.7.2 AFM

For AFM the small drop of sample placed on microscopic slide followed by drying. The images were captured using Innova SPM Atomic Force Microscope⁴⁸

2.8 Solid State Characterization

2.8.1 FT-IR spectroscopy

FT-IR of DTB, Eudragit L 100, physical mixture and DTBNPs were obtained to examine for possible interactions between drug and excipients. The samples were prepared by finely grinding in a glass mortar and pestle with potassium bromide to minimize IR scattering on the particle surface, The prepared sample was placed in the sample holder and scanned in the range of 4000 – 1000 cm⁻¹ using JASCO Plus FTIR.⁴⁰

2.8.2 Thermal analysis

With the assistance of a differential scanning calorimeter SHIMADZU, DSC-60, solid state and component interactions were examined. Analysis were performed on pure DTB, eudragit L 100, Physical mixture, and DTBNps. Sample weighing 5 mg placed on aluminum pans and sealed. The data were collected under nitrogen gas between 25 °C and 300 °C at scan rate of 10 °C/min.⁴⁰

2.8.3 Powder X-Ray Diffraction (P-XRD)

The P-XRD was employed to detect patterns of pure DTB, eudragit L100, Physical mixture and DTBNps was measured with powder X-ray diffractometer instrument

(Bruker D8 advance diffractometer). The operation data were: 2.2 kW X-ray source of Cu anode with fine focus ceramic X-ray tube operated at 40 kV voltage and 40 mA current at 1.6 kW power. All measurement was performed over the 2θ range from 10° to 70° . 2θ values and collected using LYNXEYE high speed SSD160–2 detector with 500 μm sensor.⁴⁹

2.9 *In-vitro* release study & Release kinetics determination

In-vitro cumulative release study for the DTBNps and pure DTB suspension were performed utilizing the dialysis membrane technique (Molecular weight cutoff 12000-14000). Formulation was suspended in end clipped dialysis membrane and suspended in phosphate buffer 100 mL consisting of two mediums (pH 1.2 and 6.8) containing 2% tween 80 establishing sink conditions and maintaining the temperature of $37 \pm 0.5^\circ\text{C}$ and agitated at 100 rpm. At pre-determined time points (30min, 1, 2, 3, 4, 6, 8, 12, 24h) samples was collected and sink conditions were maintained by replenishing with fresh media to maintain the sink conditions. The quantification of released drug was performed in triplicates using UV Spectroscopy at 324nm.⁵⁰ To investigate the mechanism of drug release from DTBNps they were fitted in various mathematical models which interpret drug release in function of time. Release kinetics were evaluated by the DD Solver add-in program. Add-in software DD solver reduces errors, fasten up the calculations and determine release curve precisely.⁵¹

2.10 Animals

A total of 88 female Wistar rats were procured from Skanda Life Sciences Pvt. Ltd. and used for conducting experiments. The experiments were designed following the guidelines and regulations of the committee for the purpose of control and supervision

of experiments on animals (CPCSEA). The Institutional Animal Ethics Committee (IAEC) approved the experimental procedures based on IAEC No. 02/AP/2022. During the 7-day acclimatization period, the rats were provided with unlimited access to food and water and were accommodated in standard polypropylene cages, with six rats per cage. The animal housing environment was maintained at an average temperature of $22\pm 2^{\circ}\text{C}$ with a 12-hour cycle of light and dark.

2.11 Pharmacokinetics and Biodistribution

The pharmacokinetic study was conducted using female Wistar rats ($n=6$, 220-250g body weight) to evaluate the absorption and disposition of DTB and DTBNps. Dosages of 10 mg/kg for DTB and 10 mg/kg for DTBNps were administered orally. Blood samples were collected pre-dose and at specific time points post-dose: 0.5, 1, 2, 3, 8, 12, and 24 hrs. The sampling was performed using the retro-orbital method under isoflurane anesthesia. Blood was collected into tubes containing EDTA as an anticoagulant and centrifuged at 4,000 g for 10 min to separate plasma, which was stored at -80°C until analysis.

The quantification of DTB in plasma samples was carried out using a validated LC-MS/MS method. Plasma processing involves protein precipitation by adding a mixture of acetonitrile and methanol (1:1, v/v) to the plasma. After vortex mixing and centrifugation at 15,000 rpm for 15 min, the supernatant was transferred to a new tube and analyzed.

Non-compartmental analysis was used to calculate pharmacokinetic parameters including maximum plasma concentration (C_{max}), time to reach maximum concentration (T_{max}), area under the plasma concentration-time curve (AUC), mean residence time (MRT), and the elimination half-life ($t_{1/2}$). Data analysis was performed to compare the

pharmacokinetic profiles and bioavailability between the standard and DTBNps. In addition to plasma concentration analysis, biodistribution studies were performed to determine the distribution of DTB in various tissues. Post-euthanasia, tissues including the liver, brain, heart, kidney, and spleen were collected. Tissue samples were homogenized and treated with a solution of acetonitrile and methanol to extract DTB. The homogenates were centrifuged, and the supernatants were analyzed using the same LC-MS/MS method as plasma samples. This enabled quantification of DTB concentration in these tissues, providing insight into the drug's distribution and potential target sites within the organism.⁵²

2.12 Pharmacodynamics

2.12.1 Experimental design

Animals (40 female, Wistar albino Rats), aged 50–55 days, weighing (180 ± 10 g) were categorized into 4 groups of 10 animals each.

Group I- Normal control.

Group II- DMBA treated - DMBA induced rats only.

Group III- DTBNps treated-DMBA-induced rats treated with DTBNps (10 mg/kg per day) for one month after tumor development.

Group IV- DTB treated-DMBA-induced rats treated with DTB (10 mg/kg per day) for one month after tumor development.

Following the administration of the doses, the rats were anesthetized using diethyl ether and euthanized. The collection of blood samples was performed via orbital puncture subsequently, the serum was isolated to conduct various analyses, including a complete

blood test (CBC). Breast tissue, liver, kidney, and small intestine samples were preserved in a 10% formalin solution for histopathological examinations.

2.12.2 Induction of Mammary Gland Tumors

To induce the tumors, a freshly prepared solution of 7,12-dimethylbenz(a)anthracene (DMBA) at a concentration of 20 mg/mL in olive oil was orally administered using the gavage technique. Starting from the fourth week after DMBA administration, the rats underwent weekly palpation to detect any tumor development. The initial tumor manifestation was observed in the 16th week, and by the 18th week, tumors had developed in all rats.

2.12.3 Measurement of Tumor Volume, Body Weight, and Survival Rate

To assess the efficacy of the treatments, tumor volume, body weight, and survival rate of the animals were monitored throughout the study. Tumors were induced in female Wistar rats by administering a freshly prepared solution of 7,12-Dimethylbenz(a)anthracene (DMBA) at 20 mg/mL concentration in olive oil using the gavage technique. Tumor development was initially observed in the 16th week and all rats had developed tumors by the 18th week. The dimensions of the mammary tumors were measured weekly using a Vernier caliper. Tumor volume (V) was calculated using the formula:

$$V = (L \times B^2) \div 2$$

L represents the longer diameter, and B represents the shorter diameter, measured in cm and oriented perpendicularly to each other. Concurrently, the body weights of the animals were recorded weekly using a digital scale to monitor the overall health and potential weight changes associated with the treatments. Initial body weights were recorded before the treatment, and final body weights were recorded after the treatment period. The

survival rate was calculated as the ratio of the number of surviving animals in each group to the total number of animals at the start of the experiment.

2.12.4 Hematologic examination

For the hematologic examination in our study, blood samples were collected from experimental rats via retro-orbital bleeding under anesthesia using isoflurane to minimize discomfort. Approximately 1-2 mL of blood was drawn and immediately transferred into EDTA-coated tubes to prevent coagulation. The samples were stored at 4°C and promptly transported to the laboratory for analysis. Hematologic parameters were evaluated using an automated hematology analyzer, including RBC count, hemoglobin concentration, hematocrit, WBC count, differential WBC count (lymphocytes, monocytes, eosinophils), and platelet count. Quality control was maintained by calibrating the hematology analyzer according to the manufacturer's instructions and running control samples daily to ensure accuracy and precision.

2.12.5 Histopathologic examination

For histopathological examination, breast, liver, kidney, and small intestine tissues were collected from the experimental rats after euthanasia. The tissues were immediately fixed in 10% neutral-buffered formalin for 24-48 hrs. After fixation, the tissues were processed using a standard paraffin embedding technique. Sections 4-5 micrometers in thickness were cut from the paraffin blocks using a microtome and mounted on glass slides. The sections were then deparaffinized in xylene, rehydrated through a series of graded alcohols, and stained with hematoxylin and eosin (H&E). The stained sections were dehydrated, cleared, and cover slipped using a mounting medium. Histopathological

examination was performed under a light microscope to assess morphological changes in the breast, liver, kidney, and small intestine tissues.

2.13 MTT Assay

Cytotoxicity of the test samples DTB and DTBNps was evaluated in MCF-7, MDA MB231, and 4T1 cell lines using the MTT assay. Cell lines were procured from ATCC. Stock cells were cultured in their respective media, supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), and streptomycin (100 µg/mL), in a humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated using 0.05% trypsin and centrifuged at 1000 rpm for 5 minutes. The culture media was discarded, and the cell pellet was gently resuspended in 2 ml complete media. Cells were seeded into 96-well plates at a density of 50,000 cells/well. After 24 hours, cells were treated with various concentrations of the test samples (3.125 to 100 µM) for 24 hours. Post-treatment, the media was replaced with MTT reagent (5 mg/10 ml in PBS) and incubated for 4 hours. Formazan crystals formed by viable cells were solubilized using DMSO, and the absorbance was measured at 590 nm using a microplate reader. Percent inhibition was calculated, and IC₅₀ values were determined using nonlinear regression analysis.

2.14 Stability Studies

To evaluate the physical stability a 3-month storage period at 25.0 ± 0.5 °C, 4.0 ± 0.5 °C and 40 ± 0.5 °C was used for prepared DTBNps. To determine the stability of DTBNps the physical parameters including particle size, zeta-potential and entrapment efficiency were measured before and after storage to determine their stability.⁵³

3. DATA ANALYSIS PLAN

3.1 Statistical Analysis

Statistical Analysis for Evaluation of Formulations

All formulation studies were performed in triplicate, and the mean values were presented along with their standard deviations (mean $\pm$ SD). The software employed to the experimental design and ANOVA test was Design Expert version 13.0.5 (stat-Ease Inc., Minneapolis, MN, USA). Microsoft Excel® was used for calibration and dissolution studies, while FTIR, DSC, and P-XRD graphs were analyzed with Origin8.5® software.

Statistical Analysis for Evaluation of In-vivo Studies: Pharmacokinetic parameters were estimated by applying the pk Solver® software. The results from in-vivo studies were statistically compared to determine the level of significance using GraphPad Prism 8.0® (GraphPad Inc., CA, USA), and the mean values were reported with their standard deviations.

4. RESULTS AND DISCUSSION

4.1 Authentication of drug

4.1.1 UV Spectroscopy

Wavelength for maximum absorbance was recorded. The λ_{max} of DTB was found to be 324 nm.⁵⁴

The λ_{max} of DTB is represented in figure 7. The calibration curve of DTB in pH 1.2 & 6.8 is given in Table 5 & 6 and figure 8 & 9.

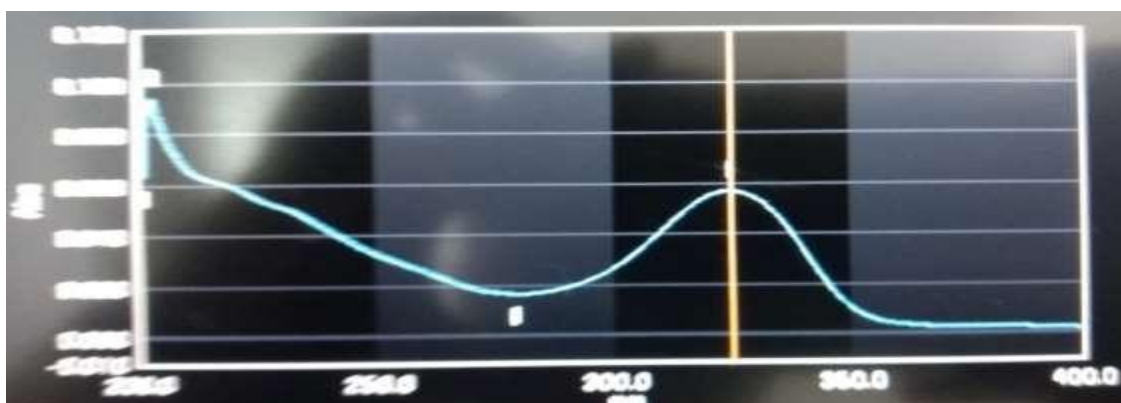


Figure 7: UV Visible Spectra of DTB

Table 5: Calibration of DTB at pH 6.8

Concentration ($\mu\text{g/ml}$)	Absorbance (nm)
1	0.13 ± 0.003
3	0.26 ± 0.002
5	0.38 ± 0.010
7	0.51 ± 0.024
9	0.61 ± 0.004

*Data shown in mean $\pm$ SD (n=3)

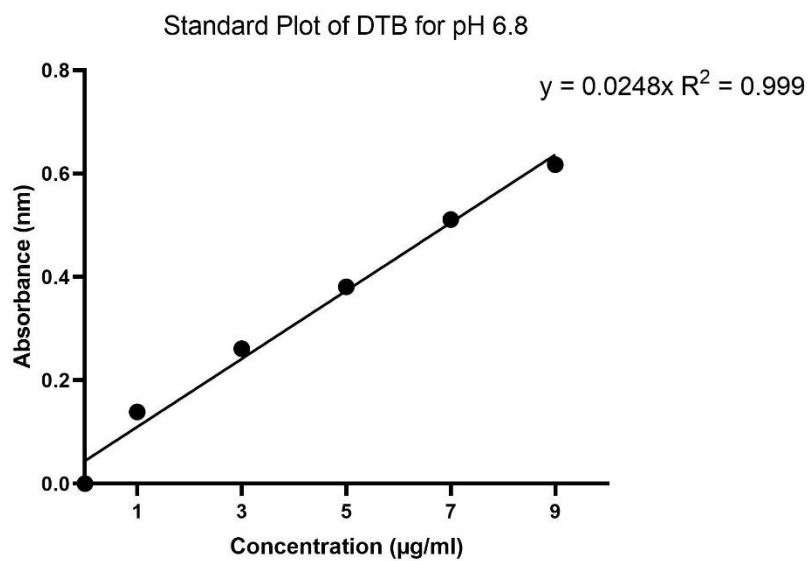


Figure 8: Standard calibration curve of DTB at pH 6.8.

Table 6: Calibration of DTB at pH 1.2

Concentration (µg/ml)	Absorbance (nm)
1	0.09 ± 0.02
3	0.2 ± 0.01
5	0.38 ± 0.04
7	0.54 ± 0.08
9	0.71 ± 0.10

*Data shown in mean ± SD (n=3)

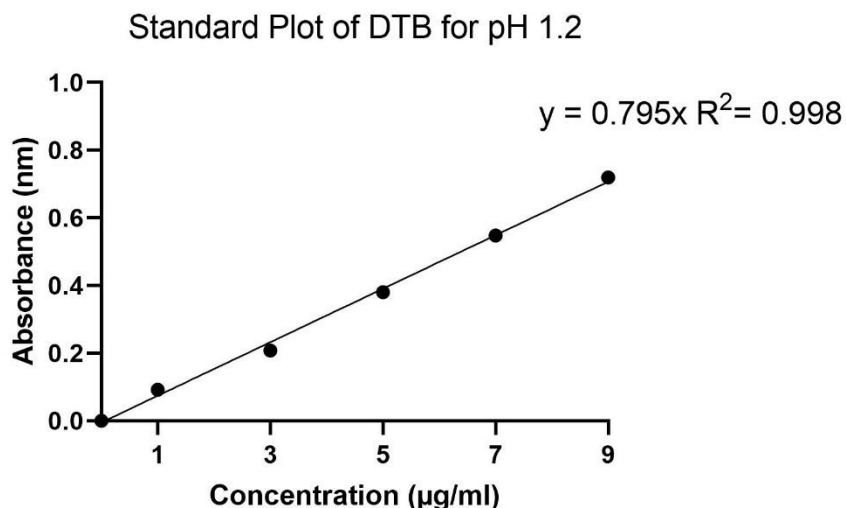


Figure 9: Standard calibration curve of DTB at pH 1.2.

The linear regression data obtained from the calibration curve showed a linear relationship over the concentration range of 1-11 µg/mL. Based on the average absorbance at 324 nm, the equation for the best line fit was generated ($y = 0.0248x$; $R^2 = 0.999$) for pH 6.8 and ($y = 0.0795x$; $R^2 = 0.999$) for pH 1.2

4.1.2 FT-IR Spectroscopy

The FT-IR spectrum shown in fig. DTB showed characteristic peaks at 3258.14 cm^{-1} attribute the presence of carboxylic acid OH stretch. A sharp dip at 1414.53 cm^{-1} indicated the C=C alkene aromatic group. The sharp stretch at 1197.58 cm^{-1} indicates the C-O of the phenol group. The sharp peak at 773.31 cm^{-1} indicates the C-Cl group halo-compound.⁵⁵

The FT-IR spectrum of DTB is given in figure 10 and corresponding wavelength is mentioned in table 7.

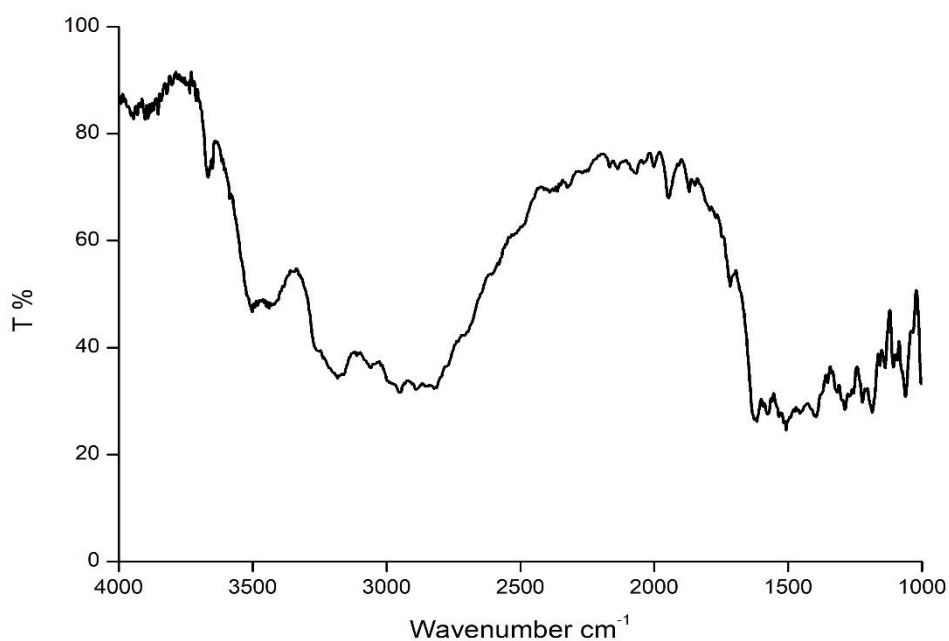


Figure 10: FTIR spectrum of DTB

Table 7: Interpretation of FTIR spectra of DTB

Peak No	Wave-number (cm ⁻¹)	Functional Group
1	3258.14	O- H
2	1577.49	-C-H
3	1414.53	HC=CH (aryl)
5	1197.58	C-O
6	773.31	C-Cl

4.1.3 Melting Point

The DSC curve of DTB shows a sharp endothermic peak that corresponds to drug melting ($T_{\text{onset}} = 283.49^{\circ}\text{C}$).⁵⁶ The DSC of DTB is given in figure 11.

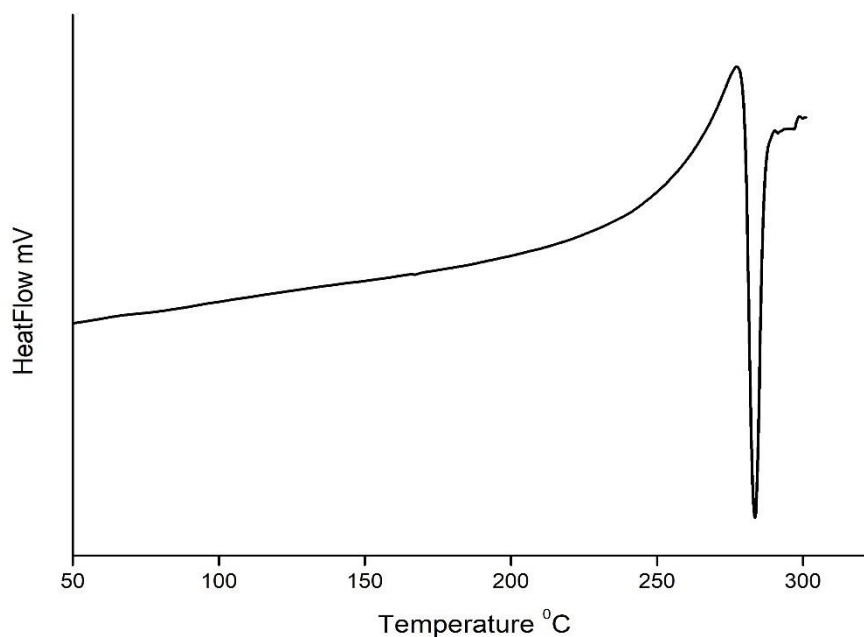


Figure 11: DSC thermogram of DTB

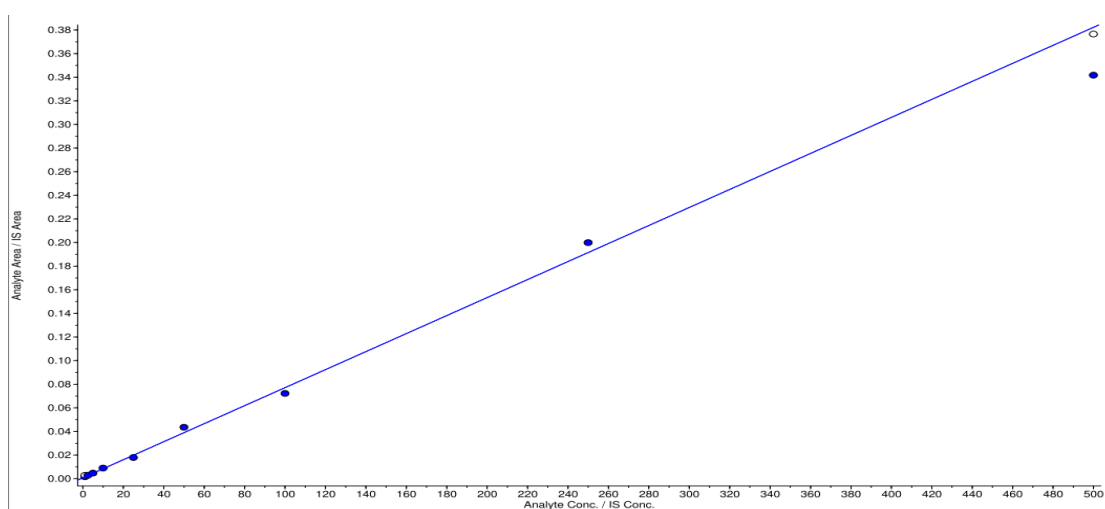
4.2 LC-MS method development

4.2.1 Selectivity

The responses observed in the blank plasma were consistently below 14.9% of the signal at the (LLOQ) of 1 ng/mL for DTB, and below 0.62% for the IS, indicating no significant interference from endogenous plasma components at the analyte's retention time. No interference from endogenous compounds was detected at the drug's retention time in any of the six evaluated blank plasma extracts, confirming the specificity of the analytical method. This indicates that the chromatographic conditions were well-optimized, ensuring accurate and selective detection of the drug without overlapping signals from biological matrix components.

Table 8: DTB linearity results

Final conc. in ng/mL	DTB peak area
Blank	00
1	0.366
2.5	4.720
5	20.130
10	39.133
25	53.155
50	74.240
100	89.069
250	92.250
500	99.325
Y Intercept	0.00126
Slope	0.0140
Regression coefficient (r^2)	0.9958

**Figure 13: Representative calibration curve**

4.2.3 Accuracy & Precision

To assess the intra-assay precision and accuracy, six duplicate samples containing DTB were analyzed at three different quality control (QC) levels within a single analytical run.

For inter-assay precision, the same three QC levels were analyzed across multiple independent runs to evaluate consistency and reproducibility over time.

The proposed method's mean accuracy ranged from 94.15% to 107.33%, while the precision (%CV) for the low (LQC), middle (MQC), and high (HQC) quality controls ranged from 1.79% to 5.44%. Table 9 summarizes these findings.

Table 9: Calculated concentrations obtained for precision and accuracy batches

Injections	Observed Concentration (ng/mL)			
	HQC	MQC	LQC	LLOQ QC
Standard	1.45980	0.63620	0.01700	0.01050
Deviation				
CV (Precision %)	2.37	1.79	2.86	5.44
Accuracy (%)	106.09	107.33	99.83	94.15

4.2.4 Recovery

The CV or RSD of the responses from replicate injections of aqueous samples for DTB peaks ranged from 0.33% to 0.67%, indicating excellent precision in the analytical method for these samples. For the extracted samples, the CV or RSD of the DTB peak responses ranged from 2.85% to 3.08%, demonstrating slightly higher variability, which is expected due to sample processing. The average recoveries of DTB at all three

concentration levels were between 79.77% and 85.17%, with a mean recovery of 82.12%, suggesting that the method is efficient in recovering the drug from plasma samples. This recovery rate supports the method's suitability for accurate quantification of DTB in biological matrices.

Table 10: Recovery of DTB

Sl. No	Observed response								
	Extra HQC	Aqueous HQC	% Recov ery	Extra MQC	Aqs MQC	% recov ery	Extra LQC	Aqs LQC	% recov ery
ME	109956	129107	85.17	105616	132416	79.77	108044	132690	81.43
AN.	2.7	5.3		8.0	5.5		2.8	6.0	
SD	31372. 14	5708.7 8	2.480	32510 .02	4376.0 2	2.60 2	32866. 70	8889. 17	2.432
% CV	2.85	0.44	2.91	3.08	0.33	3.26	3.04	0.67	2.99
Mean recovery		82.12							
SD		2.766							

4.2.5 Matrix effect

The back-calculated concentrations for the (HQC) and (LQC) levels showed mean accuracies of 99.86% and 98.76%, respectively. As these results met the acceptance criteria of 85.00% to 115.00%, the current method demonstrated no significant ionization effects.

4.2.6 Stability

To evaluate the stability of DTB in plasma, we conducted tests using 6 replicates of QC samples at both low and high concentrations. Appropriate volumes of DTB standard solutions were added to create drug-free plasma samples. The results, shown in Table 11, fell within the acceptable range, indicating that DTB demonstrates favorable stability.

Table 11: DTB QC sample stability results using LC - MS/MS

Stability	Nominal concentration ng/ml	Standard deviation	CV (Precision %)	% Accuracy
Bench-top stability	50	1.59400	2.52	102.64
Auto - sampler stability		1.40400	2.37	102.23
Sort- term		2.68200	4.38	105.54
Long term (64 days)		1.59400	2.69	102.54
Freeze-and taw- stability		1.38300	2.32	102.70

4.3 Compatibility studies using FT-IR Spectroscopy

The FT-IR spectrum shown in fig. DTB showed characteristic peaks at 3258.14 cm^{-1} attribute the presence of carboxylic acid OH stretch. A sharp dip at 1414.53 cm^{-1} indicated the C=C alkene aromatic group. The sharp stretch at 1197.58 cm^{-1} indicates the C-O of the phenol group. The sharp peak at 773.31 cm^{-1} indicates the C-Cl group halo-compound. The Eudragit L 100 showed a broader peak at 1725.01 cm^{-1} indicate the C=O aliphatic ketone. A characteristic peak at 1154.01 cm^{-1} indicated the C-O-C anhydride group.⁵⁵ A peak at 2951.52 cm^{-1} indicated the presence of -CH Alkane group which are present in the structures.⁵⁷ The physical mixture of DTB and Eudragit L 100 indicated almost similar peaks 3291.89 cm^{-1} , 1460.81 cm^{-1} , 715.46 cm^{-1} , suggesting the absence of any drug polymer interaction.

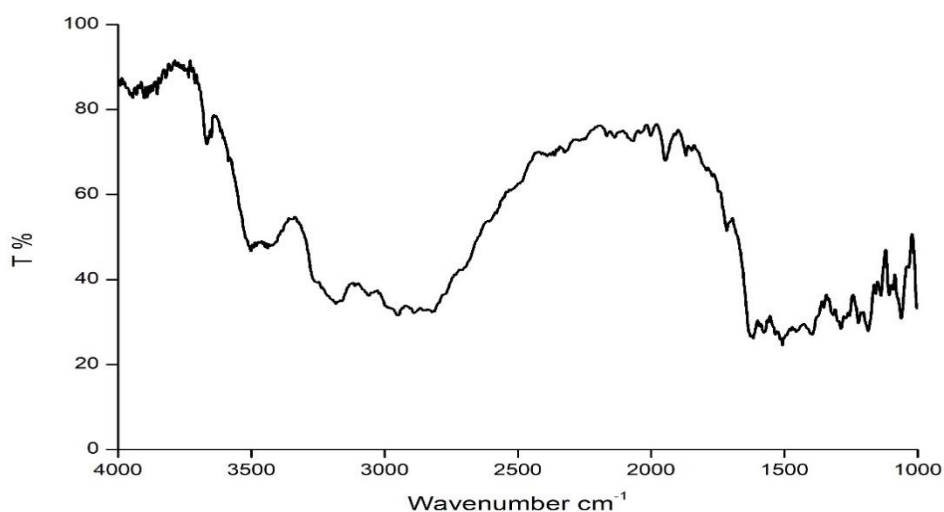


Figure 14: FT-IR spectrum of DTB

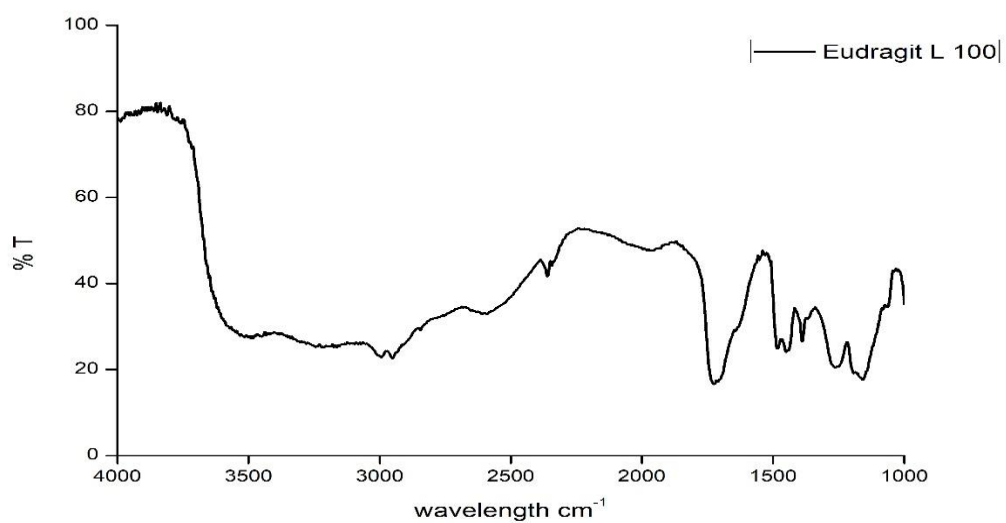


Figure 15: FT-IR spectrum of EL 100

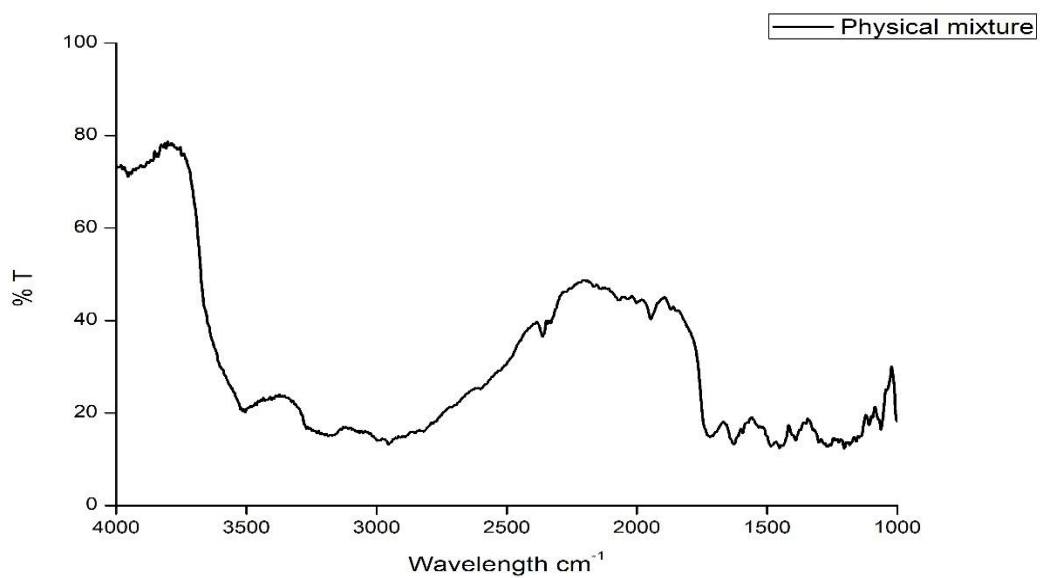


Figure 16: FT-IR spectrum of Physical mixture of DTB and Eudragit L 100

4.4 Full Factorial Design

Table 12: Full Factorial Design table

File Version	13.0.5.0		
Study Type	Factorial	Subtype	Randomized
Design Type	2 Level Factorial	Runs	8.00
Design Model	3FI	Blocks	No Blocks
Center Points	0.0000	Build Time (ms)	34.00

Table 13: Responses of 2³ Full factorial design of prepared DTBNPs.

Formulation	Response 1 Particle Size (nm)	Response 2 Zeta Potential (mV)	Response 3 Entrapment Efficiency %
F1	240.3±9.0	-24.7±0.8	84.11±1.2
F2	221.3±8.3	-19±1.1	82.47±0.9
F3	270.3±12.4	-19.8±0.9	82.32±1.7
F4	259.8±9.1	-18.6±1.8	86.01±1.1
F5	202.1±5.7	-18±1.01	93.11±0.2
F6	295.3±11.8	-21.06±1.5	86.45±1.3
F7	221.9±10.9	-20.4±1.8	90.11±0.3
F8	215.7±11.1	-21±1.3	88.78±0.9

* Data shows mean ± SD (n=3)

Table 14: Summarization of model parameters

Models	R ²	Adjusted R ²	SD	Adequate Precision	%CV	Remarks
Particle Size R1 3FI	0.9958	0.9809	3.67	26.49	1.15	Significant
Zeta Potential R2 3FI	0.9888	0.9495	0.41	17.79	2.02	Significant
Entrapment Efficiency R3 3FI	0.9878	0.9453	0.63	15.38	0.73	Significant

4.4.1 Level of Significance for Factors

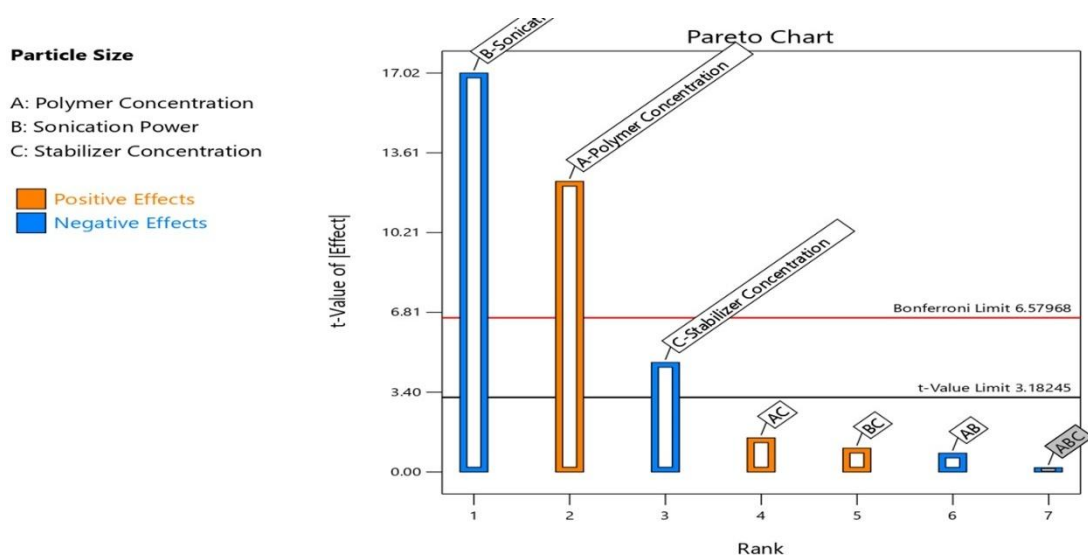


Figure 17: Pareto chart for effects of variables on particle size.

Sonication power (B) exerts the strongest negative effect on particle size, likely due to increased power which reduces size of particles. Similarly, Polymer concentration (B) increases particle size. Stabilizer concentration (C) also reduce particle size to a smaller extent.

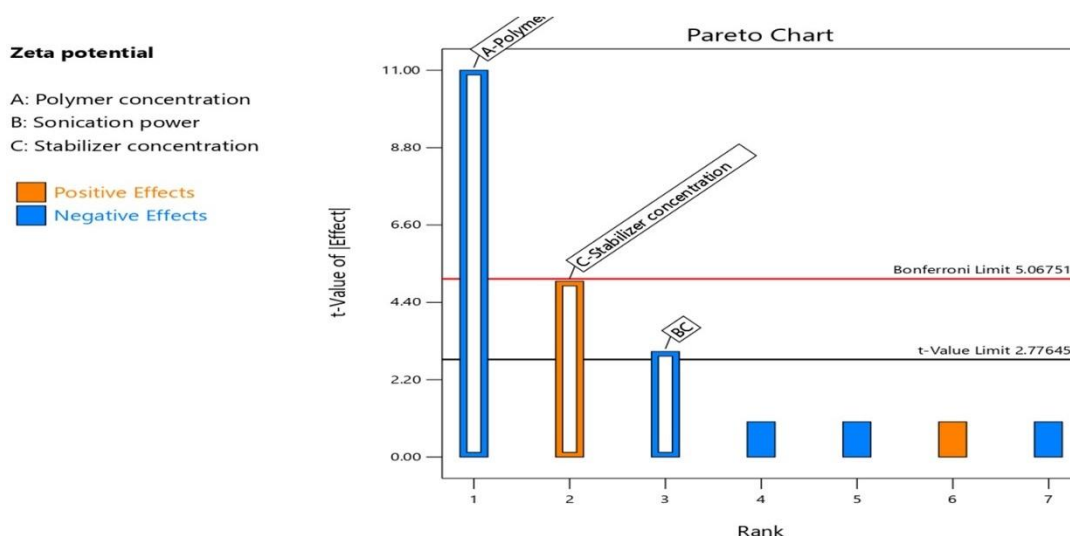


Figure 18: Pareto chart for effects of variables on zeta potential.

Polymer concentration (A) exerts the strongest positive effect on zeta potential, likely due to increased surface coverage and charge stabilization of particles. Similarly, stabilizer concentration (C) enhances zeta potential, possibly by improving steric or electrostatic stabilization. In contrast, sonication power (B) and the interaction between sonication and stabilizer (BC) show weaker, statistically insignificant effects, suggesting that their influence on zeta potential is minimal under the studied conditions.

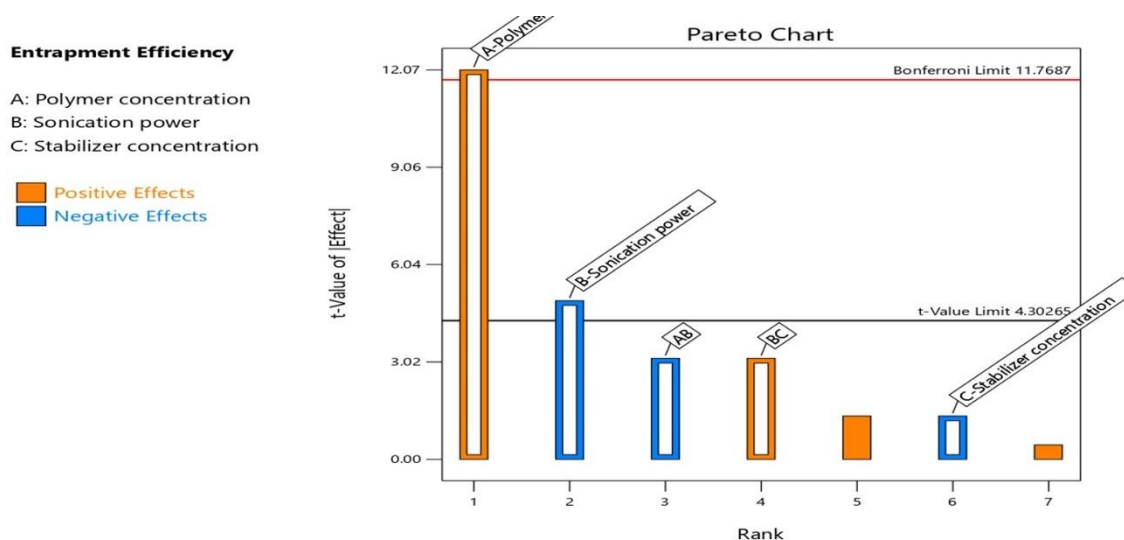


Figure 19: Pareto chart for effects of variables on Entrapment Efficiency.

The Pareto chart visually demonstrates that polymer concentration is the most critical factor positively influencing entrapment efficiency, while sonication power has a significant negative impact. Interactions and other variables have lesser, statistically insignificant effects. moderate sonication helps reduce particle size (improving stability and uniformity), excessive sonication can make particles too small. Smaller particles have a larger surface area-to-volume ratio, which can increase the rate of diffusion or leakage of the entrapped material.

4.5 Physicochemical Characterization of DTNps.

4.5.1 Influence of variables on particle size

The P.S is one of the most important factors affecting the *in vivo* efficiency in nanoscale based drug delivery system. P.S has a significant effect, which influences distribution, targeting ability, toxicity. The NPs are in suitable range. The particles are sphere in shape which can be concluded from curves. The particle size was observed in the range of 202 - 295nm. The summarization of model parameters and p values are shown (Table 15).

The size range of optimized formulation is shown (Figure. 20). The 3D surface plot shown (Figure 21a,b,c). The corresponding polynomial equation by the FFD on particle size is as given below:

$$\text{Particle Size (nm)} = +325.84 + 16.09A - 6.06B - 22.09C + 1.89AB - 1.04AC + 1.31BC - 0.2375ABC$$

Table 15: Summary of Particle Size ANOVA

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	6318.70	7	902.67	66.96	0.0148	significant
A-Polymer Concentration	2070.46	1	2070.46	153.60	0.0064	
B-Sonication Power	3902.86	1	3902.86	289.53	0.0034	
C-Stabilizer Concentration	294.03	1	294.03	21.81	0.0429	
AB	8.61	1	8.61	0.6388	0.5080	
AC	28.50	1	28.50	2.11	0.2831	
BC	13.78	1	13.78	1.02	0.4184	
ABC	0.4513	1	0.4513	0.0335	0.8717	
Curvature	1445.28	1	1445.28	107.22	0.0092	
Pure Error	26.96	2	13.48			
Cor Total	7790.94	10				

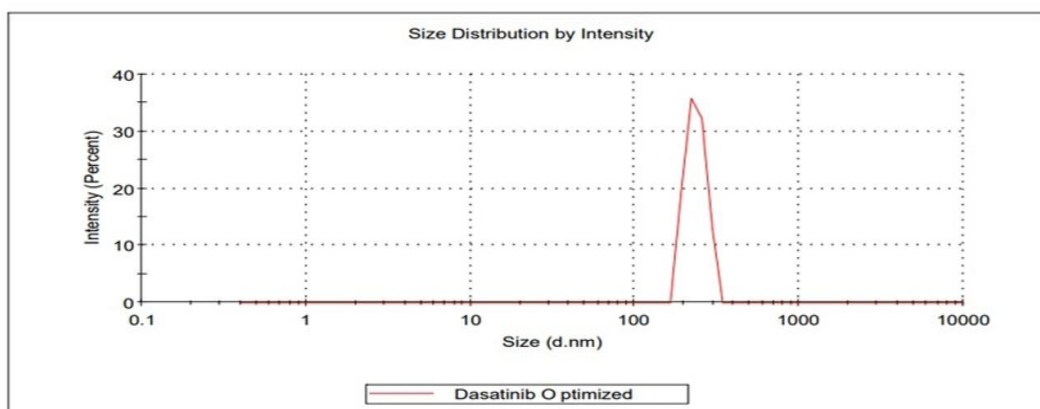


Figure 20: Particle size of DTBNps 5.

Factor Coding: Actual

Particle Size (nm)
(adjusted for curvature)

Design Points
202 295

X1 = A

X2 = C

Actual Factor
B = 40

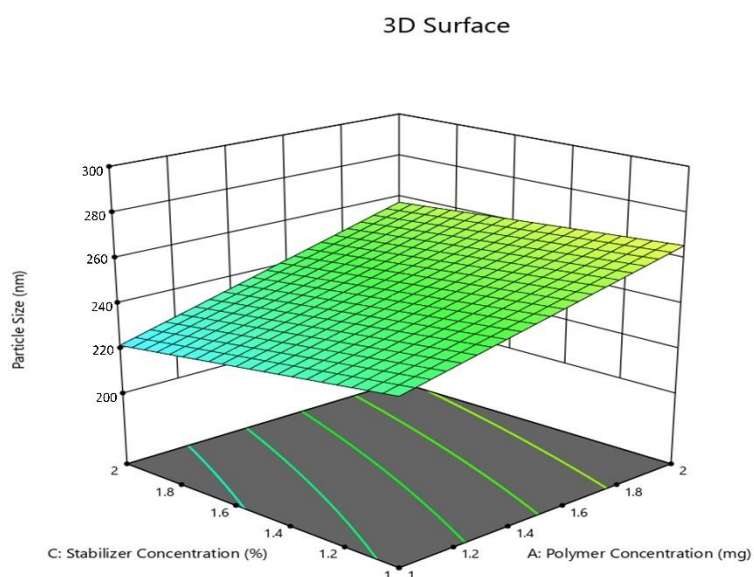


Figure 21a. 3D surface plot of Stabilizer concentration vs Polymer concentration affecting particle size of DTBNPs prepared using Emulsion solvent evaporation method.

Factor Coding: Actual

Particle Size (nm)
(adjusted for curvature)
Design Points
202 295

X1 = A
X2 = B

Actual Factor
C = 1.5

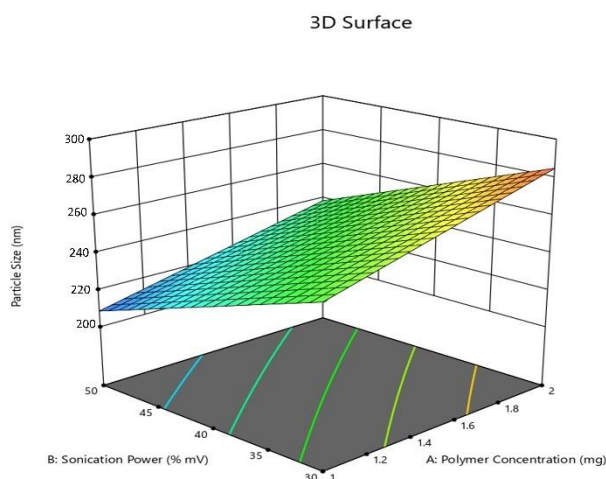


Figure 21b. 3D surface plot of Sonication power vs Polymer concentration affecting particle size of DTBNPs prepared using Emulsion solvent evaporation method.

Factor Coding: Actual

Particle Size (nm)
(adjusted for curvature)
Design Points
202 295

X1 = B
X2 = C

Actual Factor
A = 1.5

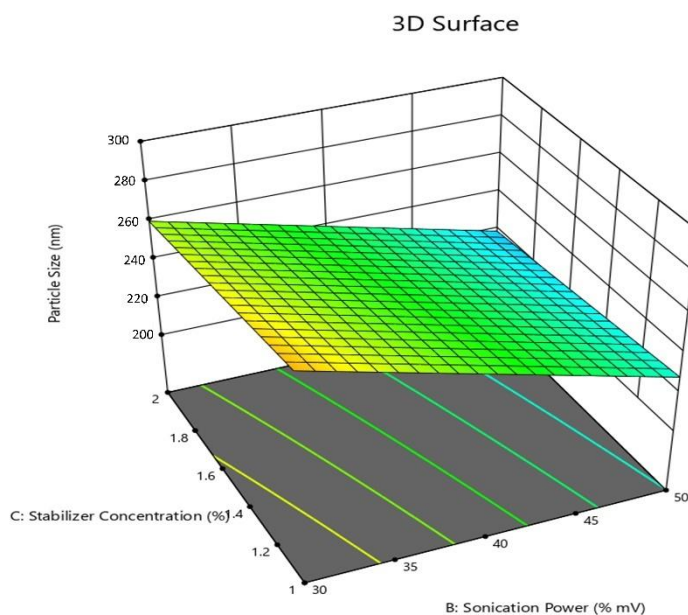


Figure 21c. 3D surface plot of Sonication power vs Stabilizer concentration affecting particle size of DTBNPs prepared using Emulsion solvent evaporation method.

The effect of sonication power was studied. It was discovered that sonication power and polymer concentration had a significant impact on the particle size. Here, sonication power is strongest factor and stabilizer concentration is lowest factor. Higher stress during the increased voltage could have led to nucleation and reduction in the particle size. At higher stress the droplet breakdown was higher which led to reduced particle size and reducing the voltage energy available for nucleation reduced which led to increase in particle size which were found similar to previous reports.⁵⁸ The effect of polymer concentration was studied. It was observed as the polymer concentration increase the particle size also increase. This phenomenon may be due to the increase in the viscosity of dispersed phase and thereby reducing the shear stress applied and thereby increase in particle size. The increase in the viscosity of dispersed phase gradually decreases the diffusion rate and increases the mixing time and final size of particles depends on net shear stress available for droplet breakdown thereby influencing the particle size as reported previously.⁵⁹ The effect of stabilizer was studied. It was noticed that as stabilizer concentration increased diameter of nanoparticles also increased. The use of stabilizers helped in reducing the particle size to spherical shape and preventing the aggregation of particles. For improving stabilization surfactant molecules cover the emulsion droplet. Hence at lowest concentration small size particles were achieved and increasing the concentration would have increased the viscosity leading to higher particle size which was earlier reported.⁶⁰

4.5.2 Influence of variables on zeta potential

Strong electrostatic attraction between the particles caused by surface charge on the particles could regulate the stability of the nanoparticulate formulation. The summarization of model parameters and p values are shown (Table 16). The zeta potential

was observed in the range of -18 mV to -24.7 mV. The zeta potential intensity of optimized formulation is shown (Figure 22). The 3D surface plot shown (Figure 23a,b,c).

The corresponding polynomial equation by the FFD on Zeta potential is as given below:

$$\text{Zeta Potential mV} = -20.32 + 0.3750A + 0.4000B - 0.9500AC - 1.32C + 87.50ABC$$

Table 16: Summary Zeta Potential ANOVA

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	30.55	7	4.36	25.18	0.0387	significant
A-Polymer Concentration	1.10	1	1.10	6.32	0.1285	
B-Sonication Power	0.3362	1	0.3362	1.94	0.2983	
C-Stabilizer Concentration	1.31	1	1.31	7.57	0.1106	
AB	7.30	1	7.30	42.09	0.0229	
AC	0.5202	1	0.5202	3.00	0.2253	
BC	13.94	1	13.94	80.42	0.0122	
ABC	6.06	1	6.06	34.93	0.0275	
Curvature	3.39	1	3.39	19.56	0.0475	
Pure Error	0.3467	2	0.1733			
Cor Total	34.29	10				

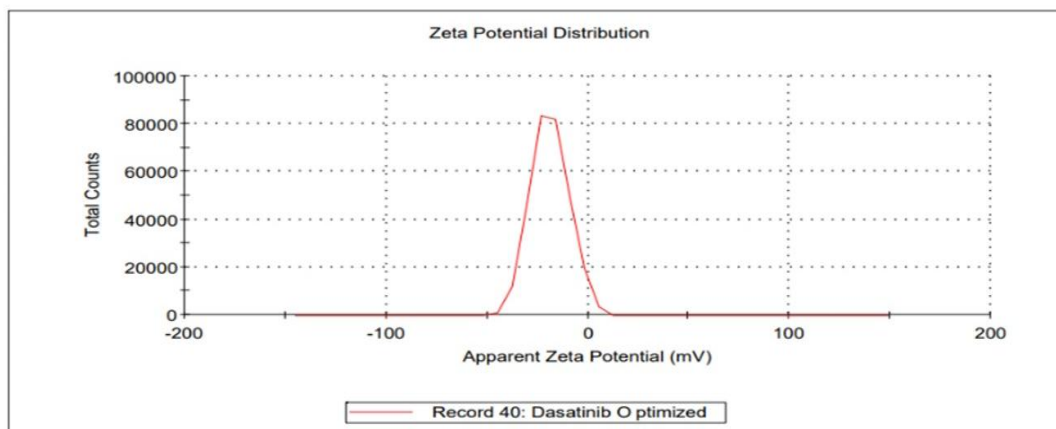


Figure 22: Zeta Potential of DTBNps 5.

Factor Coding: Actual

Zeta Potential (mV)
(adjusted for curvature)

Design Points
-24.7 -18

X1 = A
X2 = B

Actual Factor
C = 1.5

3D Surface

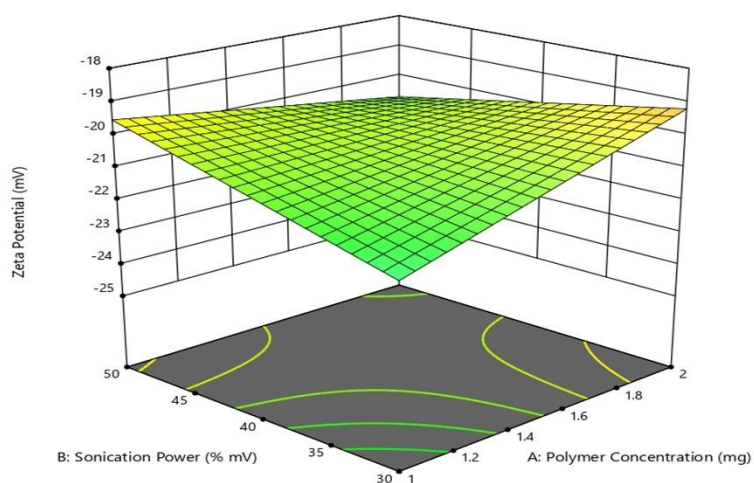


Figure 23a: 3D surface plot of Sonication power vs Polymer concentration affecting zeta potential of DTBNPs prepared using Emulsion solvent evaporation method.

Factor Coding: Actual

Zeta Potential (mV)
(adjusted for curvature)Design Points
-24.7 -18

X1 = B

X2 = C

Actual Factor

A = 1.5

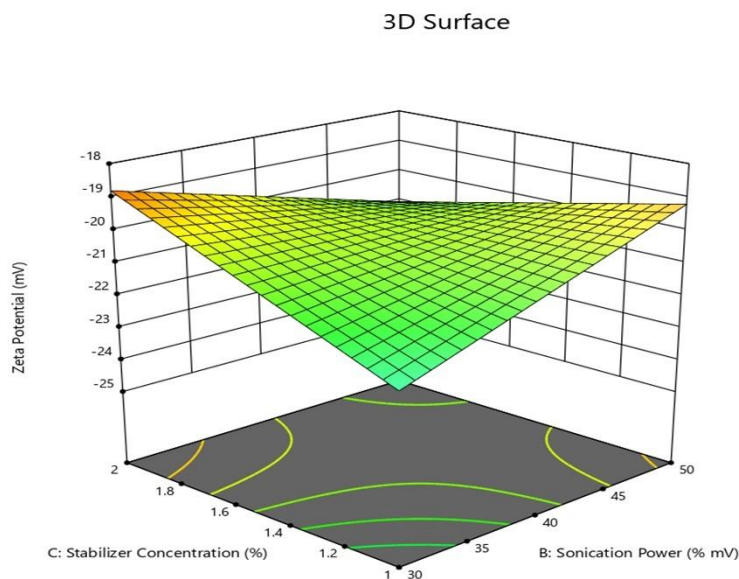


Figure 23b: 3D surface plot of Sonication power vs Stabilizer concentration affecting zeta potential of DTBNPs prepared using Emulsion solvent evaporation method.

Factor Coding: Actual

Zeta Potential (mV)
(adjusted for curvature)Design Points
-24.7 -18

X1 = A

X2 = C

Actual Factor

B = 40

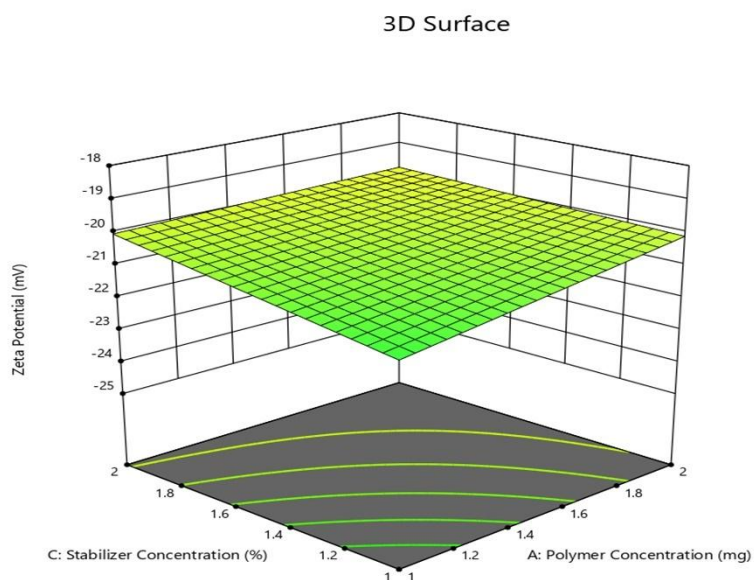


Figure 23c: 3D surface plot of Stabilizer concentration vs Polymer concentration affecting zeta potential of DTBNPs prepared using Emulsion solvent evaporation method.

The influence of sonication power on zeta potential was studied. The change in power do not account to change in electrostatic potential of nanoparticles. However any slight changes maybe observed due to the reduced size of particles which possess more surface area.⁶¹ The influence of polymer concentration was studied. As the Z.P increases the electrostatic repulsion between the particle increases. The Z.P was maximized when the amount of polymer was increased. This could be due to the terminal carboxylic acid group which may induce polar interactions with the dispersion medium. The equilibrium which is formed between the acid and the carboxylate generates the negative charges of the particles which were reported earlier.⁶² The increase in the stabilizer concentration the charge would decrease due to the neutral nature of stabilizer. The surfactant deposited on the surface of nanoparticles may hide the charge exposed by carboxylic groups. Since surfactant is in a more considerable proportion with the amount of the polymer.⁶³

4.5.3 Influence of variables on entrapment efficiency

The entrapment efficiency was in optimal range of 82-93 %. The 3D surface plot shown (Figure. 21a,b,c). The summarization of model parameters and p values are shown (Table 17). The corresponding polynomial equation by the FFD on Entrapment efficiency is as given below:

$$\text{Entrapment Efficiency (\%)} = +86.04 + 1.01A - 0.1375B + 2.31C + 0.4625A^2 + 0.5625AC - 0.6375BC - 0.8875ABC$$

Table 17: Summary of Entrapment Efficiency ANOVA

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	64.83	7	9.26	23.21	0.0419	significant
A-Polymer Concentration	8.16	1	8.16	20.45	0.0456	
B-Sonication Power	42.97	1	42.97	107.66	0.0092	
C-Stabilizer Concentration	0.1104	1	0.1104	0.2767	0.6514	
AB	2.62	1	2.62	6.57	0.1244	
AC	1.67	1	1.67	4.20	0.1771	
BC	3.18	1	3.18	7.96	0.1061	
ABC	6.12	1	6.12	15.35	0.0594	
Curvature	2.81	1	2.81	7.04	0.1175	
Pure Error	0.7982	2	0.3991			
Cor Total	68.44	10				

Factor Coding: Actual

Entrapment Efficiency (%)

(adjusted for curvature)

Design Points

82.32 93.11

X1 = A

X2 = B

Actual Factor

C = 1.5

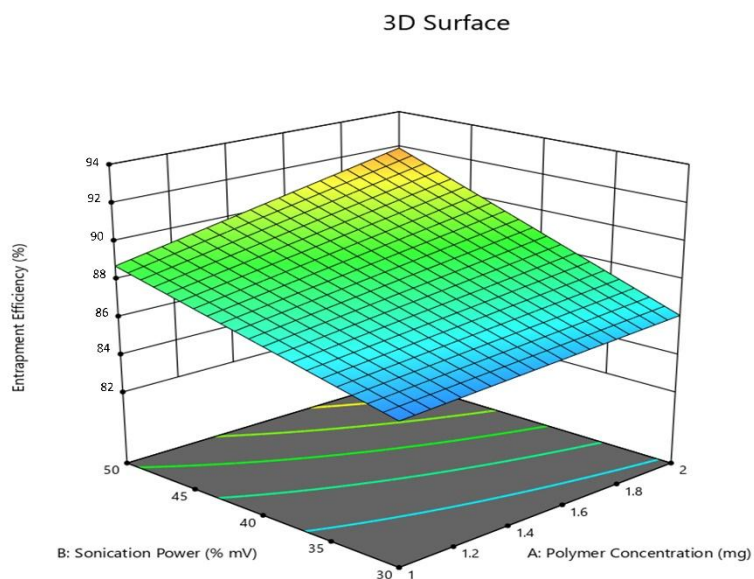


Figure 24a: 3D surface plot of a) Sonication power vs Polymer concentration affecting zeta potential prepared using Emulsion solvent evaporation method.

Factor Coding: Actual

Entrapment Efficiency (%)

(adjusted for curvature)

Design Points

82.32 93.11

X1 = B

X2 = C

Actual Factor

A = 1.5

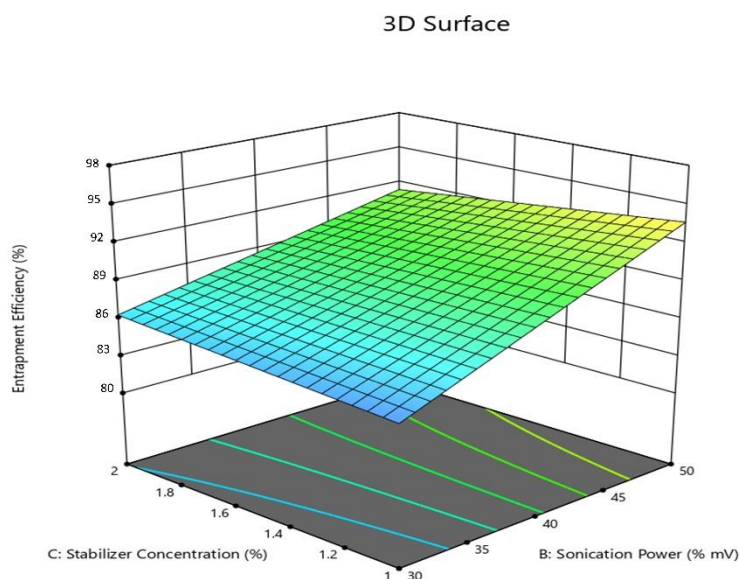


Figure 24b: 3D surface plot of Sonication power vs Stabilizer concentration affecting zeta potential prepared using Emulsion solvent evaporation method.

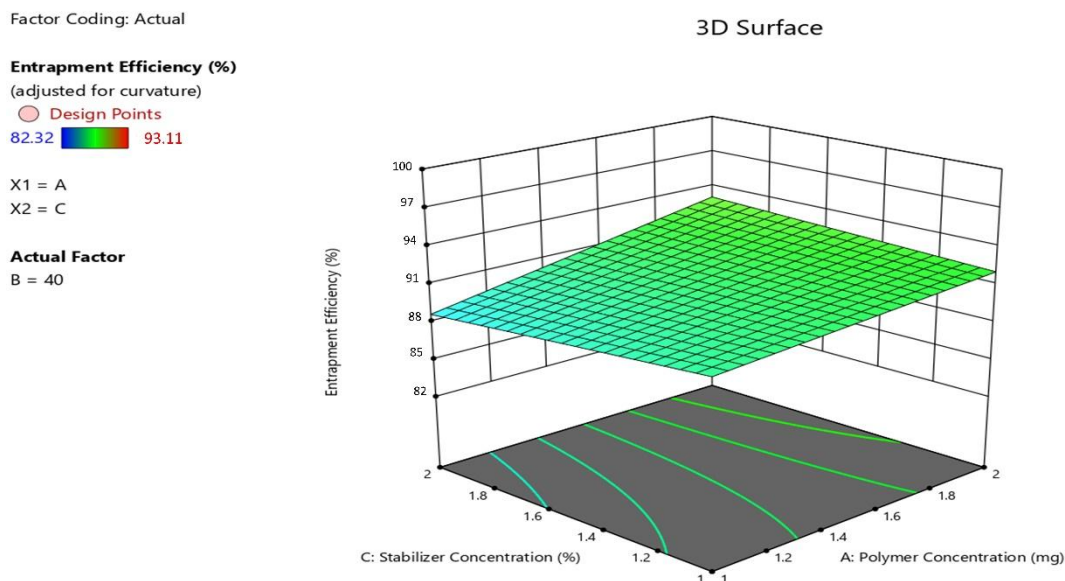


Figure 24c: 3D surface plot of Stabilizer concentration vs Polymer concentration affecting zeta potential of DTBNPs prepared using Emulsion solvent evaporation method.

The influence of sonication power was studied. It was observed that entrapment efficiency was not significantly affected by sonication power. Some effect maybe observed due to the change in particle size. The influence of polymer concentration on entrapment efficiency was studied. The consistency of organic phase increased with increasing concentration of polymer, resulting in more resistance to drug diffusion from organic to aqueous phase, which led to higher encapsulation efficiency. Additionally the increase in particle size also provide higher encapsulation efficiency. The drug diffusion out of nanoparticles at higher polymer concentration decreases since polymer precipitation time decrease thereby increasing entrapment efficiency.⁶⁴ Concentration of stabilizer had directly proportional effect on % EE. This may be due to increased viscosity of external medium as a result of thick layer of stabilizer and minimum diffusion of drug towards external aqueous medium. Correspondingly, higher

encapsulation efficiency was obtained with increasing the concentration of surfactant which might be due to stronger binding contacts between polymer and drug.⁶⁵

Table 18: Desirability chart for Full Factorial Design

Num ber	Polymer concentr ation (mg)	Sonica tion power (mV)	Stabilize r concentr ation (%)	Parti cle size (nm)	Zeta poten tial (mV)	Entrap ment Efficien cy (%)	Desira bility	
1	1.000	-1.000	-1.000	202. 000	- 18.87 5	92.750	0.550	Selec ted
2	1.000	-1.000	-1.000	202. 000	- 18.87 5	92.750	0.550	
3	1.000	-1.000	-0.993	202. 069	- 18.86 8	92.741	0.548	
4	1.000	-1.000	-0.981	202. 180	- 18.85 6	92.726	0.545	
5	0.992	-1.000	-1.000	202. 145	- 18.86 4	92.718	0.544	

6	1.000	-1.000	-0.973	202. 253	- 18.84 8	92.717	0.543	
7	1.000	-0.988	-1.000	202. 561	- 18.87 2	92.712	0.542	
8	1.000	-1.000	-0.965	202. 331	- 18.84 0	92.706	0.541	
9	1.000	-1.000	-0.950	202. 472	- 18.82 5	92.688	0.538	
10	1.000	-0.978	-0.999	203. 047	- 18.86 8	92.679	0.536	

Based on the desirability values generated by the software, the formulation **DTBNp5** was selected as the optimised formulation in the present study. The desirability value obtained for the optimized formulation was identical to that of the experimental formulations. This result confirms that the model predictions align precisely with the experimental outcomes for the selected responses and formulation, indicating excellent model reliability and predictive accuracy.

4.6 DTBNps Morphology

4.6.1 SEM

The microstructures of DTBNps 5 batch nanoparticles were subjected for SEM imaging. The morphology of the formulation was seen as spherical and homogenous shape having smooth surface as shown (Figure. 25).



Figure 25. SEM image of DST-NPs 5.

4.6.2 AFM

The 2D surface topographic AFM image of optimized formulation depicted a smooth surfaced nanoparticles without any agglomerations or perforations as shown (Fig. 26 a, b). This was mainly achieved because of uniform covering of polymer.

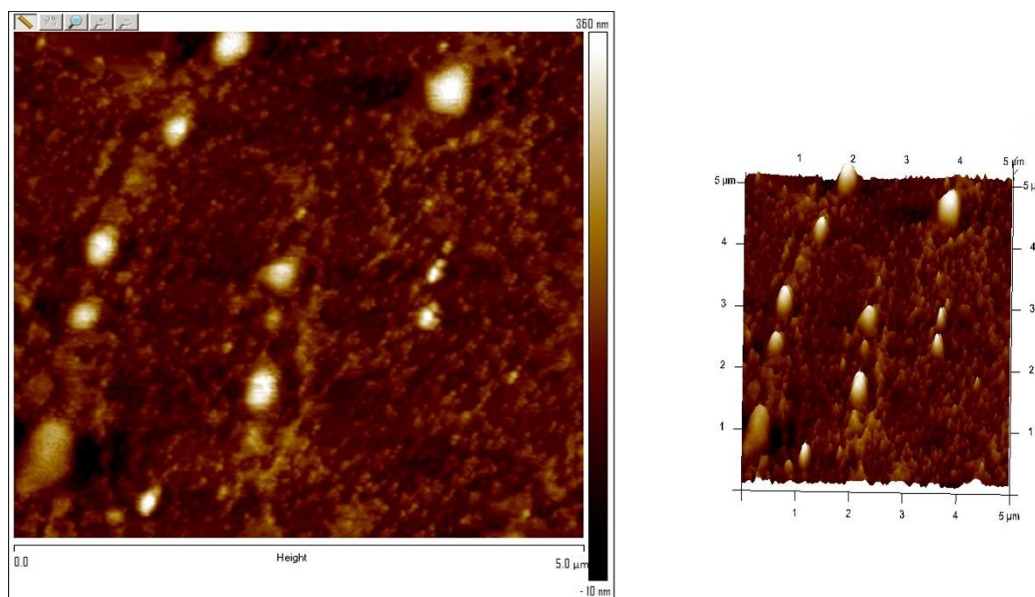


Figure 26: 2D & 3D AFM image of DTBNPs 5.

4.7 Solid State Characterization

4.7.1 FT-IR Spectroscopy

The FT-IR spectrum shown (Figure. 27), DTB showed characteristic peaks at 3258.14 cm^{-1} attribute the presence of carboxylic acid OH stretch. A sharp dip at 1414.53 cm^{-1} indicated the C=C alkene aromatic group. The sharp stretch at 1197.58 cm^{-1} indicates the C-O of the phenol group. The sharp peak at 773.31 cm^{-1} indicates the C-Cl group halo-compound. The Eudragit L 100 showed a broader peak at 1725.01 cm^{-1} indicate the C=O aliphatic ketone. A characteristic peak at 1154.01 cm^{-1} indicated the C-O-C anhydride group. A peak at 2951.52 cm^{-1} indicated the presence of -CH Alkane group which are present in the structures. The physical mixture of DTB and Eudragit L 100 indicated almost similar peaks 3291.89 cm^{-1} , 1460.81 cm^{-1} , 715.46 cm^{-1} , suggesting the

absence of any drug polymer interaction. Further the absence of major peaks of drug in DTBNps attribute the encapsulation of maximum drug in polymer.⁶⁶

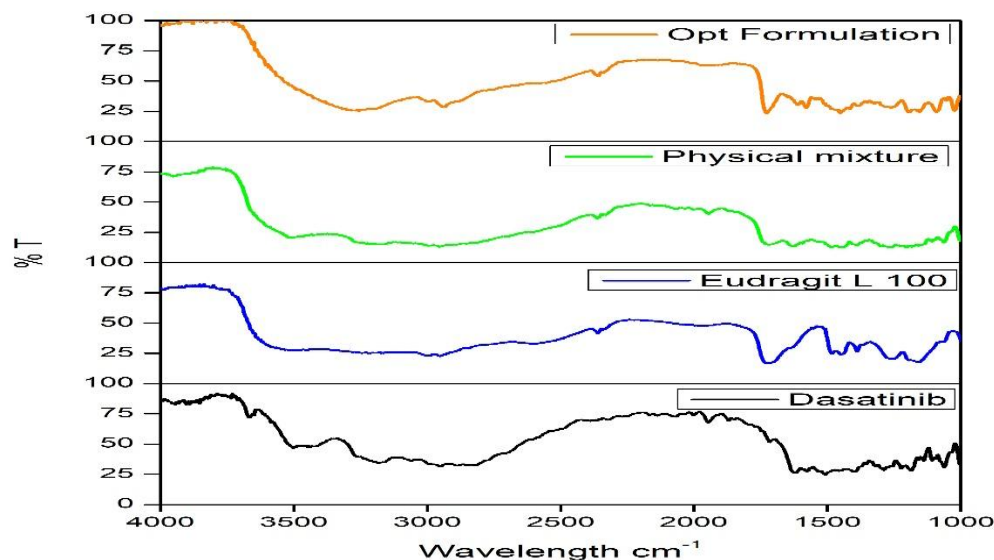


Figure 27: FT-IR overlay of DTB, Eudragit L 100, Physical mixture of DTB & Eudragit L 100 and DTBNps 5.

4.7.2 Thermal Analysis

The thermogram of DTB, Eudragit L 100, physical mixture, DTB loaded nanoparticles are demonstrated (Figure 28). The DSC curve for pure DTB exhibited single sharp endothermic melting peak at 283.49 °C, typical of a drug in the crystalline form.⁵⁶ Eudragit L 100 displayed single endothermic melting peak at 214.06 °C.⁶⁷ DTB melting peak was moved towards slightly lesser temperature (280.66 °C) in physical mixture. DTB melting peak was found to be absent in DST-NPs, indicate the absence of the crystalline nature of the drug.⁶⁸ It can be concluded that drug has successfully encapsulated in the polymer matrix.

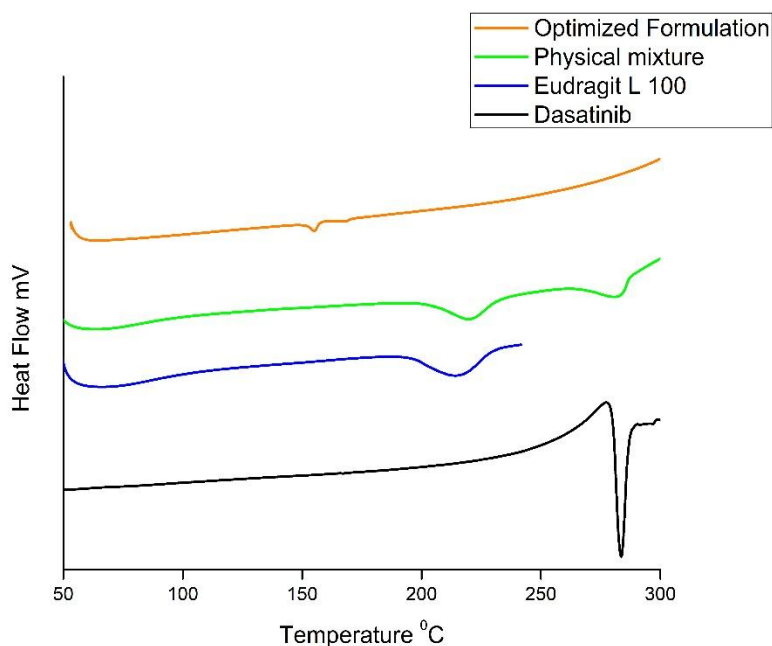


Figure 28: DSC overlay of DTB, Eudragit L 100, Physical mixture of DTB & Eudragit L 100 and DTBNps 5.

4.7.3 XRD Analysis

XRD overlay spectrum of optimized dasatinib loaded nanoparticles along with physical mixture and pure DTB is shown (Figure.29). DTB exhibited the crystalline nature with relatively intense peaks at 2θ angles of 11.3° , 12.2° , 13.1° , 16.6° , 24.4° , 25.31° , 27.82° .⁶⁹ Eudragit L 100 and Physical mixture diffraction patterns displayed several smaller diffraction peaks. DTBNpsspectrum abridged their intensities and revealed broad diffraction peaks indicating the conversion of crystalline nature of DTB into amorphous nature.⁷⁰

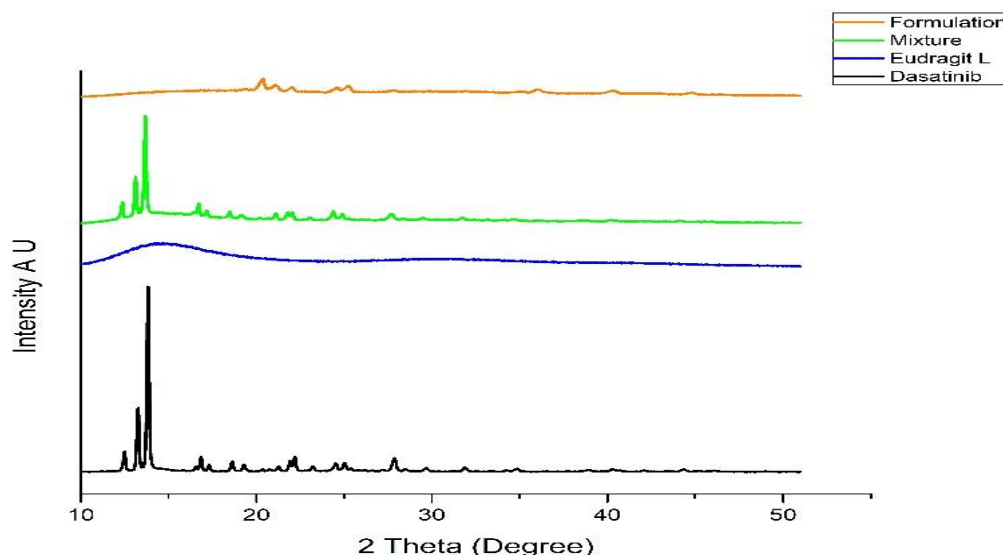


Figure 29: XRD overlay of DTB, Eudragit L 100, Physical mixture of DTB & Eudragit L 100 and DTBNps 5.

4.8 *In-vitro* dissolution Studies and release kinetics

The primary investigation of the present study was to examine how Eudragit L 100 affected the drug release profile from nanoparticles (NPs). In (Figure. 30) the lower release profile of pure DTB is primarily due to its poor solubility and the absence of a controlled release system. Without the benefit of a nanoparticulate carrier, pure DTB releases slowly, as it lacks mechanisms like enhanced surface area or controlled diffusion that nanoparticles offer. In contrast, the DTBNps show significantly higher drug release profiles. This is due to the nanoparticles improving the drug's solubility and providing a larger surface area, which accelerates drug release into the medium. The nanoparticle matrix also facilitates controlled and sustained release of DTB.

The primary investigation of present study was to examine how Eudragit L 100 affected. Among the formulations, DTBNps 5 exhibits the highest drug release profile. This can be attributed to the optimized formulation of DTBNp5, which may have smaller particle sizes, increasing the surface area for drug diffusion. Additionally, DTBNps 5 polymer

composition (Eudragit) likely allows for more efficient encapsulation and gradual degradation, promoting sustained drug release. These combined factors make DTBNps 5 the most efficient in releasing the drug over time, highlighting its potential as the best formulation for controlled drug delivery. The drug was released in accordance with the Kosmeyer-Peppas release format as shown in table 19, with r^2 value of 0.9881 and $n = 0.405$. Fickian diffusion-based n value.⁷¹⁻⁷²

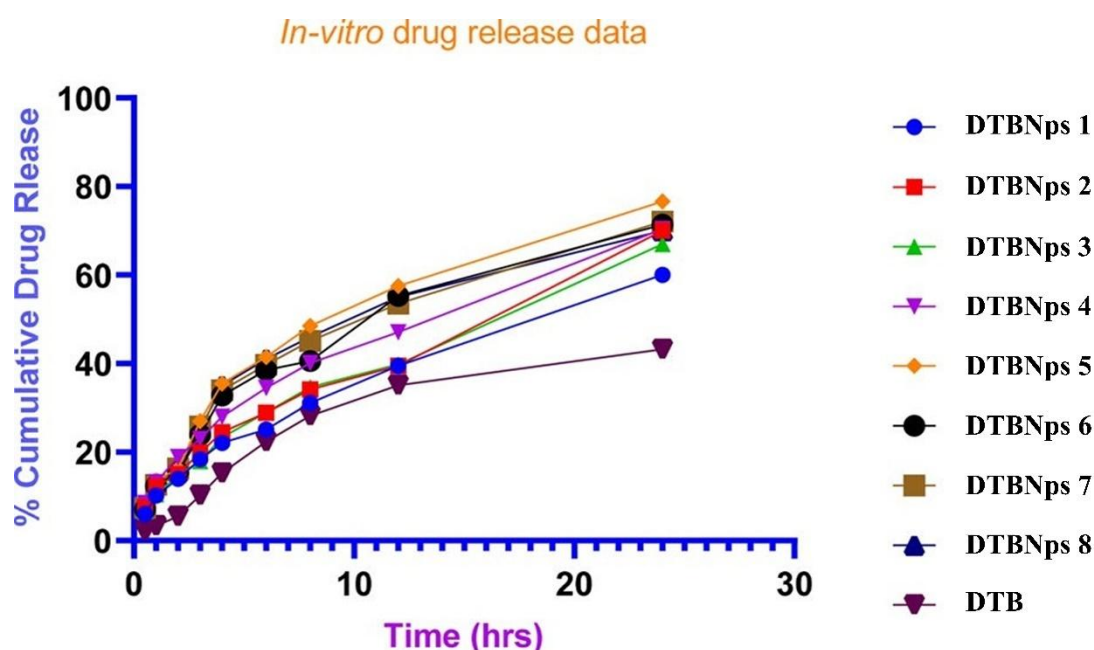


Figure 30: *In vitro* cumulative drug release of DTB-NP1 to DTB-NP8 compared to DTB.

Table 19: Release Kinetics of DTBNps 5.

Release order	R ² value
---------------	----------------------

Zero Order	0.3288
First Order	0.6475
Higuchi	0.9604
Kosmeyer Peppas	0.9881 (n = 0.405)
Hixon Crowell	0.5562

4.9 Pharmacokinetics and Biodistribution of DTBNPs

The pharmacokinetic profiles of DTB and DTBNPs were characterized following oral administration in two groups of rats, dosed with DTB and DTBNPs at 10 mg/kg each, respectively. Blood samples were collected at predetermined time points at 0, 0.5, 1, 2, 3, 8, 12, and 24 hrs post-dosing, and DTB concentrations were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

DTB exhibited rapid absorption in both groups (Figure 31). The maximum plasma concentration ($C_{\max}$) was observed to be 8.50 ng/mL at 4 hours ($T_{\max}$) in Group 1 (pure DTB) and 12.60 ng/mL at 2 hours ($T_{\max}$) in Group 2 (DST-NPs), indicating a faster absorption rate in DST-NPs. The area under the plasma concentration-time curve (AUC) from time zero to the last measurable concentration (AUC_{0-t}) was 66.06 ng/mLh for pure DTB and 71.23 ng/mLh for DST-NPs, reflecting a higher exposure in DTBNPs (Table 20). The elimination half-life ($t_{1/2}$) was slightly shorter in DTBNPs (3.15 hours) compared to pure DTB (3.97 hours). The mean residence time (MRT) was also lower in DTBNPs (5.23 hours) compared to pure DTB (7.26 hours), consistent with the faster kinetics observed. The total body clearance (Cl/F) and volume of distribution (V_z/F) were calculated, with DTBNPs demonstrating more efficient distribution and elimination properties (Table 20).

Tissue distribution studies were conducted to determine the concentration of DTB in the liver, brain, heart, kidney, and spleen at 24 hours post-administration (Table 21). In pure DTB, the highest concentrations were found in the spleen (6.59 ng/10mg tissue) and kidney (5.06 ng/10mg tissue), with no detectable levels in the brain. In DST-NPs, similar patterns were observed, with slightly lower concentrations in the spleen (5.83 ng/10mg tissue) and kidney (2.87 ng/10mg tissue).

Table 20: Pharmacokinetics parameters of DTB vs DTBNps 5

Parameter	DTB	DTBNPs 5
$C_{\max}$ (ng/ml)	8.50 ± 0.85	$12.60 \pm 1.26^{***}$
$T_{\max}$ (hr)	4.00 ± 0.40	$2.00 \pm 0.20^{***}$
$t_{1/2}$ (hr)	3.97 ± 0.40	$3.15 \pm 0.32^{**}$
AUC_{0-24h} (ng./ml/hr)	66.06 ± 6.61	$71.23 \pm 7.12^*$

All results are means of measurements from six rats. Values are expressed as mean $\pm$ SD.

p values apply to differences between DTB and DTBNPs. $***p < 0.001$, $**p < 0.01$, $*p < 0.05$ when compared to DTB.

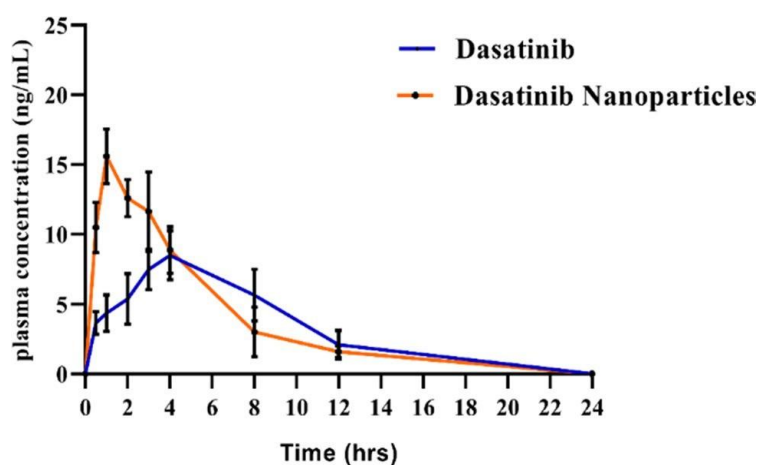


Figure 31: Compartmental distribution of oral solution and formulation (DTBNPs 5) at different time intervals in blood plasma concentration versus time in albino Wistar rats.

Table 21: Biodistribution of DTB and DTBNPs in different tissues.

Group	Tissue	Drug Concentration ng/ml
		Mean $\pm$ SD
DTB	Liver	2.31 $\pm$ 0.45
	Brain	0
	Heart	0
	Kidney	5.06 $\pm$ 1.2
	Spleen	6.59 $\pm$ 4.82
DTBNPs	Liver	2.03 $\pm$ 0.28*
	Brain	0
	Heart	3.97 $\pm$ 0.75***
	Kidney	2.87 $\pm$ 1.79*
	Spleen	5.83 $\pm$ 2.03*

All results are means of measurements from six rats. Values are expressed as mean $\pm$ SD.

p values apply to differences between DTB and DTBNPs. ****p* < 0.001, **p* < 0.05 when compared to DTB.

4.10 Pharmacodynamics

4.10.1 Measurement of Tumor Volume, Body Weight, and Survival Rate

The body weight, fraction survival, and tumor volume were measured once treatment started for 4 weeks. The body weight changes of the animals in different groups were monitored to indicate overall health and treatment-related toxicity. In the DMBA-treated

group, there was a significant decrease in body weight, dropping from 170 ± 15 g at the beginning of the treatment to 140 ± 15 g by week 4, reflecting the severe impact of tumor burden and lack of treatment efficacy. In contrast, the groups treated with DTBNps and pure DST exhibited more stable body weights, ending at 165 ± 10 g and 150 ± 12 g, respectively, by week 4. The stability in body weight for DTBNps further supports the therapeutic advantage of the nanoparticle formulation, mitigating the adverse effects commonly associated with cancer progression and treatment (Figure. 32b). The survival fraction of the animals was calculated to evaluate the potential life-extending benefits of the treatments. In the DMBA-treated group, the survival rate dropped sharply, with only 45% of the animals surviving by week 4. This high mortality rate underscores the lethality of untreated tumor growth. DTBNps and DST exhibited improved survival rates, with DTBNps showing a 75% survival rate and DST showing a 60% survival rate by the end of the study. The higher survival rate in DTBNps not only slow tumor progression but also contribute to overall better health outcomes compared to pure DST treatment. The tumor volumes of the animals in different groups were measured to assess the effectiveness of the various treatments (Figure. 32c). In the DMBA-treated group, tumor growth was rapid, with a significant increase observed from week 1 (173 ± 15 mm³) to week 4 (764 ± 120 mm³). This indicates the aggressive nature of tumor progression in untreated conditions. In contrast, the groups treated with DTBNps and pure DTB exhibited markedly slower tumor growth rates. By week 4, the tumor volumes for DTBNps and DTB were 350 ± 85 mm³ and 570 ± 100 mm³, respectively (Figure. 32a). The reduced tumor volume in DTBNps suggests that the nanoparticle formulation significantly enhances the therapeutic efficacy of DTB, likely due to improved drug delivery and sustained release properties.⁷² The enhanced performance of the nanoparticle formulation can be attributed to several factors: improved bioavailability,

sustained release, and targeted delivery.⁷³ Nanoparticles enhanced the bioavailability of DTB, allowing for more efficient drug delivery to tumor sites.

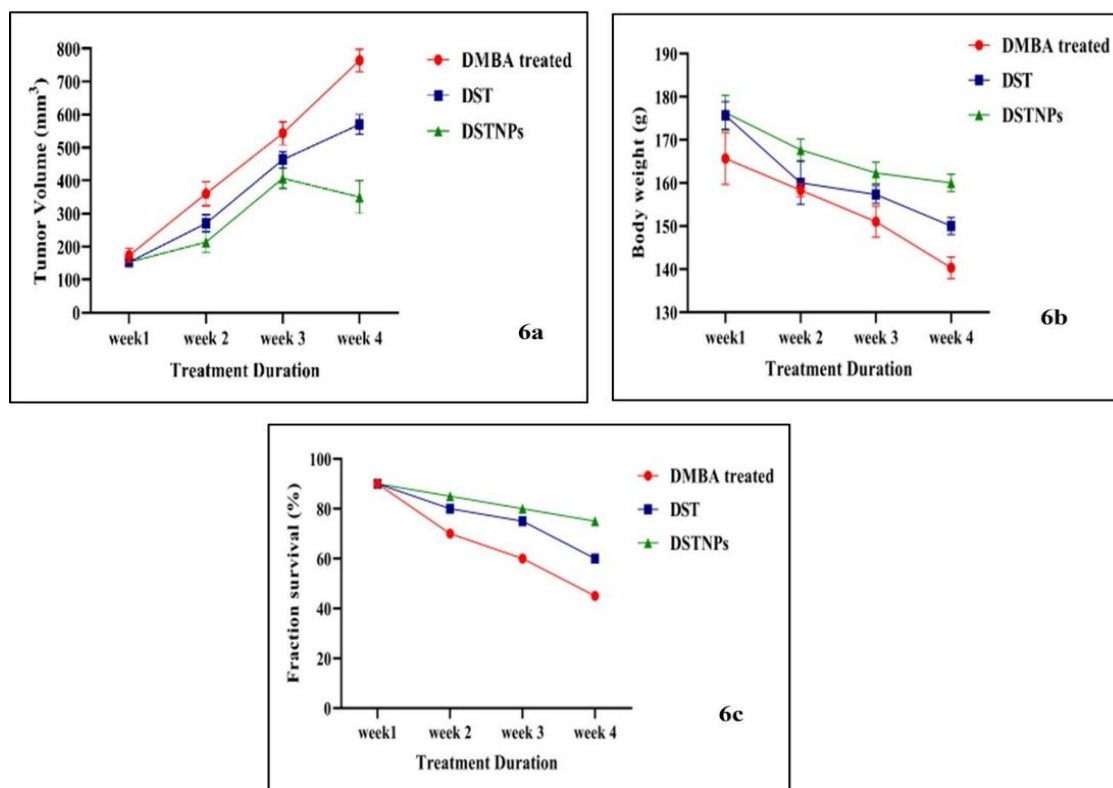


Figure 32: Tumor volume progression in different treatment groups, b: Body weight changes in different treatment groups, and c: Survival rates in different treatment groups.

4.10.2 Hematologic examination

The hematologic parameters were evaluated to assess the impact of the various treatments on the blood profiles of the experimental rats. The Complete Blood Count (CBC) analyses included measurements of hemoglobin (Hgb) levels, red blood cell (RBC) count, white blood cell (WBC) count, and differential counts shown in Table 22. The normal control group exhibited an average hemoglobin level of 15.2 g/dL, while the DMBA-treated group showed a significant reduction in hemoglobin levels, averaging 11.9 g/dL. Rats treated with DTB nanoparticles had near-normal hemoglobin levels at

14.7 g/dL, and those treated with pure DTB showed hemoglobin levels of 13.4 g/dL, indicating partial restoration. Hematocrit levels were highest in the normal control group at 42%, with the DMBA-treated group having the lowest at 30%. The DTBNps treated group had a hematocrit level of 40%, close to normal, while the DTB-treated group had a level of 38%. White blood cell counts followed a similar pattern: the normal control group had 8.4×10^9 cells/L, the DMBA-treated group had 5.8×10^9 cells/L, the DTBNps treated group had 7.9×10^9 cells/L, and the DTB-treated group had 6.3×10^9 cells/L. The red blood cell counts were 6.52×10^{12} cells/L in the normal control group, 8.31×10^{12} cells/L in the DMBA-treated group, 6.93×10^{12} cells/L in the DTBNps treated group, and 7.91×10^{12} cells/L in the DTB-treated group. Differential counts revealed that the normal control group had 28% segmenters, 65% lymphocytes, 5% monocytes, and 2% eosinophils, with a platelet count of 6.07×10^{13} cells/L. The DMBA-treated group had 19% segmenters, 73% lymphocytes, 2% monocytes, and 2% eosinophils, with a platelet count of 4.94×10^{13} cells/L. The DTBNps treated group had 26% segmenters, 70% lymphocytes, 5% monocytes, and 2% eosinophils, with a platelet count of 5.91×10^{13} cells/L, and the DTB-treated group had 24% segmenters, 66% lymphocytes, 4% monocytes, and 2% eosinophils, with a platelet count of 5.01×10^{13} cells/L. These results indicate that treatment with DTBNps significantly ameliorated the hematologic alterations induced by DMBA, approaching the hematologic profile of the normal control group.

Table 22: Hematologic examination

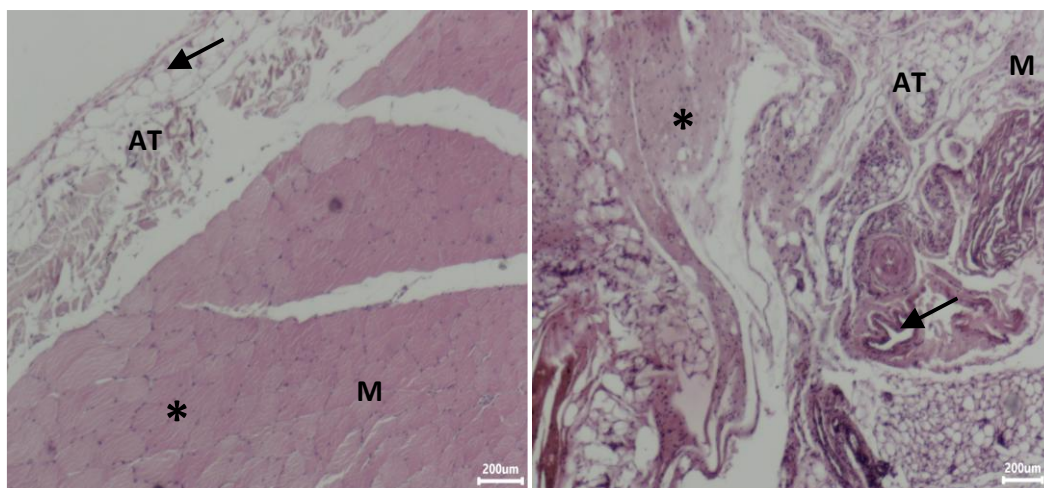
Blood component	Normal control	DMBA treated	DTBNPs	DTB

Hemoglobin (g/dL)	15.2 ± 0.8	11.9 ± 0.7#	14.7 ± 0.6*	13.4 ± 0.5
Hematocrit (%)	42 ± 2.1	30 ± 1.8##	40 ± 2.3*	38 ± 2.0
WBC (cells/L)	$8.4 \times 10^9 \pm 0.5$ $\times 10^9$	$5.8 \times 10^9 \pm 0.4$ $\times 10^9##$	$7.9 \times 10^9 \pm 0.6$ $\times 10^9*$	$6.3 \times 10^9 \pm 0.5$ $\times 10^9$
RBC (cells/L)	$6.52 \times 10^{12} \pm$ 0.3×10^{12}	$8.31 \times 10^{12} \pm$ 0.4×10^{12}	$6.93 \times 10^{12} \pm$ 03×10^{12}	$7.91 \times 10^{12} \pm$ 0.3×10^{12}
Differential count (%)				
Segmenters	28 ± 2	19 ± 1#	26 ± 2*	24 ± 2
Lymphocytes	65 ± 3	73 ± 4##	70 ± 3	66 ± 3
Monocytes	5 ± 1	2 ± 0.5#	5 ± 1*	4 ± 0.5
Eosinophils	2 ± 0.2	2 ± 0.2	2 ± 0.2	2 ± 0.2
Platelet count (cells/L)	$6.07 \times 10^{13} \pm$ 0.3×10^{13}	$4.94 \times 10^{13} \pm$ $0.2 \times 10^{13}#$	$5.91 \times 10^{13} \pm$ $0.3 \times 10^{13}*$	$5.01 \times 10^{13} \pm$ 0.2×10^{13}

Data are presented as mean ± SD and analyzed using a t-test; a p-value < 0.05 was considered statistically significant. Symbols indicate significant differences: # for DMBA treated vs. normal control, and * for DTBNPs vs. DMBA treated.

4.10.3 Histopathology examination

Histopathological examination of the mammary gland in the control group showed normal architecture predominantly composed of adipose tissue (AT) without staining, with blue-staining oval nuclei placed peripherally resembling signet ring cells (arrow). The smooth muscle layer (M) exhibited pink-staining cytoplasm and oval-shaped blue-staining nuclei (asterisk) (Figure. 33A). In contrast, the disease group revealed significant architectural disruption, characterized by the infiltration of microinvasive cancer cells (asterisk) and papillary tumor cells with projections (arrow), along with squamous epithelial cell transformation and the presence of cell nests (Figure. 33B). Treatment with DTBNps restored the mammary gland architecture to a state similar to the control group, maintaining the normal appearance of adipose tissue and smooth muscle layers (Figure. 33C). The standard group treated with DTB also showed a normal architecture, with the addition of stratified squamous epithelial cells with keratin layers in the surrounding skin, and spindle-shaped fibrous connective tissues in the dermis layer (Figure. 33D).



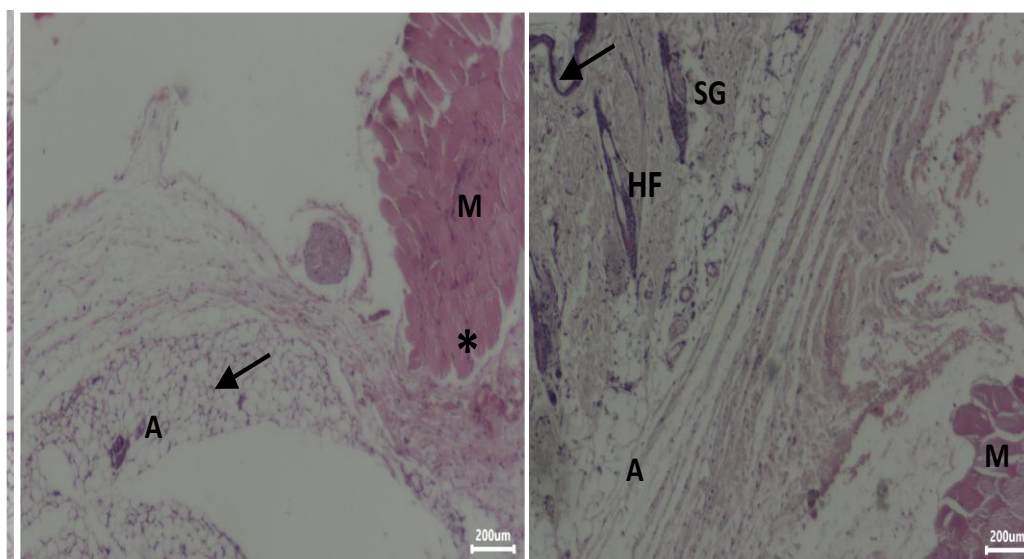
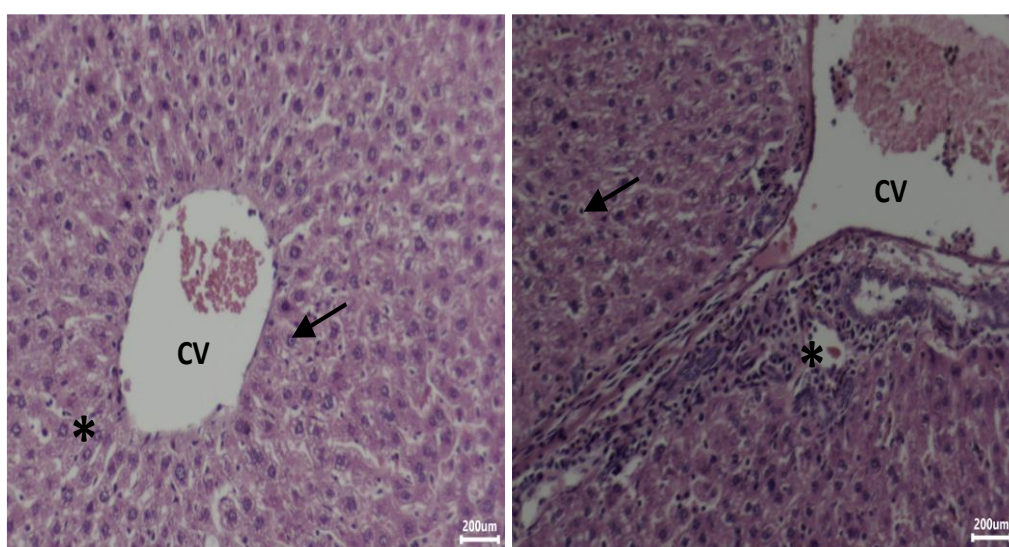


Figure 33: Histopathological examinations of breast sections stained with H&E stain: (a) Normal Control, (b) DMBA treated (c) DTBNps administered and (d) DTB administered.

The liver tissue in the control group displayed a typical arrangement of hepatocytes with pink-staining cytoplasm and vacuolated blue-staining nuclei (arrow), arranged hexagonally around the central vein (CV) containing red blood cells, with Kupffer cells seen in the sinusoidal spaces (asterisk) (Figure. 34A). The disease group exhibited hepatocyte vacuolar degeneration, nuclear condensation, and pyknosis (arrow), along with infiltration by tumor cells of varying sizes and shapes (asterisk). The normal hexagonal arrangement of hepatocytes around the central vein was disrupted by the presence of mononuclear cells (Figure. 34B). Treatment with DTBNps resulted in the restoration of the normal hepatocyte architecture, resembling the control group with well-organized hexagonal hepatocytes around the central vein and Kupffer cells in the sinusoidal spaces (Figure. 34C). The standard DTB treatment group also showed normal hepatocyte arrangement, with minimal cell infiltration around the central vein (Figure 34D).



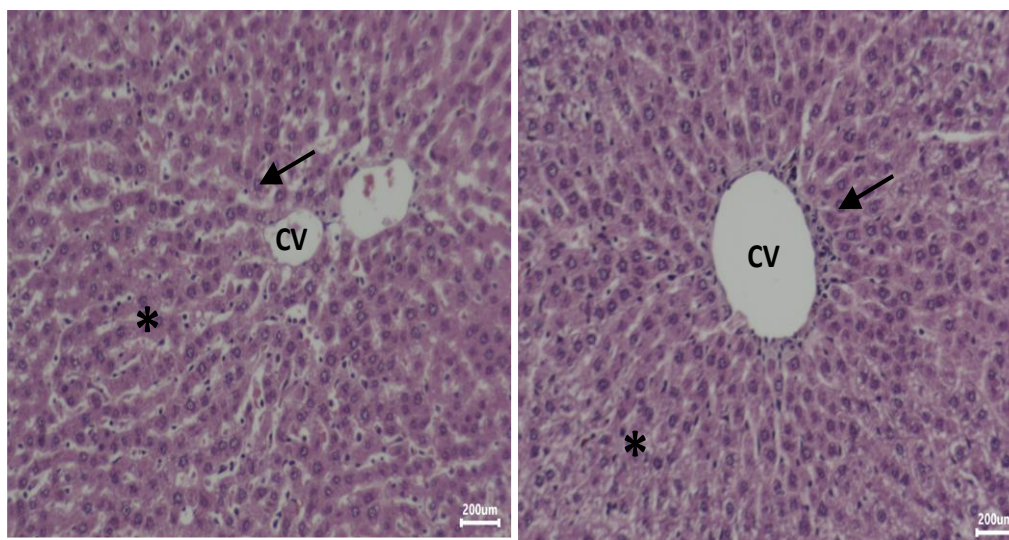


Figure 34: Histopathological examinations of liver sections stained with H&E stain: (a) Normal Control, (b) DMBA treated (c) DTBNps administered and (d) DTB administered.

The intestinal tissue in the control group showed normal villi architecture lined with columnar epithelial cells containing blue-staining oval nuclei, with numerous goblet cells (arrow). The lamina propria (LP) contained glands (asterisk) and mononuclear cells, and the smooth muscle layer (M) exhibited pink-staining cytoplasm with oval-shaped blue-staining nuclei (Figure. 35A). The disease group demonstrated a loss of villi architecture, with fusion of villi, loss of columnar epithelial cells, and hyperplasia of goblet cells (arrow). The lamina propria was infiltrated by mononuclear and tumor cells along with glands (asterisk) (Figure. 35B). Treatment with DTBNps restored the normal architecture of the intestine, resembling the control group with well-structured villi and intact columnar epithelial cells and goblet cells (Figure. 35C). The standard DTB treatment group, however, exhibited some fusion of villi (arrow) but maintained a similar overall structure to the control group with intact glands and mononuclear cells in the lamina propria (Figure. 35D). These findings suggest that treatment with DTBNps effectively

restores the normal architecture of mammary gland, liver, and intestinal tissues in a manner comparable to standard DTB treatment, demonstrating potential therapeutic efficacy in the management of DMBA-induced breast cancer and associated tissue changes.

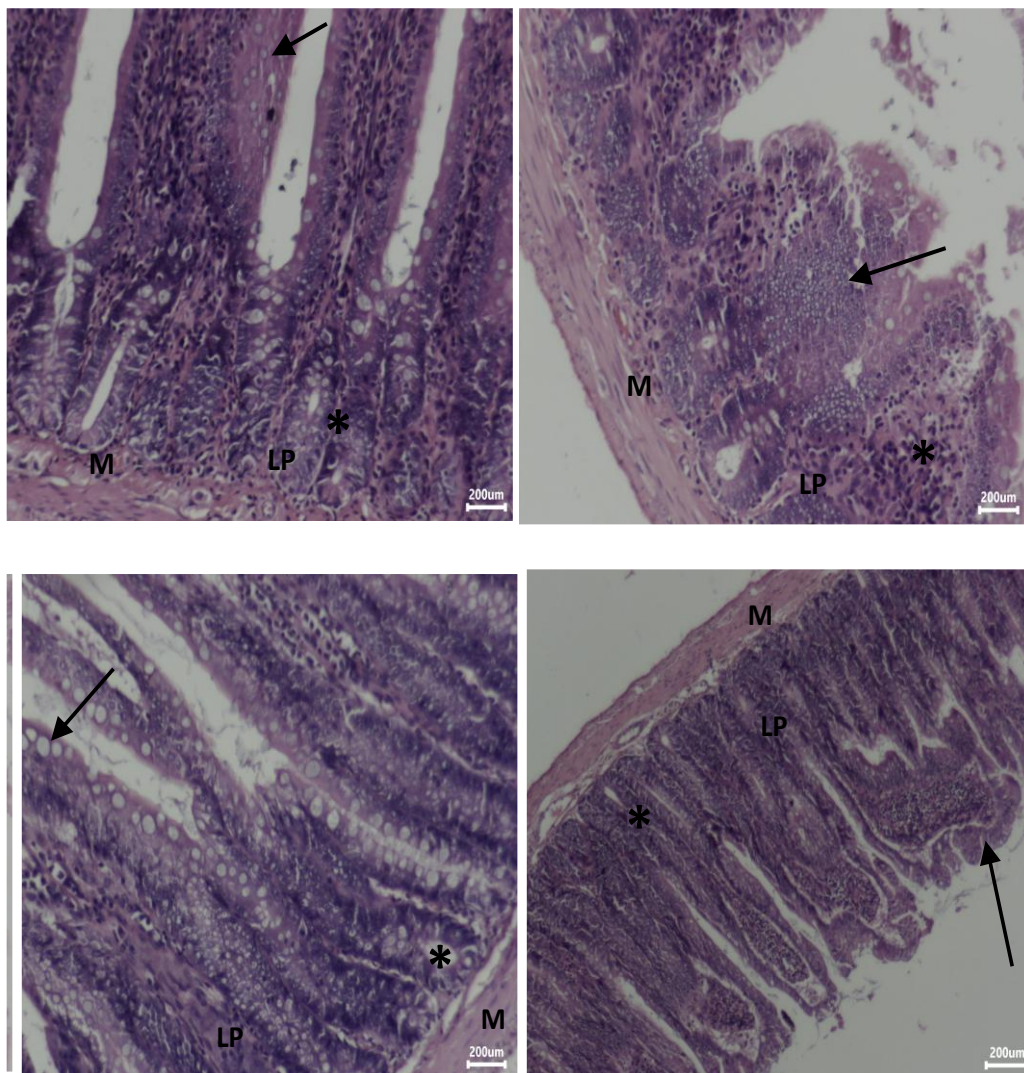


Figure 35: Histopathological examinations of small intestine sections stained with H&E stain: (a) Normal Control, (b) DMBA treated (c) DTBNps administered and (d) DTB administered.

4.11 MTT Assay

The cytotoxic effects of DTB and DTBNps were assessed by calculating the percentage inhibition of cell viability and determining the IC₅₀ values across MCF-7, MDA-MB231, and 4T1 cell lines. In MDA-MB231 cells, DTB showed an IC₅₀ value of 80.74 μ M, whereas DSTNps exhibited a more potent anticancer activity with an IC₅₀ value of 43.32 μ M. The standard anticancer drug, Doxorubicin, had a significantly lower IC₅₀ value of 9.3 μ M, indicating higher efficacy. Similarly, in MCF-7 cells, DTB demonstrated an IC₅₀ value of 51.45 μ M, and DST-NP showed an IC₅₀ value of 34.87 μ M. Doxorubicin again showed a lower IC₅₀ value of 18.05 μ M, highlighting its stronger anticancer activity. In 4T1 cells, DTB showed an IC₅₀ value of 75.20 μ M, while DTBNps exhibited an IC₅₀ value of 40.45 μ M. Doxorubicin had an IC₅₀ value of 12.00 μ M in 4T1 cells (Figure 36).

The cell viability data, representing the percentage of viable cells after treatment, further supports the IC₅₀ findings. In MDA-MB231 cells treated with 3.125 μ M of DTB, the cell viability was approximately 94.08%, and for 100 μ M, it decreased to 39.73%. DTBNps showed similar trends with 91.76% viability at 3.125 μ M and 29.71% at 100 μ M. Doxorubicin-treated MDA-MB231 cells had a viability of 86.08% at 3.125 μ M, decreasing to 9.51% at 100 μ M (Figure 37). For MCF-7 cells, DTB treated cells exhibited 93.62% viability at 3.125 μ M, reducing to 34.16% at 100 μ M. DTBNps treated cells had 90.35% viability at 3.125 μ M and 27.42% at 100 μ M. Doxorubicin-treated MCF-7 cells showed 91.05% viability at 3.125 μ M and 24.52% at 100 μ M (Figure 38). In 4T1 cells, DTB treated cells exhibited 92.00% viability at 3.125 μ M, decreasing to 42.50% at 100 μ M. DTBNps treated cells showed 89.50% viability at 3.125 μ M and 32.80% at 100 μ M. Doxorubicin-treated 4T1 cells had 85.00% viability at 3.125 μ M, decreasing to 10.00% at 100 μ M (Figure 39). These results indicate that both DTB and DTBNps possess anticancer properties, with DTBNps showing greater potency across all three cell lines

when compared to DTB. Doxorubicin, as expected, demonstrated the highest cytotoxicity among the tested compounds. The decreasing cell viability percentages with increasing concentrations of the test compounds confirm their cytotoxic effects, correlating well with the IC₅₀ values obtained. Previous studies have demonstrated the enhanced efficacy of nanoparticle-based drug delivery systems in cancer treatment. A study reported that paclitaxel-loaded nanoparticles showed significantly lower IC₅₀ values and higher cell viability reduction in breast cancer cell lines when compared to free paclitaxel.⁷³ Similarly, a study found that curcumin-loaded nanoparticles exhibited better cytotoxic effects in lung cancer cells than free curcumin⁷⁴ These findings are consistent with our results, where DTBNps showed better cytotoxicity than free DTB. The improved efficacy of DTBNps can be attributed to several factors. Nanoparticles can enhance drug solubility, stability, and bioavailability, leading to better cellular uptake and retention. Additionally, nanoparticles can facilitate targeted drug delivery, reducing off-target effects and increasing drug concentration at the tumor site.⁷⁵

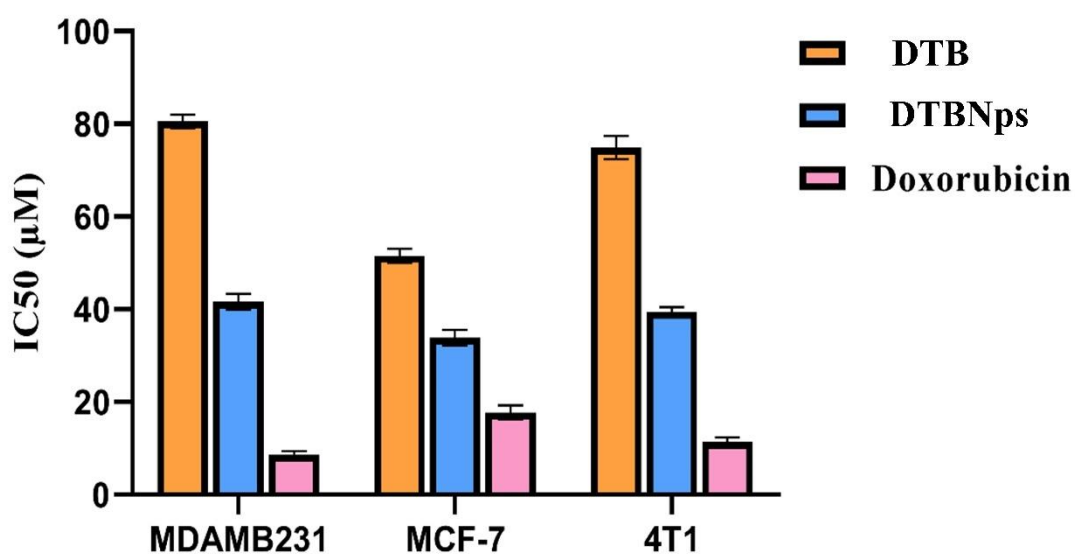


Figure 36: IC₅₀ value of DTB, DTBNps, and Doxorubicin in three different cell lines.

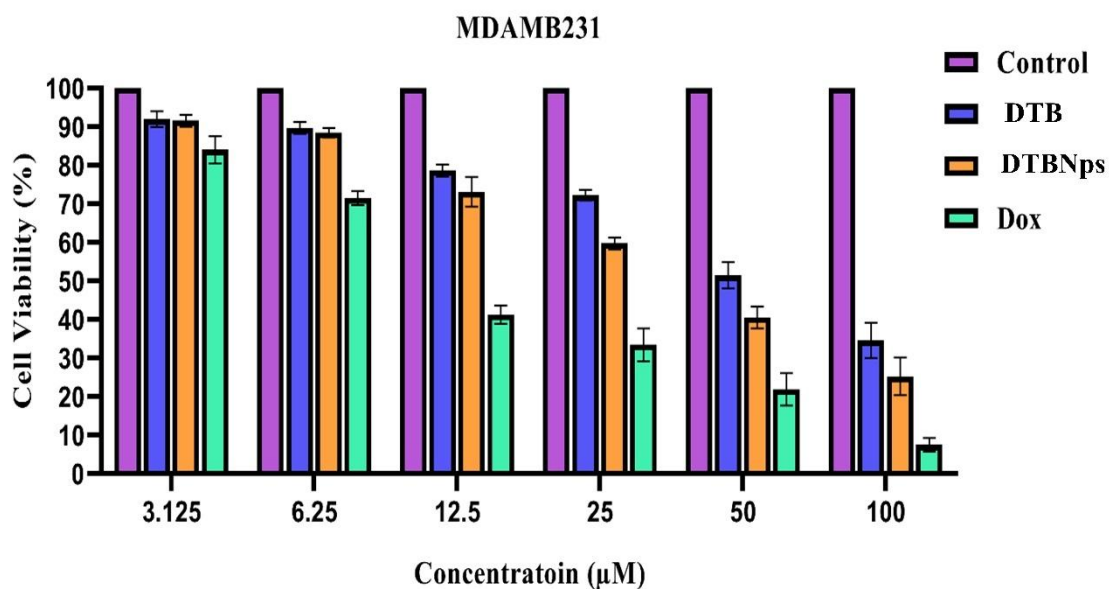


Figure 37: Cell viability of DTB, DTBNps, and Doxorubicin in MDAMB231 cell line.

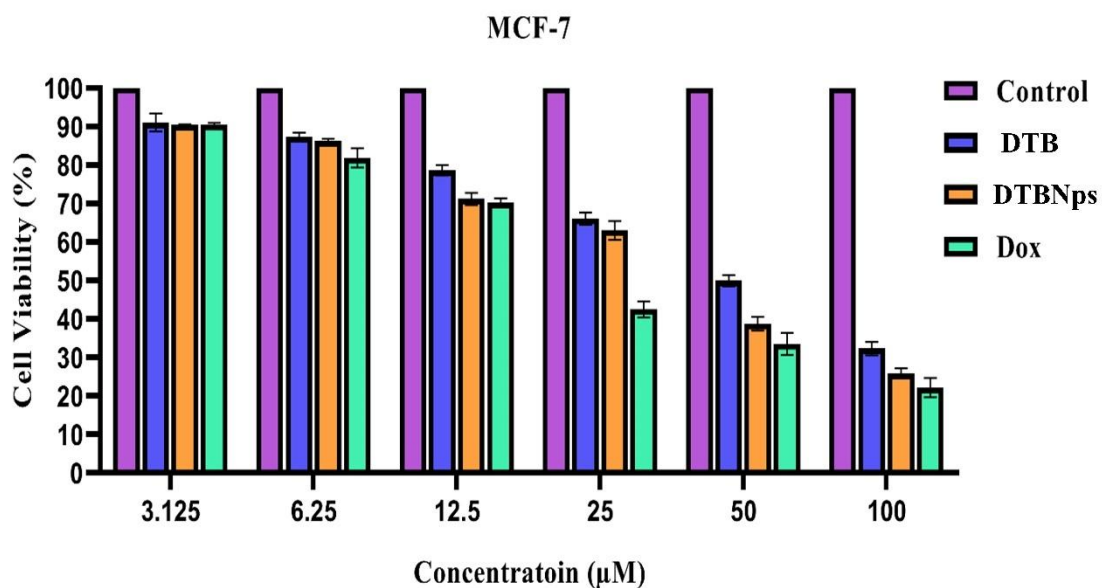


Figure 38: Cell viability of DTB, DTBNps, and Doxorubicin in MCF-7 cell line.

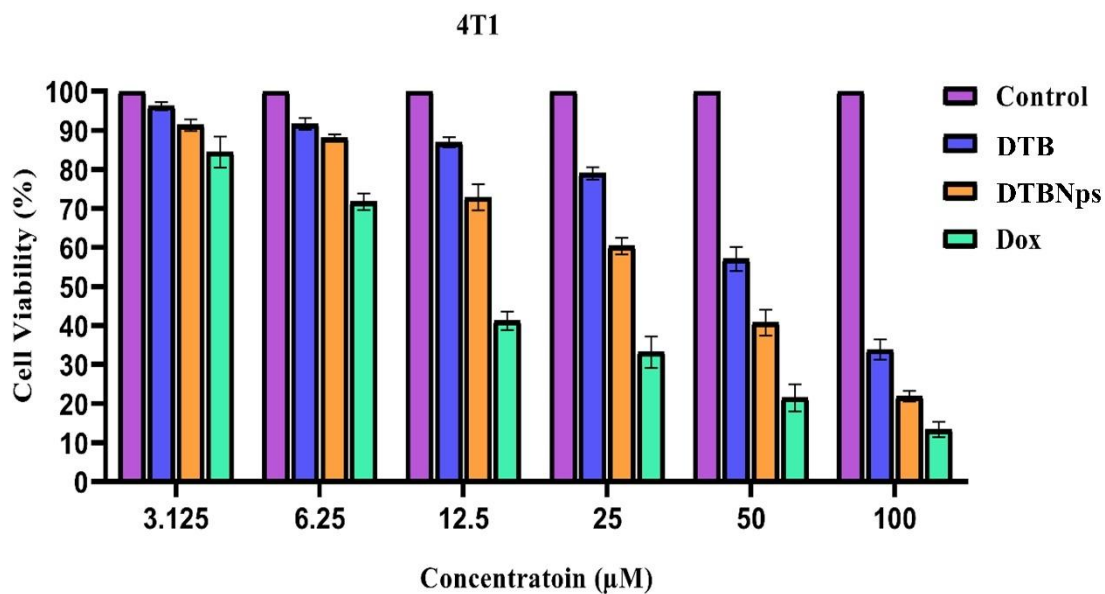


Figure 39: Cell viability of DTB, DTBNps, and Doxorubicin in 4T1 cell line.

4.12 Stability

The physical stability of DTBNps was investigated for 3 months at $25 \pm 1^\circ\text{C}$, $4.0 \pm 1^\circ\text{C}$, and $40 \pm 1^\circ\text{C}$ respectively. The results showed in Table. 23 that there was no significant difference in particle size, zeta potential and entrapment efficiency between the two conditions studied. The prepared nanoparticles were stabilized for an extended period, which agreed with our results. It could be concluded that polymeric DTBNps were stable for 3 months at the three temperatures that were tested.⁷⁶

Table 23: Physical stability of DTNps.

Storage Time	Parameter	$40 \pm 1^\circ\text{C}$	$25 \pm 1^\circ\text{C}$	$40 \pm 1^\circ\text{C}$
0 Month	PS (nm)	202.1 ± 5.7	202.3 ± 5.8	202.6 ± 6.0
	ZP (mV)	-18.0 ± 1.01	-17.9 ± 1.05	-18.1 ± 1.02
	EE (%)	93.11 ± 0.2	93.08 ± 0.3	93.05 ± 0.2
3 Months	PS (nm)	201.4 ± 5.3	204.7 ± 5.9	288.4 ± 6.0
	ZP (mV)	-18.21 ± 1.7	-18.45 ± 1.2	-18.50 ± 1.3
	EE (%)	92.94 ± 1.1	91.99 ± 1.5	91.56 ± 1.3

5. SUMMARY

DTB an anticancer drug utilized in the treatment of leukaemia. The drug has exhibited potential action against Breast cancer from recent studies. However the solubility, low bioavailability and systemic side effects hinders its use clinically. The most often reported side effects include headache, nausea, pleural effusion, hair loss. To reduce these side effects the drug bioavailability should also be increased. This can be achieved by slow release and also by side reduction. The nanoparticulate approach was considered to increase the effectiveness for treatment of cancer. In the present investigation DTB nanoparticles were developed and evaluated for its use in Breast cancer.

The DTBNps was successfully formulated using emulsion solvent evaporation technique. A 3 factor 2 level factorial design was used for optimization of nanoparticles and evaluation was done for 8 batches as generated by Design Expert software.

The results obtained fitted to different polynomial models and the best-fit model was selected on the basis of different statistical parameters. The response surface plots thus generated from the Design-Expert software were demonstrated the relationship between the independent and dependant variables. The optimized batch of nanoparticles generated based on desirability was chosen based on the maximum entrapment efficiency and smaller particle size. Zeta sizer were used to evaluate the particle size, zeta potential and EE of nanoparticle formulation. Entrapment efficiency of nanoparticles was determined by using high speed centrifugation technique. The surface morphology of the nanoparticles was confirmed by using the SEM and AFM analysis.

The characterization and compatibility of the drug with other excipients were confirmed by using FTIR. The crystalline properties and thermal behaviour of the freeze-dried powders of optimized nanoparticle formulation were performed by FT-IR, XRD and DSC

analysis. In vitro release from optimized DTBNps batch was determined by using a dynamic dialysis membrane method and the release mechanism was determined by fitting the results of drug release into different drug release kinetic models.

The DTBNps have shown enhanced efficacy in boosting the bioavailability of DTB. The concentration of the drug in nanoform was significantly higher than pure form. The in vitro cytotoxicity of DTB, DTBNps & DXB was evaluated against the MCF 7, MDA MB 231 and 4T1 cell lines by MTT assay. The nanoparticles shown better activity against all cell lines. It was demonstrated from the anti-cancer study that administration of DTBNp into tumor-bearing mice up to four weeks resulted in tumor regression, and histopathological examination of tissues of mice demonstrated lesser side effects. The results of in vitro and in vivo studies thereby proposing, DTBNps to be a promising drug delivery system for Breast cancer treatment.

6. CONCLUSION

In the present research work, an attempt was made to improve the bioavailability of DTB by side reduction. Successful preparation and optimization of DTB loaded nanoparticles were achieved using the 2^3 factorial design. FTIR analyses revealed that there was no interaction between the drug and the excipients used in the formulation. The DSC, XRD, FT-IR study demonstrated the successful loading of the drug. The optimized formulation (F5) shows the desirable particle size and greater entrapment efficiency. SEM and AFM study for DTBNps reveals the spherical shape of nanoparticles. In vitro drug release has shown a biphasic burst release with a release mechanism of diffusion controlled over 24 h. DTBNps have demonstrated significant efficacy “in enhancing the bioavailability of DTB. The in vitro activity of DTBNps was confirmed by the MTT assay in MCF 7, MDA M 231, 4T1 cell lines in comparison to free DTB. The histopathological examination demonstrated the biocompatibility of the developed DTBNps. In vivo anticancer study has shown potential therapeutic effect. Overall, developed Nanoparticulate drug delivery system has promising potential in the prevention and treatment of Breast cancer. Further studies using advanced preclinical models are required to prove the efficacy of this nanoparticulate formulation for Breast cancer treatment.

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7. ANNEXURE

PRESENTATIONS				
Sl. No.	Date	Type	Presented At	Title
1	20 th – 22 nd January 2023	Poster	72 nd Indian Pharmaceutical Congress, Nagpur	An experimental study on nanoparticulate drug delivery system of anticancer drug.
2	18 th to 19 th October 2023	Oral	Convention on Discovery and Development – CDD 2023	A Quality by Design approach on Dasatinib Laden nanoparticulate Drug Delivery System: Formulation and <i>In-vitro</i> Evaluation.

PUBLICATIONS				
Sl. No.	Date	Name of the Journal	Title	Indexing
1	15 th November 2024	International Journal of Pharmaceutical Quality Assurance	Development and Validation of a Bioanalytical LC-MS/MS Method for Detection of Dasatinib in Rat Plasma	Scopus [Q3] IF – 0.5
2	19 th November 2024	Journal of Biomaterials Science, Polymer Edition	Development, optimization, and characterization of Eudragit-based nanoparticles for Dasatinib delivery	Scopus [Q2] Web of Science IF – 3.7



KLE ACADEMY OF HIGHER EDUCATION & RESEARCH, BELAGAVI
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2nd Block, Rajajinagar, Bengaluru - 560 010, Karnataka, India.

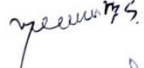
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Ref. No. KLE/COPBLR/

Date :

INSTITUTIONAL ANIMAL ETHICS COMMITTEE CERTIFICATE

This is to certify that the project proposal no. 02/AP/2022 entitled "Characterization of Designed Nanoparticulate Drug Delivery System of Dasatinib for Cancer Therapy" submitted by Mr.Hemanth. G has been approved /recommended by the IAEC of KLE College of Pharmacy,Bengaluru in its meeting dated 17/10/2022 and has been sanctioned 78Albino Wistar Rats under this proposal for a duration of next 12 months.

Authorized by	Name	Signature	Date
Chairman:	Dr.Rajamma A.J		
Member Secretary	Dr.Hariprasad M.G		21/10/22
Main Nominee of CPCSEA	Dr.Giridhar.P		



72nd Indian Pharmaceutical Congress, Nagpur

Certificate

It is our pleasure to certify that,

HEMANTH G presented a Poster in Scientific Session entitled **"AN EXPERIMENTAL STUDY ON NANOPARTICULATE DRUG DELIVERY SYSTEM OF ANTICANCER DRUG"** in the 72nd Indian Pharmaceutical Congress held at Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur during January 20-22, 2023.

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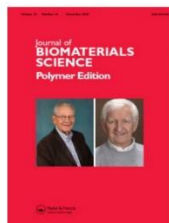
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Prof/Dr/Mr/Ms. <u>Hemanth .g</u> of <u>KLE college of pharmacy, Bangalore</u> has participated and presented (Oral/Poster) entitled <u>A Quality by design approach on Dasatinib-Laden Nanoparticle Drug Delivery System: Formulation and In-Vitro Evaluation.</u> in "Convention on Discovery and Development - CDD 2023" organised by College of Pharmaceutical Sciences, Dayananda Sagar University in association with Karnataka Drugs and Pharmaceuticals Manufacturer's Association, and Bangalore Bioinnovation Centre.			
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"Development, optimization, and characterization of Eudragit-based nanoparticles for Dasatinib delivery"

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ABSTRACT

This study focused on developing and evaluating dasatinib-loaded nanoparticles (DST-NPs) using Eudragit L100 as a polymer matrix for enhanced breast cancer treatment. The optimized formulation exhibited a particle size of 202.1 ± 5.7 nm, a zeta potential of -18 ± 1.01 mV, and an entrapment efficiency of $93.11 \pm 0.2\%$. In-vitro release studies demonstrated sustained drug release from DST-NPs, following Fickian diffusion. Pharmacokinetic studies in rats revealed higher C_{max} and AUC_{0-t} for DST-NPs compared to pure DST, indicating improved bioavailability. Tissue distribution studies showed enhanced targeting of DST-NPs, with higher concentrations in the liver and spleen. *In vivo* efficacy in a DMBA-induced mammary carcinoma model demonstrated that DST-NPs significantly reduced tumor volume, maintained stable body weight, and improved survival rates compared to pure DST. Hematologic analysis indicated a favorable blood profile with DST-NPs, and histopathological examinations confirmed the restoration of normal mammary gland and liver architecture. MTT assays showed higher cytotoxicity of DST-NPs against MCF-7, MDA-MB231, and 4T1 cell lines, with lower IC₅₀ values than pure DST. Stability studies indicated that DST-NPs maintained their properties over six months at various storage conditions. These findings highlight the potential of DST-NPs as an effective nanocarrier system for cancer therapy.

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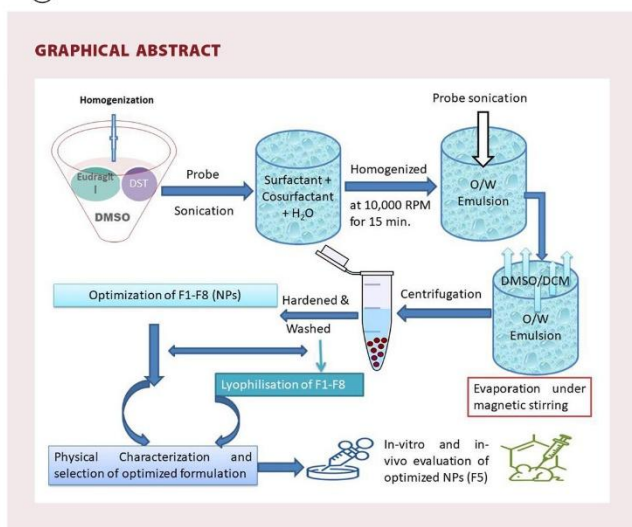
KEYWORDS

Cancer; nanoparticles;
 Dasatinib; DST-NPs;
 Eudragit

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Introduction

Dasatinib (DST) is a second-generation tyrosine kinase inhibitor, recognized for its efficacy in targeting multiple tyrosine kinases associated with tumor cell proliferation. It is particularly effective against the SRC family, BCR-ABL, receptor tyrosine kinases (c-KIT, PDGFR, DDR1), and TEC class kinases [1]. DST serves as a first-line treatment for chronic myelogenous leukemia, with potency 300 times greater than imatinib in inhibiting BCR/ABL activity [2,3]. Recent studies highlight DST's ability to induce apoptosis and inhibit the proliferation, migration, and invasion of cancer cells [4]. Despite these benefits, DST's oral bioavailability ranges from only 14 to 34%, primarily due to its poor solubility in water [5]. As a BCS (biopharmaceutical classification) class II drugs are characterized by high permeability but low solubility, DST's bioavailability is significantly influenced by its solubility [6]. Additionally, DST has a short terminal half-life of 3-5 h and is sensitive to pH changes, which further complicates its effective delivery [7]. Adverse effects associated with DST also necessitate the development of a more efficient drug delivery system [8].

The development of novel drug delivery systems is crucial for enhancing the efficacy of contemporary anticancer therapies. Nano Drug Delivery Systems (NDDS) have shown improved therapeutic outcomes with advancements in nanomedicine [9]. By modifying NDDS carriers, it is possible to achieve active targeting and environmental responsiveness [10]. CD44, a type I transmembrane glycoprotein, is highly expressed in endothelial, mesenchymal, and mesoderm-derived cells, playing a key role in cell migration, proliferation, and adhesion through its interaction with the extracellular matrix. It is particularly overexpressed in various tumor cells, including breast cancer, and has been identified as a marker for cancer stem cells (CSCs), contributing to drug resistance, metastasis, and tumor recurrence. Due to its pivotal role in tumor biology, CD44 is frequently targeted in cancer therapies to develop active targeting drug

delivery systems. In this study, the nanoparticles could be optimized for enhanced tumor targeting by incorporating CD44-targeting ligands, such as hyaluronic acid, to improve selective uptake in CD44-positive tumor cells, thereby enhancing therapeutic efficacy and minimizing off-target effects. The potential for further functionalization of DST-NPs makes this approach highly relevant for targeting CD44-overexpressing cancer cells [11].

Methacrylic acid-based polymers from the Eudragit® family are utilized to create polymeric nanoparticles (NPs). Over the past decade, Eudragit® NPs have demonstrated their potential to encapsulate and enhance the solubility and bioavailability of poorly soluble drugs. The chemical modification of these polymers allows for the specific delivery of drugs, thanks to their variable solubility and release behavior at different pH levels [12–14]. Recent studies such as the one done by Mehandole et al. [15] have also looked at developing Dasatinib oral nanoparticles, expand knowledge on enhancing bioavailability and stability. However, our study is exceptional in that we design and prepare Eudragit®-based nanoparticles with emphasis on active targeting through the CD44 pathway, which prevails in a majority of cancer cells. This approach affords better tumour selectivity, minimises toxicity, and has better bioavailability than simple oral formulations. Furthermore, the present formulation also shows controlled release and environmental sensitivity; this equally poses a solution to the short half-life of Dasatinib and response to pH. Our *in vivo* outcomes from this study also partially support the therapeutic application of this system by showing a significant decrease in tumor volume and negligible influence on neighbouring healthy tissues as compared with previously described nanoparticle-based therapies.

Considering the existing challenges with DST, it is essential to develop a dosage form that can enhance its solubility and dissolution rate. This research aims to formulate and optimize a nanoparticle-based drug delivery system using Eudragit® to improve the biopharmaceutical performance of DST.

Materials and methods

DST was generously provided as a gift sample by MSN Laboratories Pvt Ltd, Hyderabad. Eudragit L 100 was procured from Evonik Pvt Ltd, Mumbai, and Poloxamer 407 was supplied by Yarrow Chem, Bangalore. Dimethyl sulfoxide (DMSO) and dichloromethane (DCM) were obtained from Thermo Fisher Scientific, Mumbai. Deionized water was purified using a Milli-Q water purification system (Millipore, Bedford, MA). Cell lines MCF-7, MDA-MB-231, and 4T1 were purchased from ATCC. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and MTT reagent were acquired from Gibco, Thermo Fisher. DMBA was procured from TCI, India. All other reagents and chemicals used were of analytical grade.

A total of 88 female Wistar rats were procured from Skanda Life Sciences Pvt. Ltd. for the experiments. All experimental procedures were conducted in accordance with the guidelines and regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The study was approved by the Institutional Animal Ethics Committee (IAEC) under IAEC No. 02/AP/2022.

Optimization of nanoparticles by response variables effects (2^3 factorial designs)

To maximize the synthesis of DST-NPs, a factorial design was employed. The influence of sonication power, surfactant concentration, and polymer concentration on the physicochemical characteristics of the produced nanoparticles was investigated. Specifically, the effect of three independent variables—Eudragit L100 concentration (X1), Poloxamer 407 concentration (X2), and sonication power (X3)—on the dependent variables (particle size, zeta potential, and entrapment efficiency) was examined using a full 2^3 factorial design, encompassing a total of 8 experiments (Table 1). Throughout the experiments, DST levels remained constant. Each factor was explored at two levels, denoted by -1 (low) and 1 (high). The levels and parameters were selected based on the literature review and results from previous experiments conducted in our lab. The full factorial design, responses, design summary, and significant values were evaluated using Design Expert 12 software [15–16].

Preparation of DST-loaded nanoparticles

Nanoparticles (NPs) were formulated using a single-emulsion (o/w) solvent evaporation method, following the parameters specified in the experimental design. Briefly, precise amounts of DST and Eudragit L were weighed and added to the organic phase, consisting of DMSO and DCM. This organic phase was then added dropwise (0.5 ml/min) to a beaker containing deionized water with specific proportions of Poloxamer 407 under probe sonication (Labmann). The resultant solution was further homogenized (IKA T18 Ultra Turrax) to form a nanoemulsion. The particles were separated by centrifugation at 10,000 rpm (Remi, PR 24, Mumbai, India) and washed three times with water. The nano-formulation was freeze-dried into powder form using a vacuum freeze dryer with mannitol (2%) as a cryoprotectant (Alpha Christ) and stored for further studies [17–18].

Characterization of DST-NPs

The produced nanoparticles were characterized and optimized by evaluating zeta potential (ZP), particle size (PS), entrapment efficiency (EE), and nanoparticle morphology using scanning electron microscopy (SEM). Additional analyses included X-ray powder diffraction (XRPD) and *in vitro* drug release tests [19].

Table 1. Composition of DST-NPs by using 2^3 full factorial design (actual and coded labels).

Run	Drug:Polymer Ratio (X1)	Surfactant concentration (w/w %) (X2)	Sonication power (w/w %) (X3)
F1	1:1 (-1)	1 (-1)	30 (-1)
F2	1:1 (-1)	2 (1)	30 (-1)
F3	1:1 (-1)	1 (-1)	50 (1)
F4	1:1 (-1)	2 (1)	50 (1)
F5	1:2 (1)	1 (-1)	30 (-1)
F6	1:2 (1)	2 (1)	30 (-1)
F7	1:2 (1)	1 (-1)	50 (1)
F8	1:2 (1)	2 (1)	50 (1)

Zeta potential, particle size, and entrapment efficiency

The particle size and zeta potential of the prepared DST-NPs were determined using dynamic light scattering (DLS) at 25°C with a quartz cell at ambient temperature (Malvern Zetasizer Instruments). Zeta potential is a key characteristic that affects the physical stability of a colloidal system [19].

Entrapment efficiency was estimated using an indirect technique. Briefly, DST-NPs were dissolved in DMSO and stirred for 1 h, followed by sonication using an ultrasonic cleaner (GT Sonic Ultra Sonic Cleaner) for 10 min. The solution was then filtered using a 0.25 µm PDF syringe filter and subjected to centrifugation at 15,000 rpm for 15 min (Remi, PR 24 Microprocessor Research Centrifuge, India). The supernatant, containing the free drug, was analyzed by UV spectroscopy at 324 nm. Measurements were performed in triplicates. The entrapment efficiency was calculated using the following formula [19]:

$$\text{Entrapment Efficiency \%} = \frac{\text{Total drug} - \text{Free drug in supernatant}}{\text{Total drug}} \times 100$$

Surface morphology of DST-NPs

The surface texture of the optimized nanoparticles was observed using Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). The DST-NPs were carefully cast on glass slides and degassed under vacuum to eliminate contaminants. A thin layer of a gold-palladium alloy was sputter-coated onto the nanoparticles to enhance conductivity and improve image quality. After drying, the nanoparticle surface morphology was captured at suitable magnifications using a Zeiss Gemini 300 Scanning Electron Microscope (SEM) [20].

For Atomic Force Microscopy (AFM), a small drop of the sample was placed on a microscopic slide and allowed to dry. The images were captured using an Innova SPM Atomic Force Microscope [21].

X-Ray powder diffraction (XRPD)

XRPD was employed to detect patterns of pure DST-loaded nanoparticles using a powder X-ray diffractometer (Bruker D8 Advance Diffractometer). The operation parameters were as follows: 2.2 kW X-ray source with a Cu anode, fine focus ceramic X-ray tube operated at 40 kV voltage and 40 mA current, and 1.6 kW power. All measurements were performed over the 2θ range from 10° to 70°. The 2θ values were collected using a LYNXEYE high-speed SSD160–2 detector with a 500 µm sensor [22].

In-vitro release study and release kinetics determination

In-vitro cumulative release studies for the optimized formulation and pure DST suspension were performed using the dialysis membrane technique (molecular weight

cut-off 12,000–14,000). The formulation was suspended in an end-clipped dialysis membrane and immersed in 100 mL of phosphate buffer containing 2% Tween 80 to establish sink conditions. The study was conducted in two media (pH 1.2 and 6.8) at a temperature of $37 \pm 0.5^\circ\text{C}$ with agitation at 100 rpm. At predetermined time points (30 min, 1, 2, 3, 4, 6, 8, 12, 24 h), samples were collected, and sink conditions were maintained by replenishing the medium with fresh buffer. The quantification of the released drug was performed in triplicates using UV spectroscopy at 324 nm [23].

To investigate the mechanism of drug release from DST-NPs, various mathematical models were applied to interpret drug release as a function of time. Release kinetics were evaluated using the DD Solver add-in program, which reduces errors, accelerates calculations, and precisely determines the release curve [24].

Pharmacokinetics and biodistribution

The pharmacokinetic study was conducted using female Wistar rats ($n=6$, 220–250 g body weight) to evaluate the absorption and disposition of DST and DST-NPs. Dosages of 10 mg/kg for DST and 10 mg/kg for DST-NPs were administered orally. Blood samples were collected pre-dose and at specific time points post-dose: 0.5, 1, 2, 3, 8, 12, and 24 h. Sampling was performed using the retro-orbital method under isoflurane anesthesia. Blood was collected into tubes containing EDTA as an anticoagulant and centrifuged at 4000 g for 10 min to separate plasma, which was then stored at -80°C until analysis [25].

The quantification of DST in plasma samples was carried out using a validated LC-MS/MS method. The LC-MS/MS system was equipped with an ESI+ ion source and operated under multiple reaction monitoring (MRM) conditions, using specific transitions for DST and the internal standard, verapamil [26].

Pharmacodynamics study

Forty female Wistar albino rats, aged 50–55 days and weighing 180 ± 10 g, were categorized into four groups of ten animals each. The groups were designated as follows: Group I (normal control), Group II (7,12-dimethylbenz[a]anthracene (DMBA)-induced rats), Group III (DMBA-induced rats treated with DST-loaded nanoparticles at 10 mg/kg per day for one month after tumor development), and Group IV (DMBA-induced rats treated with DST at 10 mg/kg per day for one month after tumor development).

Following the administration of the doses, the rats were anesthetized using diethyl ether and euthanized. Blood samples were collected *via* orbital puncture, and serum was isolated for various analyses, including a complete blood count (CBC). Breast tissue, liver, kidney, and small intestine samples were preserved in a 10% formalin solution for histopathological examinations [27].

Induction of mammary gland tumors

To induce tumors, a freshly prepared solution of DMBA at a concentration of 20 mg/mL in olive oil was administered orally using the gavage technique. Starting from the

fourth week after DMBA administration, the rats underwent weekly palpation to detect any tumor development. The initial tumor manifestation was observed in the 16th week, and by the 18th week, tumors had developed in all rats [28].

Measurement of tumor volume, body weight, and survival rate

To assess the efficacy of the treatments, tumor volume, body weight, and survival rate of the animals were monitored throughout the study. Tumors were induced in female Wistar rats by administering a freshly prepared solution of DMBA at a concentration of 20 mg/mL in olive oil using the gavage technique. Tumor development was initially observed in the 16th week, and by the 18th week, all rats had developed tumors.

The dimensions of the mammary tumors were measured weekly using a Vernier caliper. Tumor volume (V) was calculated using the formula:

$$V = \frac{(L \times B^2)}{2}$$

where L represents the longer diameter and B represents the shorter diameter, measured in centimeters and oriented perpendicularly to each other. Concurrently, the body weights of the animals were recorded weekly using a digital scale to monitor the overall health and potential weight changes associated with the treatments. Initial body weights were recorded before the treatment, and final body weights were recorded after the treatment period. The survival rate was calculated as the ratio of the number of surviving animals in each group to the total number of animals at the start of the experiment [29].

Hematologic examination

For the hematologic examination in this study, blood samples were collected from experimental rats *via* retro-orbital bleeding under isoflurane anesthesia to minimize discomfort. Approximately 1-2 mL of blood was drawn and immediately transferred into EDTA-coated tubes to prevent coagulation. The samples were stored at 4°C and promptly transported to the laboratory for analysis. Hematologic parameters were evaluated using an automated hematology analyzer, including RBC count, hemoglobin concentration, hematocrit, WBC count, differential WBC count (lymphocytes, monocytes, eosinophils), and platelet count [30].

Histopathologic examination

For histopathologic examination, breast, liver, kidney, and small intestine tissues were collected from the experimental rats after euthanasia. The tissues were immediately fixed in 10% neutral-buffered formalin for 24–48 h. After fixation, the tissues were processed using a standard paraffin embedding technique. Sections 4–5 micrometers in thickness were cut from the paraffin blocks using a microtome and mounted on glass slides. The sections were then deparaffinized in xylene, rehydrated through a

series of graded alcohols, and stained with hematoxylin and eosin (H&E). The stained sections were dehydrated, cleared, and cover-slipped using a mounting medium. Histopathologic examination was performed under a light microscope to assess morphological changes in the breast, liver, kidney, small intestine, spleen, brain, and heart [31].

MTT assay

The cytotoxicity of the test samples DST and DST-NPs was evaluated in MCF-7, MDA-MB-231, and 4T1 cell lines using the MTT assay. The cell lines were procured from ATCC. Stock cells were cultured in their respective media, supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), and streptomycin (100 µg/mL), in a humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated using 0.05% trypsin and centrifuged at 1000 rpm for 5 min. The culture media was discarded, and the cell pellet was gently resuspended in 2 mL of complete media. Cells were seeded into 96-well plates at a density of 50,000 cells/well. After 24 h, the cells were treated with various concentrations of the test samples (3.125 to 100 µM) for 24 h. Post-treatment, the media was replaced with MTT reagent (5 mg/10 mL in PBS) and incubated for 4 h. Formazan crystals formed by viable cells were solubilized using DMSO, and the absorbance was measured at 590 nm using a microplate reader. Percent inhibition was calculated, and IC₅₀ values were determined using nonlinear regression analysis [32].

Stability studies

The stability of the prepared DST-NPs was evaluated over a 6-month storage period at three different conditions: 25 ± 0.5°C (room temperature), 4 ± 0.5°C (refrigerated), and 40 ± 0.5°C (accelerated). The stability study was conducted in accordance with ICH guidelines. Physical parameters including particle size, zeta potential, and entrapment efficiency were measured before and after the storage period to assess any changes. These parameters were analyzed to determine the stability of DST-NPs over the specified storage conditions [33].

Results

Optimization of nanoparticles by response variables effects (2³ factorial designs)

A two-level, three-factor full factorial design was employed to assess the interaction, quadratic, and main effects of individual factors on the chosen responses. Regression equations were generated for each response. Experimental runs were conducted as shown in Table 1. The 3D response surface plots were utilized to illustrate the interactions between variables and factors. ANOVA was used to analyze the data and determine the quantitative effects of the factors.

Particle size, zeta potential, and entrapment efficiency

Strong electrostatic attraction between the particles, caused by the surface charge, regulates the stability of the nanoparticulate formulation. The observed zeta potential ranged from -18 mV to -24.7 mV (Table 3). The zeta potential intensity of the optimized formulation is shown in Figure 1.

The entrapment efficiency was within the optimal range of 80–93% (Table 2). A summary of the model parameters and p value is provided in Table 3.

The composition of the optimized batch of DST-NPs included a drug ratio of 1:2, sonication power of 30 mV, and a stabilizer concentration of 1% (as listed in Table 1). Experimental values for particle size ($202.1 \pm 5.7\text{ nm}$), zeta potential ($-18 \pm 1.01\text{ mV}$), and entrapment efficiency ($93.11 \pm 0.2\%$).

Based on the results, the F5 formulation was selected as the optimized formulation due to its favorable particle size ($202.1 \pm 5.7\text{ nm}$), zeta potential ($-18 \pm 1.01\text{ mV}$), and the highest entrapment efficiency ($93.11 \pm 0.2\%$). Additionally, the model parameters for particle size, zeta potential, and entrapment efficiency were statistically significant,

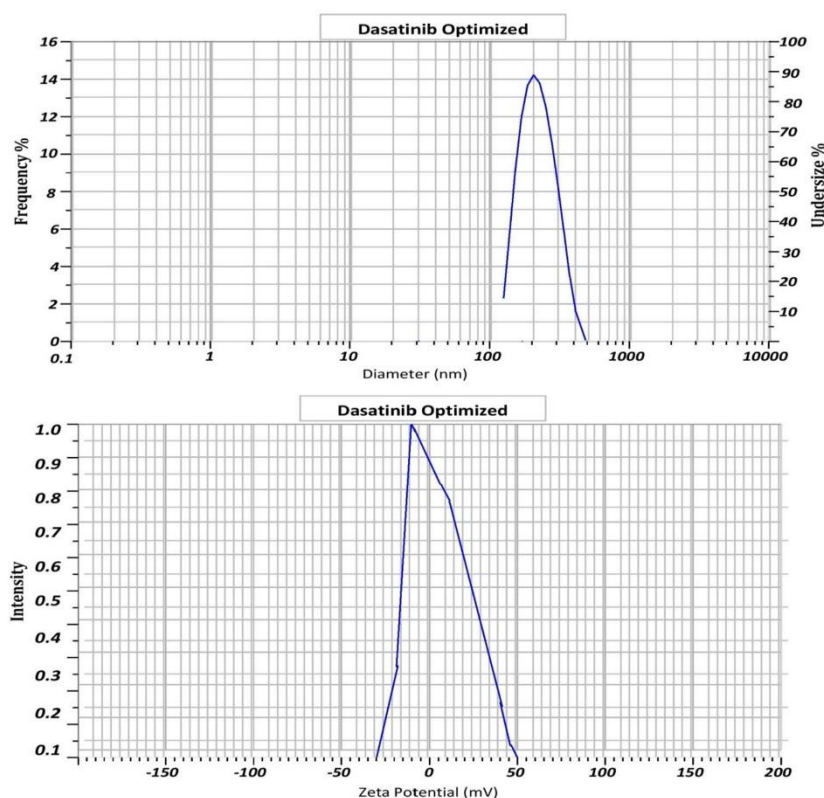


Figure 1. Particle size distribution and zeta potential intensity of DST-NPs (F5).

Table 2. Responses of 2³ full factorial design of prepared DST-NPs.

Run	Particle Size (nm)	Zeta Potential (mV)	Entrapment Efficiency (%)
F1	240.3 ± 9.0	-24.7 ± 0.8	84.11 ± 1.2
F2	221.3 ± 8.3	-19 ± 1.1	82.47 ± 0.9
F3	270.3 ± 12.4	-19.8 ± 0.9	82.32 ± 1.7
F4	259.8 ± 9.1	-18.6 ± 1.8	86.01 ± 1.1
F5	202.1 ± 5.7	-18 ± 1.01	93.11 ± 0.2
F6	295.3 ± 11.8	-21.06 ± 1.5	86.45 ± 1.3
F7	221.9 ± 10.9	-20.4 ± 1.8	90.11 ± 0.3
F8	215.7 ± 11.1	-21 ± 1.3	88.78 ± 0.9

Table 3. Summary of model parameters (F5).

Models	R ²	Adjusted R ²	SD	Adequate Precision	%CV	p-value	Remarks
Particle Size	0.9958	0.9809	3.67	26.49	1.15	< 0.0034	Significant
Zeta Potential	0.9888	0.9495	0.41	17.79	2.02	< 0.0046	Significant
Entrapment Efficiency	0.9878	0.9453	0.63	15.38	0.73	< 0.0023	Significant

with high R² values (0.9958, 0.9888, and 0.9878, respectively) and low %CV, indicating the reliability and precision of the formulation. The model parameters were calculated using **Design-Expert** software, which fits the experimental data to various statistical models (such as linear, quadratic, or cubic) and determines the best-fitting model based on these statistical parameters.

Surface morphology of DST-NPs

The microstructures of the F5 batch nanoparticles were subjected to SEM imaging to assess their morphology. The SEM images revealed that the formulation exhibited a spherical and homogeneous shape with a smooth surface, as shown in Figure 2 (a). This indicates a well-defined and uniform particle formation, crucial for consistent drug delivery.

The 2D surface topographic AFM image of the optimized formulation further depicted nanoparticles with smooth surfaces without any agglomerations or perforations, as shown in Figure 2 (b). This uniform surface texture can be attributed to the even distribution of the polymer coating, which helps to maintain the structural integrity and stability of the nanoparticles.

X-Ray powder diffraction (XRPD)

The XRPD overlay spectrum of optimized DST-NPs, along with the physical mixture and pure DST, is shown in Figure 3. The pure DST exhibited crystalline nature with relatively intense peaks at 2θ angles of 11.3°, 12.2°, 13.1°, 16.6°, 24.4°, 25.31°, and 27.82°. The diffraction patterns of Eudragit L 100 and the physical mixture displayed several smaller diffusion peaks. In contrast, the DST-NPs spectrum showed reduced peak intensities and broad diffusion peaks, indicating the conversion of the crystalline nature of DST into an amorphous form [34].

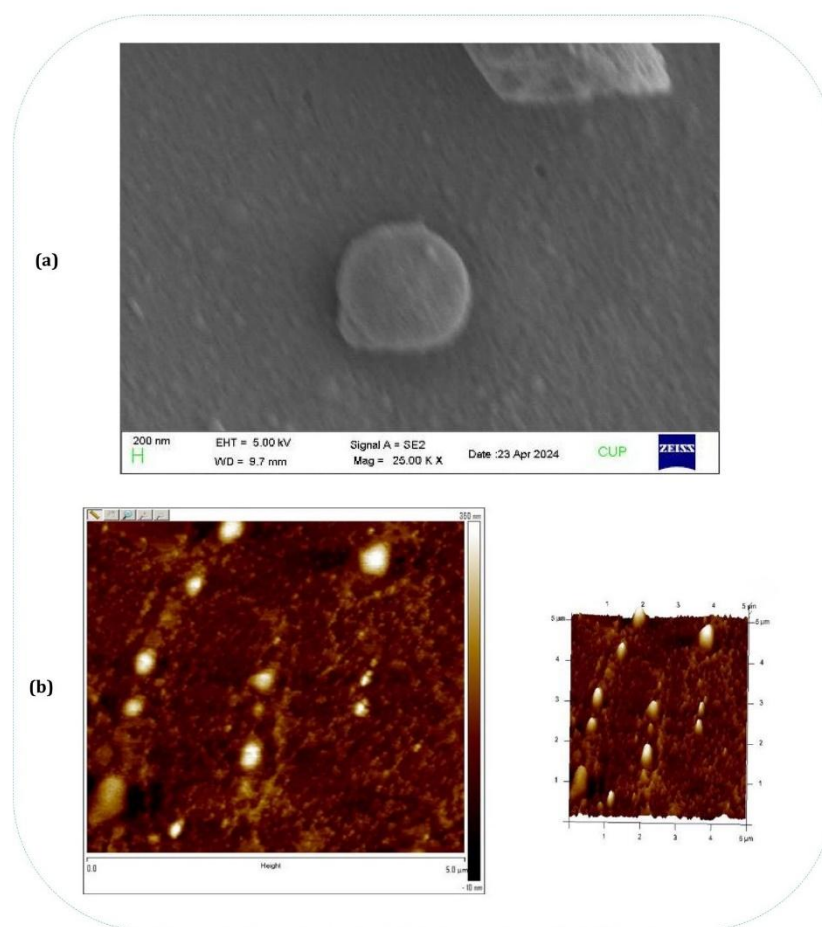


Figure 2. (a) SEM image of DST-NPs (F5) (b) 2D & 3D AFM image of DST-NPs (F5).

In-vitro release study & release kinetics determination

The primary investigation of the present study was to examine how Eudragit L 100 affected the drug release profile from nanoparticles (NPs). In Figure 4, the lower release profile of pure DST (free drug) is primarily due to its poor solubility and the absence of a controlled release system. Without the benefit of a nanoparticulate carrier, pure DST releases slowly, as it lacks mechanisms like enhanced surface area or controlled diffusion that nanoparticles offer.

In contrast, the DST-loaded nanoparticles (DST-NPs) show significantly higher drug release profiles. This is due to the nanoparticles improving the drug's solubility and providing a larger surface area, which accelerates drug release into the medium. The nanoparticle matrix also facilitates controlled and sustained release of DST.

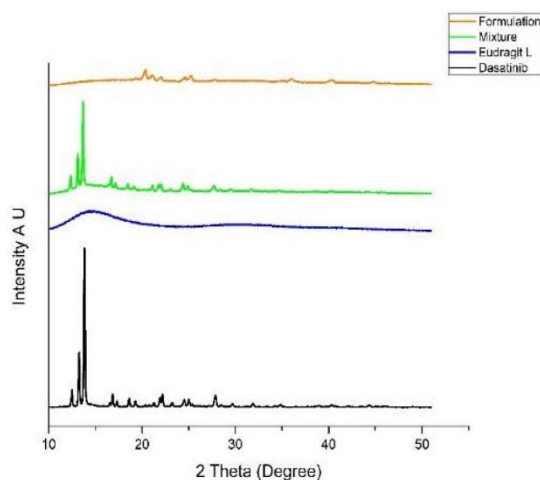


Figure 3. XRPD overlay spectrum of optimized DST-NPs (F5), physical mixture, and pure DST.

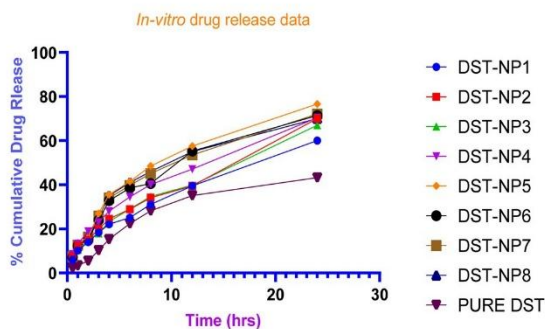


Figure 4. *In vitro* cumulative drug release of DST-NP1 to DST-NP8.

Among the formulations, F5 exhibits the highest drug release profile. This can be attributed to the optimized formulation of F5, which may have smaller particle sizes, increasing the surface area for drug diffusion. Additionally, F5's polymer composition (Eudragit) likely allows for more efficient encapsulation and gradual degradation, promoting sustained drug release. These combined factors make F5 the most efficient in releasing the drug over time, highlighting its potential as the best formulation for controlled drug delivery. Below are the equations corresponding to the release kinetics models presented in Table 4:

$$\text{Zero order: } Q = Q_0 + k_0 \cdot t$$

Where, Q is the amount of drug released, Q_0 is the initial amount of drug, k_0 is the zero-order release constant, and t is time.

Table 4. Release kinetics of DST-NPs.

Release order	R ² value
Zero Order	0.3288 ± 0.001
First Order	0.6475 ± 0.001
Higuchi	0.9604 ± 0.002
Kosmeyer Peppas	0.9881 ± 0.002 (<i>n</i> = 0.405)
Hixon Crowell	0.5562 ± 0.001

$$\text{First Order : } \ln(Q_0 - Q) = \ln(Q_0) - k_1 \cdot t$$

Where, k_1 is the first-order release constant.

$$\text{Higuchi Model : } Q = k_H \cdot t^{1/2}$$

Where, k_H is the Higuchi release constant.

$$\text{Korsmeyer - Peppas : } \frac{Q_t}{Q_\infty} = k_K \cdot t^n$$

Where Q_t is the amount of drug released at time t , Q_∞ is the total drug released, k_K is the release rate constant, and n is the release exponent.

$$\text{Hixon - Crowell : } Q_0^{1/3} - Q^{1/3} = k_{HC} \cdot t$$

Where k_{HC} is the Hixon-Crowell constant.

Table 4 presents the release kinetics data for the optimized formulation F5, where the Kosmeyer-Peppas model exhibited the best fit ($R^2 = 0.9881 \pm 0.002$) and $n = 0.405$, suggesting diffusion-controlled release.

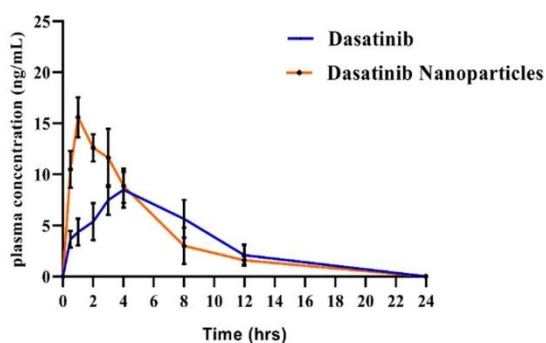
Physical stability of DST-NPs (F5)

The physical stability of F5 was investigated for 3 months at $25 \pm 1^\circ\text{C}$, $4.0 \pm 1^\circ\text{C}$, and $40 \pm 1^\circ\text{C}$, respectively. The results showed no significant difference in particle size (PS), zeta potential (ZP), and entrapment efficiency (EE) between the two conditions studied ($25 \pm 1^\circ\text{C}$ and $4.0 \pm 1^\circ\text{C}$). It could be concluded that polymeric F5 were stable for 6 months at the two temperatures that were tested. The updated Table 8 includes the stability results at $40 \pm 1^\circ\text{C}$. At the 0-month point, the DST-NPs were freshly prepared, and minimal variations were observed across temperature conditions due to the lack of exposure to environmental stress. Since degradation or physical changes typically occur over time, the differences at this early stage were expected to be minimal.

Table 5 shows that F5 maintain their physical stability at $40 \pm 1^\circ\text{C}$ for 6 months, with only minor variations in particle size, zeta potential, and entrapment efficiency, indicating good stability across all tested conditions.

Table 5. Physical stability of DST-NPs (F5).

Storage Time	Parameter	4.0 ± 1 °C	25 ± 1 °C	40 ± 1 °C
0 Month	PS (nm)	202.1 ± 5.7	202.3 ± 5.8	202.6 ± 6.0
	ZP (mV)	-18.0 ± 1.01	-17.9 ± 1.05	-18.1 ± 1.02
	EE (%)	93.11 ± 0.2	93.08 ± 0.3	93.05 ± 0.2
3 Months	PS (nm)	201.4 ± 5.3	284.7 ± 5.9	288.4 ± 6.0
	ZP (mV)	-18.21 ± 1.7	-18.45 ± 1.2	-18.50 ± 1.3
	EE (%)	92.94 ± 1.1	91.99 ± 1.5	91.56 ± 1.3
6 Months	PS (nm)	280.2 ± 10.0	280.1 ± 10.6	280.8 ± 10.5
	ZP (mV)	-18.34 ± 1.2	-18.55 ± 1.4	-18.60 ± 1.5
	EE (%)	92.56 ± 1.1	90.78 ± 1.6	90.23 ± 1.4

**Figure 5.** Compartmental distribution of oral solution and formulation (F5) at different time intervals in blood plasma concentration versus time in albino Wistar rats.

Pharmacokinetics and biodistribution

DST exhibited rapid absorption in both groups (Figure 5). The maximum plasma concentration (C_{max}) was observed to be 8.50 ng/mL at 4 h (T_{max}) in Group 1 (pure DST) and 12.60 ng/mL at 2 h (T_{max}) in Group 2 (DST-NPs), indicating a faster absorption rate in DST-NPs. The area under the plasma concentration-time curve (AUC) from time zero to the last measurable concentration (AUC_{0-t}) was 66.06 ng/mLh for pure DST and 71.23 ng/mLh for DST-NPs, reflecting a higher exposure in DST-NPs.

The elimination half-life (t_{1/2}) was slightly higher in DST-NPs (6.15 h) compared to pure DST (3.97 h). The mean residence time (MRT) was also higher in DST-NPs (9.23 h) compared to pure DST (7.26 h), consistent with the slower kinetics observed. The total body clearance (Cl/F) and volume of distribution (V_z/F) were calculated, with DST-NPs demonstrating more efficient distribution and elimination properties.

Tissue distribution studies were conducted to determine the concentration of DST in the liver, brain, heart, kidney, and spleen at 24 h post-administration (Table 6). In pure DST, the highest concentrations were found in the spleen (6.59 ng/10 mg tissue) and kidney (5.06 ng/10 mg tissue), with no detectable levels in the brain. In DST-NPs, similar patterns were observed, with slightly lower concentrations in the spleen (5.83 ng/10 mg tissue) and kidney (2.87 ng/10 mg tissue).

Table 6. Biodistribution of DST and DSTNPs in different tissues.

Group	Tissue (n=3)	DST (ng/10mg tissue)	
		Mean	SD
DST	Liver	2.31	0.45
	Brain	0	0
	Heart	0	0
	Kidney	5.06	1.2
	Spleen	6.59	4.82
	Mammary	2.13	0.3
DST-NPs	Liver	2.03	0.28
	Brain	0	0
	Heart	3.97	0.75
	Kidney	2.87	1.79
	Spleen	5.83	2.03
	Mammary	1.11	0.2

Pharmacodynamics study

Measurement of tumor volume, body weight, and survival rate

In the DMBA-treated group, there was a significant decrease in body weight, dropping from 170 ± 15 g at the beginning of the treatment to 140 ± 15 g by week 4, reflecting the severe impact of tumor burden and lack of treatment efficacy. In contrast, the groups treated with F5 and pure DST exhibited more stable body weights, ending at 165 ± 10 g and 150 ± 12 g, respectively, by week 4. The stability in body weight for F5 further supports the therapeutic advantage of the nanoparticle formulation, mitigating the adverse effects commonly associated with cancer progression and treatment (Figure 6b).

In the DMBA-treated group, the survival rate dropped sharply, with only 45% of the animals surviving by week 4. This high mortality rate underscores the lethality of untreated tumor growth. F5 and pure DST exhibited improved survival rates, with F5 showing a 75% survival rate and pure DST showing a 60% survival rate by the end of the study. The higher survival rate in the F5 group not only slowed tumor progression but also contributed to overall better health outcomes compared to pure DST treatment. The tumor volumes of the animals in different groups were measured to assess the effectiveness of the various treatments (Figure 6c). In the DMBA-treated group, tumor growth was rapid, with a significant increase observed from week 1 ($173 \pm 15 \text{ mm}^3$) to week 4 ($764 \pm 120 \text{ mm}^3$). This indicates the aggressive nature of tumor progression in untreated conditions. In contrast, the groups treated with F5 and pure DST exhibited markedly slower tumor growth rates. By week 4, the tumor volumes for DST-NPs (F5) and pure DST were $350 \pm 85 \text{ mm}^3$ and $570 \pm 100 \text{ mm}^3$, respectively (Figure 6a).

A one-way ANOVA was conducted to compare the effects of different treatments (DMBA-treated, DST, and DSTNPs) on tumor volume, body weight, and survival rates over the four-week treatment duration. The analysis revealed significant differences in tumor volume among the treatment groups at all-time points ($p < 0.001$), indicating that DSTNPs significantly reduced tumor volume compared to both DST and DMBA-treated groups. Additionally, body weight analysis showed significant variation ($p < 0.01$) between the groups, with the DMBA-treated group exhibiting a notable decrease in body weight compared to the DST and DSTNPs groups, which maintained stable body weights

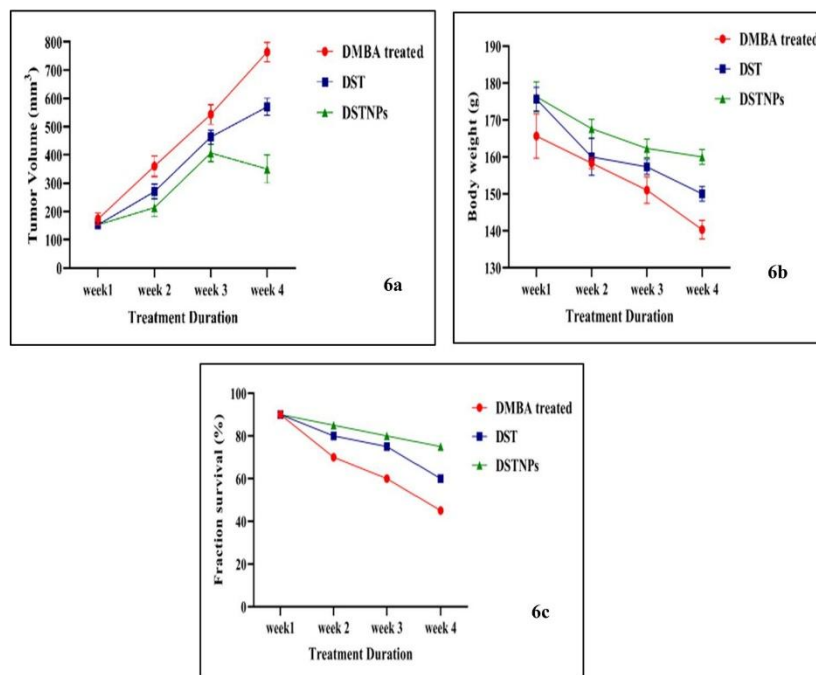


Figure 6. **a:** Tumor volume progression in different treatment groups, **b:** Body weight changes in different treatment groups, and **c:** Survival rates in different treatment groups.

throughout the treatment. Furthermore, the survival analysis indicated significant differences ($p < 0.001$) in survival rates among the groups, with DSTNPs demonstrating improved survival compared to the DMBA-treated group. These findings underscore the potential of DSTNPs as an effective treatment option in reducing tumor growth and improving overall survival in the DMBA-induced mammary carcinoma model.

Hematologic examination

The hematologic parameters were evaluated to assess the impact of the various treatments on the blood profiles of the experimental rats. The Complete Blood Count (CBC) analyses included measurements of hemoglobin (Hgb) levels, red blood cell (RBC) count, white blood cell (WBC) count, and differential counts. The results are shown in Table 7.

Histopathologic examination

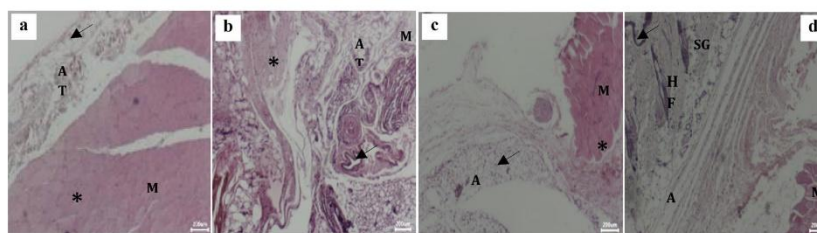
Histopathological examination of the mammary gland in the control group showed normal architecture predominantly composed of adipose tissue (AT) without staining, with blue-staining oval nuclei placed peripherally resembling signet ring cells (arrow). The smooth muscle layer (M) exhibited pink-staining cytoplasm and oval-shaped

Table 7. Hematologic examination of DST and F5 in various animal groups.

Blood component	Normal control	DMBA treated	DST-NPs (F5)	DST
Hemoglobin (g/dL)	15.2	11.9	14.7	13.4
Hematocrit (%)	42	30	40	38
WBC (cells/L)	8.4×10^9	5.8×10^9	7.9×10^9	6.3×10^9
RBC (cells/L)	6.52×10^{12}	8.31×10^{12}	6.93×10^{12}	7.91×10^{12}
Differential count (%)				
Segmenters	28	19	26	24
Lymphocytes	65	73	70	66
Monocytes	5	2	5	4
Eosinophils	2	2	2	2
Platelet count (cells/L)	6.07×10^{13}	4.94×10^{13}	5.91×10^{13}	5.01×10^{13}

Table 8. IC50 Values of DST, DST-NPs (F5), and Doxorubicin in various cell lines.

Cell Line	Treatment	IC50 Value (μ M)
MDA-MB231	DST	80.74
	DST-NP	43.32
	Doxorubicin	9.3
MCF-7	DST	51.45
	DST-NP	34.87
	Doxorubicin	18.05
4T1	DST	75.20
	DST-NP	40.45
	Doxorubicin	12.00

**Figure 7.** Histopathological examination of mammary Gland (a) control group: Normal architecture with adipose tissue (at) and smooth muscle layer (M), (b) disease group: Significant architectural disruption with microinvasive cancer cells (asterisk) and papillary tumor cells (arrow), (c) F5 treated group: Restored architecture (A) similar to control, and (d) DST treated group: Normal architecture with stratified squamous epithelial cells and spindle-shaped fibrous connective tissues (SG= submuscosal Gland and HF= hyperkeratosis focus).

blue-staining nuclei (asterisk) (Figure 7a). In contrast, the disease group revealed significant architectural disruption, characterized by the infiltration of microinvasive cancer cells (asterisk) and papillary tumor cells with projections (arrow), along with squamous epithelial cell transformation and the presence of cell nests (Figure 7b).

Treatment with DST-NPs (F5) restored the mammary gland architecture to a state similar to the control group, maintaining the normal appearance of adipose tissue and smooth muscle layers (Figure 7c). The standard group treated with DST also showed a normal architecture, with the addition of stratified squamous epithelial cells with keratin layers in the surrounding skin, and spindle-shaped fibrous connective tissues in the dermis layer (Figure 7d).

Liver tissue

The liver tissue in the control group displayed a typical arrangement of hepatocytes with pink-staining cytoplasm and vacuolated blue-staining nuclei (arrow), arranged hexagonally around the central vein (CV) containing red blood cells, with Kupffer cells seen in the sinusoidal spaces (asterisk) (Figure 8a). The disease group exhibited hepatocyte vacuolar degeneration, nuclear condensation, and pyknosis (arrow), along with infiltration by tumor cells of varying sizes and shapes (asterisk). The normal hexagonal arrangement of hepatocytes around the central vein was disrupted by the presence of mononuclear cells (Figure 8b).

Treatment with F5 resulted in the restoration of the normal hepatocyte architecture, resembling the control group with well-organized hexagonal hepatocytes around the central vein and Kupffer cells in the sinusoidal spaces (Figure 8c). The standard DST treatment group also showed normal hepatocyte arrangement, with minimal cell infiltration around the central vein (Figure 8d).

This histopathological analysis of the liver tissue further supports the therapeutic potential of F5, demonstrating their ability to preserve normal tissue architecture and reduce tumor-induced damage.

MTT assay results

The cytotoxic effects of DST and F5 were assessed by calculating the percentage inhibition of cell viability and determining the IC₅₀ values across MCF-7, MDA-MB231, and 4T1 cell lines shown in Table 8 and Figure 9.

Doxorubicin exhibited significantly lower IC₅₀ values compared to DST and F5, indicating its higher efficacy as an anticancer drug. Encapsulation within nanoparticles can improve the solubility and stability of DST, particularly in the intracellular environment. This enhancement ensures that a higher proportion of the drug remains active and available to exert its cytotoxic effects on cancer cells. F5 demonstrated more potent anticancer activity compared to DST alone in all cell lines tested.

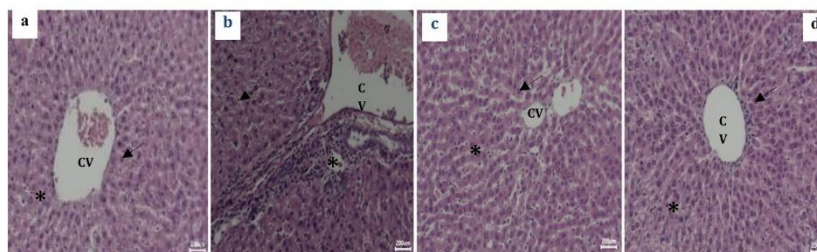


Figure 8. Histopathological examination of liver tissue- **(a)** control group: Typical arrangement of hepatocytes around the Central vein (CV) with Kupffer cells in the sinusoidal spaces (asterisk), **(b)** disease group: Hepatocyte vacuolar degeneration, nuclear condensation, pyknosis (arrow), and tumor cell infiltration (asterisk), **(c)** F5 treated group: Restored normal hepatocyte architecture similar to the control group, and **(d)** DST treated group: Normal hepatocyte arrangement with minimal cell infiltration around the Central vein.

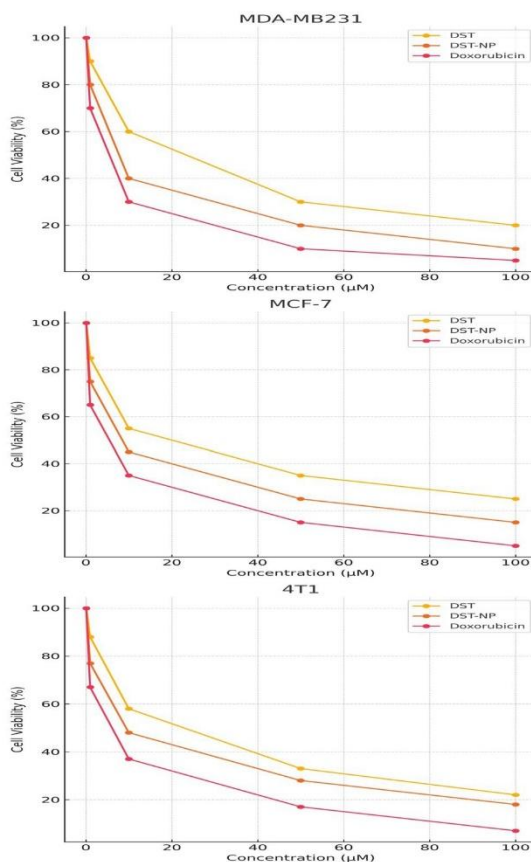


Figure 9. IC50 Values of DST, DST-NPs (F5), and Doxorubicin in various cell lines.

Discussion

The present study aimed to develop and evaluate F5 using Eudragit L100 as the polymer matrix. The findings from various characterization techniques and *in vivo* studies provide substantial evidence for the enhanced performance and potential therapeutic benefits of F5 compared to pure DST. The optimized formulation (F5) exhibited a particle size of 202.1 ± 5.7 nm, a zeta potential of -18 ± 1.01 mV, and an entrapment efficiency of $93.11 \pm 0.2\%$. The SEM and AFM images confirmed that the nanoparticles were spherical and homogenous with a smooth surface, which is essential for uniform drug release and stability (Figure 2a and 2b). The XRPD analysis showed a transition from the crystalline nature of pure DST to an amorphous form in the nanoparticles, suggesting improved solubility and bioavailability [34,35]. The *in-vitro* release studies demonstrated that F5 had a slower drug release rate compared to pure DST, with a burst release in the first hour followed by sustained release [36, 37]. This pattern is attributed to the entrapment of DST in the polymer matrix,

which controls the drug release over time. The release kinetics followed the Korsmeyer-Peppas model with an r^2 value of 0.9881 and an n value of 0.405, indicating Fickian diffusion as the primary mechanism (Table 4). Pharmacokinetic studies revealed that F5 had a higher maximum plasma concentration (C_{max}) and area under the curve (AUC_{0-t}) compared to pure DST, indicating enhanced bioavailability [38]. The nanoparticles showed a faster absorption rate, with a T_{max} of 2 h compared to 4 h for pure DST. The elimination half-life ($t_{1/2}$) and mean residence time (MRT) were higher for F5, suggesting more bioavailability and distribution (Table 7) [39].

Tissue distribution studies further confirmed the improved targeting and distribution of F5, with higher concentrations observed in the liver and spleen, and no detectable levels in the brain, reducing potential neurotoxicity risks [40] (Figure 6).

The *in vivo* efficacy of F5 was evaluated in a DMBA-induced mammary carcinoma model. F5-treated groups exhibited more stable body weights, higher survival rates, and significantly reduced tumor volumes compared to the DMBA-treated and pure DST groups. These results underscore the therapeutic advantage of the nanoparticle formulation in mitigating cancer progression and improving overall health outcomes (Figure 6). Hematologic analyses indicated that F5 had a less detrimental impact on the blood profile compared to pure DST, with higher hemoglobin levels, RBC counts, and WBC counts observed in the nanoparticle-treated groups (Table 7). Histopathological examinations showed that F5 restored normal mammary gland and liver architectures, further supporting their therapeutic efficacy and safety (Figures 7 and 8). The MTT assay demonstrated that F5 exhibited higher cytotoxicity against MCF-7, MDA-MB231, and 4T1 cell lines compared to pure DST, with lower IC50 values indicating higher potency [41]. This finding is consistent with the enhanced cellular uptake and sustained release properties of the nanoparticles.

The stability studies showed that DST-NPs maintained their particle size, zeta potential, and entrapment efficiency over six months at different storage conditions, including $4.0 \pm 1.0^\circ\text{C}$, $25 \pm 1.0^\circ\text{C}$, and $40 \pm 1.0^\circ\text{C}$. This stability is crucial for the practical application and long-term storage of the nanoparticle formulation (Table 5).

Conclusion

In conclusion, the developed DST-NPs (F5) demonstrated superior characteristics in terms of drug release, bioavailability, pharmacokinetics, and therapeutic efficacy compared to pure DST. The nanoparticles showed significant potential for enhancing the treatment of breast cancer by improving drug solubility, stability, and targeted delivery. These findings support the continued development and clinical evaluation of F5 as a promising nanocarrier system for cancer therapy.

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Authors' contributions

HG designed and optimizes the study and developed the methodology. HG, AP, performed the experiments, collection and interpretation data. HG, AP, HMG, MAMR and, SG wrote the manuscript. AP contributed to manuscript revision and provided supervision. HG, AP, HMG, MAMR and, SG read and approved the final manuscript.

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Data availability statement

The datasets of research were collected from experiments and analysis of variables during current study. These datasets are available from the corresponding author on reasonable request.

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RESEARCH ARTICLE

Development and Validation of a Bioanalytical LC-MS/MS Method for Detection of Dasatinib in Rat Plasma

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ABSTRACT

This work presents a novel method for detection of concentration of Dasatinib in rat plasma using Verapamil as internal standard by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method's pharmacokinetic application in rats was also evaluated. Separation was achieved using AB Sciex 4000 instrument, equipped with a Chromasol Gamet C18 polar analytical column (50 × 4.6 mm, 4 µm, 120 Å). The column oven was maintained at 45 ± 2°C, while the autosampler was kept at 10 ± 2°C. The mobile phase consisted of a 60:40 v/v mixture of water and methanol, delivered at flow rate of 0.8 mL/min, with 2 µL an injection volume. The liquid chromatographic procedure was carried out over a four-minute duration. The mass spectrometer operated in positive electrospray ionization molecular structure (+ESI) mode, employing multiple reaction monitoring (MRM) to detect the mass-to-charge ratio transitions for Dasatinib (m/z 488.1 → 401.0) and Verapamil (internal standard, m/z 455.6 → 165.3). were determined using multiple reaction monitoring (MRM). The concentration range for Dasatinib was 500 to 1 ng/mL, with a correlation coefficient of 0.9996. The accuracy and precision for MQC, HQC, LLQC, and LQC were 106.09 %, 107.33%, 99.83%, and 94.15%, respectively. The developed LC-MS/MS method is simple, precise, sensitive and reproducible. Henceforth it can be used to determine dasatinib.

Keywords: Dasatinib, Verapamil, Rat Plasma, LC MS/MS, Development and validation.

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INTRODUCTION

Dasatinib (BMS-354825), chemically known as N-(2-chloro-6-methylphenyl)-2-[[6-[4-(hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl]amino]-5-thiazolecarboxamide monohydrate (Fig. 1), is an effective treatment for chronic myeloid leukemia (CML) in cases where imatinib has failed (Y. Sun et al 2017). As an innovative, orally administered, multitargeted kinase inhibitor, dasatinib is effective against break point cluster region–abelson (BCR-ABL) and SRC family kinases (SFKs). Unlike imatinib and its derivative nilotinib (AMN107), dasatinib can bind to multiple conformations (both active and inactive) of the ABL kinase (J. Horinkova et al 2019) (D. Leveque et al 2020) (L. Zhu & L. Chen 2019). It is effective against 21 out of 22 BCR-ABL mutations known to cause resistance to imatinib and also strongly inhibits SFKs involved in imatinib resistance (Y. Wang et al 2020). In spite of that dasatinib is associated with several side effects including headache, nausea, diarrhea, fatigue, peripheral edema and fever. The other popular adverse events reported are diarrhea and gastrointestinal bleeding. Given these potential side effects, it is crucial to develop a reliable analytical method for determining dasatinib levels for therapeutic drug monitoring (M.N. Fomier et al 2011) (P.G. Morris et al

2018) (T.R. Vaidya, S& Ait-Oudhia 2022) (M.A. Fernandez-Peralbo et al 2014).

Bioanalysis involves studying analytes in biological samples, such as biomarkers, medications, and metabolites, through stages like data reporting, sample retrieval, and analysis. The process starts with collecting samples from clinical or preclinical research and sending them to a lab. Crucial to this is sample preparation, which ensures accuracy and reliability by removing impurities from the sample matrix. Techniques like protein precipitation, solid-phase extraction, and liquid-liquid extraction are used to isolate analytes from complex biological matrices. Proper preparation maintains the integrity of analytes and ensures the precision of bioanalytical data (European Pharmacopoeia) (The State Pharmacopoeia of Ukraine) (The State Pharmacopoeia of Ukraine).

In recent years, various research facilities have reported the utilization of high-throughput bioanalytical procedures for the assessment of antileukemia drugs. Limited number of analytical methods have been reported for dasatinib (S. De Francia et al 2009), which include high-performance thin-layer chromatography [HPTLC], high-performance liquid chromatography [HPLC], radioactive labeling methods, and a liquid chromatography–mass spectrometry

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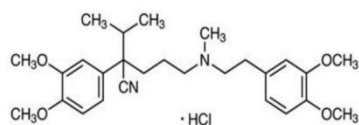


Figure 1: Molecular Structure of Dasatinib [LC-MS] (M. Clynes & R. O'Connor 2009) (M.Z. Shi et al 2019) method for plasma determination. Most approaches used to quantify pharmaceuticals have concentrated on individual agent isolation and have been

regularly applied to plasma determination or pharmaceutical analysis of the agent. The high-performance liquid chromatography-ultraviolet [HPLC-UV] (E. Kralj et al 2012) method has restricted sensitivity and specificity and required a relatively long analysis duration to achieve sufficient chromatographic separation. In contrast, the selectivity and selectivity of tandem mass spectrometry [MS/MS] (N.A. Lankheet et al 2013) have led to a growing trend of developing fast analytical methods in recent years. These advancements in MS/MS technology are particularly beneficial for creating more efficient and accurate bioanalytical procedures for antileukemia drugs like dasatinib (I. Andriamanana et al 2013) (A. Haouala et al 2009). Our objective was to develop and optimize a straightforward, user-friendly, sensitive, and specific LC-MS method for detecting dasatinib in rat plasma. This method can be effectively used in pharmacokinetic studies of dasatinib in rats following intragastric administration.

MATERIALS AND METHOD

Chemicals and reagents

Dasatinib and Verapamil reference samples were generously supplied by MSN Labs Hyderabad. Water were obtained from Thomas baker, All the chemicals, methanol, Propan-2-ol, acetonitrile suitable for LCMS analysis, were obtained from the chemical division of Rankem. The Institutional Animal Ethics Committee (IAEC) approved the experimental procedure based on IAEC No. 02/AP/2022.

Condition of liquid chromatography and mass spectroscopy:

The liquid chromatography and mass spectrometry analysis was performed using an AB Sciex 4000 instrument, equipped with a Chromasol Gamet C18 polar analytical column (50 × 4.6 mm, 4 µm, 120 Å). The column oven was maintained at 45 ± 2°C, while the autosampler was kept at 10 ± 2°C. The mobile phase consisted of a 60:40 v/v mixture of water and methanol, delivered at a flow rate of 0.8 mL/min, with an injection volume of 2 µL. The liquid chromatographic procedure was carried out over a 4 minute duration. The mass spectrometer operated in positive electrospray ionization molecular structure (+ESI) mode, employing multiple reaction monitoring (MRM) to detect the mass-to-charge ratio transitions for Dasatinib (m/z 488.1 → 401.0) and Verapamil (internal standard, m/z 455.6 → 165.3). This

Table 1: Dasatinib linearity results

Final conc. in ng/mL	Dasatinib peak area
Blank	00
1	0.366
2.5	4.720
5	20.130
10	39.133
25	53.155
50	74.240
100	89.069
250	92.250
500	99.325
Y Intercept	0.00126
Slope	0.0140
Regression coefficient (r ²)	0.9958

setup ensured precise and efficient separation and detection of the analytes, as illustrated in Figures 2 and 3. Calibration Standards and Quality Control Samples

Individual stock solutions of dasatinib (2 mg/mL) and Verapamil (IS) (2 mg/mL) were prepared in acetonitrile. Working solutions for calibration and controls were drawn up from these stock solutions by dilution with acetonitrile. A working standard solution of IS at 1.0 mg/mL was prepared by diluting the IS stock solution with acetonitrile. Quality control (QC) samples were similarly prepared to the calibration standards, with concentrations of 4000, 2000, and 30 ng/mL. All solutions were stocked at 2-8°C and brought to room temperature before use.

Dasatinib calibration standards were produced by spiking blank rat plasma with appropriate amounts of the working solutions. Calibration plots were constructed in the range of 5000–1 ng/mL for dasatinib in rat plasma, with specific concentrations of 1, 2.5, 5, 10, 25, 50, 100, 250, and 500 ng/mL. QC samples were formulated in the similar manner as the calibration standards, with plasma concentrations of 3, 200, and 400 ng/mL. Both the analytical standards and QC samples were stocked at -20°C.

Bio-analytical Method Validation:

Method validation was performed according to ICH M10 guidelines.

Selectivity

We utilized blank samples from minimum of six independent sources/ lots to evaluate selectivity. The blank sample/s are processed without including an internal standard (IS) or analyte.

Linearity

Preparing a calibration curve using the same biological matrix as the samples is essential. Separate calibration curves are required for each analyte to be measured. The method's range refers to the concentration interval within which accuracy, precision, and linearity have been demonstrated.

Accuracy and Precision

In the time of method development, it is crucial to verify the technique's suitability for validation by analyzing replicate QCs at varying in concentrations within the assay range. This involves analyzing replicate QCs at varying

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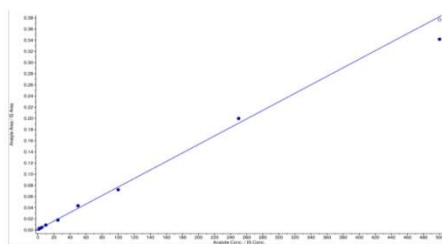


Figure 2: Representative calibration curve concentrations. Method validation studies must include minimum of six independent runs, each with a calibration

curve and several sample concentrations measured in repetition, to calibrate accuracy and precision.

Recovery

Six sets of QC samples either thawed or freshly prepared from the deep freezer. The IS was added to these samples before the injection. A 100% extraction of the analyte was accomplished by processing blank matrix samples from one single lot. These samples then injected alongside six sets of quality control dilutions at low, middle, and high concentrations. An IS was included in the process. At each QC level and for the internal standard, the %CV of recovery should be below 15.00%. The overall mean recovery %CV must be below 20.00% for each QC levels.

Matrix Effect

The matrix effect refers to a disparity in analyte reactivity caused due to interfering and sometimes undetectable components within sample matrix. To evaluate this, eight different batches of screened plasma were used to prepare two replicates of blank plasma samples. The LQC concentration was spiked with the IS using one set of eight independent blank matrices, while the HQC concentration was spiked with IS using a different set. The analysis was performed with spiked analyte(s) and IS added to the reconstitution solution to create a set of aqueous sample(s) which matched the final LQC and HQC concentrations.

Stability

Stability tests a crucial part to ensure that analyte concentration remain consistent all over sample

Table 2: Calculated concentrations obtained for precision and accuracy batches

Injections	Observed Concentration (ng/mL)			
	HQC	MQC	LQC	LLOQ QC
Standard	1.459	0.63620	0.01700	0.01050
Deviation	80			
CV	2.37	1.79	2.86	5.44
(Precision %)				
Accuracy	106.0	107.33	99.83	94.15
(%)	9			

preparation, analysis, processing and storage. To evaluate

the analyte's stability within the matrix, quality controls at both low and high concentrations are utilized. When storage conditions are established at time zero, aliquots of the quality controls are analyzed at specified intervals. It is essential to perform minimum of triple stability tests for each concentration level, time point and storage condition. According to FDA, these stability measures are recommended for biological studies. Any alteration in the analyte can affect chromatographic behavior, adding complexity to the method development process.

Application of Bio-Analytical Method to Pharmacokinetics Study:

A cohort of six Wistar rats male, weighing between 220 to 250 g, was used for the pharmacokinetic experiments. The rats were housed in ventilated cages with adequate food and water for seven days prior to the study. They were fasted overnight before the administration of the dose. A pharmacokinetic investigation of molnupiravir was conducted using this cohort. The animal study protocol was approved by the Institute of Animal Ethics Committee (registered number 02/AP/2022). A single dose of 10mg/kg Dasatinib tablet powder was administered orally to the rats. Blood samples were collected at 0, 0.5, 1, 2, 3, 6, 12 and 24 hrs hours post-dose. At each time interval, 5 mL of blood was collected into K2 EDTA vacutainer containers. Additionally, a predose sample was obtained to evaluated the potential plasma interference. The gathered samples were subjected to centrifugation to

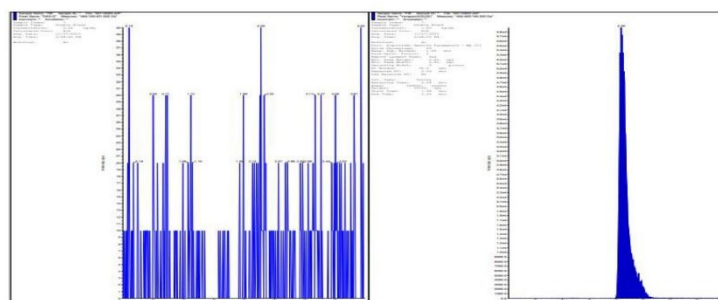


Figure 3: Representative ion chromatogram for the blank plasma (a) and LLOQ (b) with a 2 µL injection volume.

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Table 3: Recovery of Dasatinib

S.No	Observed response								
	Extra HQC	Aqs HQC	% Recovery	Extra MQC	Aqs MQC	% recovery	Extra LQC	Aqs LQC	% recovery
MEAN .	1099562.7	1291075.3	85.17	1056168.0	1324165.5	79.77	1080442.8	1326906.0	81.43
S D	31372.14	5708.78	2.480	32510.02	4376.02	2.602	32866.70	8889.17	2.432
% CV	2.85	0.44	2.91	3.08	0.33	3.26	3.04	0.67	2.99
Mean recovery	82.12								
SD	2.766								

Table 4: Dasatinib QC sample stability results using LC-MS/MS.

Stability	Nominal concentration ng/ml	Standard deviation	CV (Precision %)	% Accuracy
Bench top stability	50	1.59400	2.52	102.64
Auto sampler stability		1.40400	2.37	102.23
Short term		2.68200	4.38	105.54
Long-term (64 days)		1.59400	2.69	102.54
Freeze and thaw stability		1.38300	2.32	102.70

obtain plasma, and was stored at -80°C. Four concentrations of spiked plasma and quality control (QC) samples were analyzed. The pharmacokinetics of Dasatinib were calculated using WinNonlin (Version 5.2).

RESULTS AND DISCUSSION

Selectivity

No peaks from endogenous compounds were observed at the drug's retention time in any of the six evaluated blank plasma extracts. The responses in blank plasma were consistently below 14.9% of the signal at the LLOQ of 1 ng/mL for Dasatinib (DAS) and below 0.62% for the internal standard (IS). Figure 3 presents a representative ion chromatogram for the blank plasma (a) and LLOQ (b) with a 2 µL injection volume.

Linearity:

The seven-point calibration curve demonstrated linearity over the concentration range of 1-500 ng/mL. The calibration model, chosen based on the data, employed quadratic regression with a $1/x^2$ weighting factor. Within this concentration range, the regression coefficient (r^2) of the calibration curves consistently exceeded 0.9958, accuracy of 88-110 across the tested concentration. Quality control samples passed the accuracy of $\pm 15\%$ of theoretical value.

Accuracy and Precision

To evaluate the intra-assay precision and accuracy, six duplicates containing molnupiravir were analyzed at three different (QC) levels. The inter-assay precision was determined by analyzing these three levels of QC samples in independent runs. The proposed method's mean accuracy ranged from 94.15% to 107.33%, while the precision (%CV) for the low (LQC), middle (MQC), and high (HQC) quality controls ranged from 1.79% to 5.44%. Table 2 summarizes these findings.

Recovery

Coefficient of variation (CV) or relative standard deviations (RSD) of the responses of Dasatinib peaks of replicate injections of aqueous samples are varying

between 0.33 % to 0.67 %. Coefficient of variation (CV) or relative standard deviations (RSD) of the response of

Table 5: Pharmacokinetic parameters from the noncompartmental model analysis

PK Parameter	Unit	Value
$t_{1/2}$	h	3.97
T_{max}	h	4.00
C_{max}	ng/ml	8.50
AUC	ng/ml*h	66.06

Dasatinib peaks of extracted samples are varying between 2.85 % to 3.08%. The average recoveries of the Dasatinib at all three concentration levels ranged from 79.77 % to 85.17 %. The mean recovery of Dasatinib is 82.12 %.

Matrix effect

The back-calculated concentrations for the high-quality control (HQC) and low-quality control (LQC) levels showed mean accuracies of 99.86% and 98.76%, respectively. As these results met the acceptance criteria of 85.00% to 115.00%, the current method demonstrated no significant ionization effects.

Stability

To evaluate stability of Dasatinib in plasma, tests were conducted using six replicates of quality control samples at both low and high concentrations. Appropriate volumes of Dasatinib standard solutions were added to create drug-free plasma samples. The results, shown in Table 4, fell within the acceptable range, indicating that Dasatinib demonstrates favorable stability.

Pharmacokinetic Application:

The method was attempted on a pharmacokinetic study in rats. The main pharmacokinetic parameters which followed noncompartmental model analysis been summarized in Table 4. The mean plasma concentration-time curve following the oral administration of a single 10 mg/kg dose of dasatinib is presented in Figure 4.

CONCLUSION

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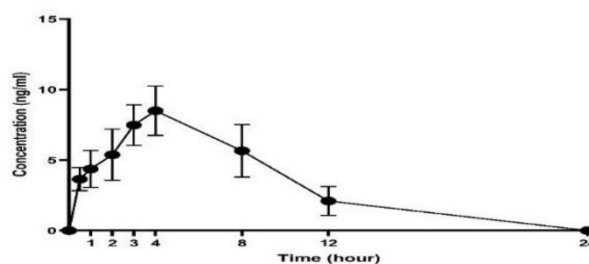


Figure 4: The mean plasma concentration–time curve following the oral administration of dasatinib.

A specific, straightforward, and sensitive LC-MS method with gradient elution were developed and validated for determining dasatinib in rat plasma in the concentration range of 1-500 ng/ml. This method was validated to meet the requirements for the pharmacokinetic determination of dasatinib in rat plasma.

Acknowledgments

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