



Silybum Marianum-based Nanoformulations for Enhanced Hepatoprotective and Anticancer Activity

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Abstract

Purpose The poor solubility, low bioavailability, and rapid degradation of bioactive compounds such as silybin and curcumin limit their therapeutic potential. This study aims to develop a novel nanoformulation by coencapsulating silybin and curcumin with *Silybum marianum* seed extract, a natural encapsulant, to increase their stability, bioavailability, and therapeutic efficacy for hepatoprotection and anticancer applications.

Methods Silybin and curcumin were encapsulated via a nanoprecipitation method. The formulation was characterized for its particle size, zeta potential, encapsulation efficiency, and in vitro release kinetics. Molecular docking studies were conducted to evaluate interactions with key proteins (Nrf2, COX-2, TNF- α , P53, and AKT1) involved in oxidative stress, inflammation, and cancer. Antimicrobial activity was assessed against mastitis-related pathogens via the agar well diffusion and broth dilution methods. Cytotoxicity was evaluated in the MCF-7 (breast cancer) and HepG2 (liver cancer) cell lines via the MTT assay.

Results The nanoformulation resulted in a mean particle size of 191.00 ± 2.25 nm, a zeta potential of -32.03 ± 1.63 mV, and high encapsulation efficiencies for silybin ($85.5 \pm 2.1\%$) and curcumin ($82.3 \pm 1.8\%$). Sustained release profiles were observed, with 98.1% and 89.1% cumulative release for silybin and curcumin, respectively, over 48 h. Molecular docking revealed strong binding affinities, particularly for silybin with AKT1 (-11.9 kcal/mol) and for curcumin with COX-2 (-8.4 kcal/mol). The formulation demonstrated enhanced antimicrobial activity, with a minimum inhibitory concentration (MIC) of 20.90 ± 5.20 μ g/mL against *E. coli*, and superior anticancer efficacy, with an IC₅₀ of 81.11 ± 1.95 μ g/mL in MCF-7 cells.

Conclusion This study presents a novel, natural nanoformulation with dual hepatoprotective and anticancer potential. The use of *Silybum marianum* seed extract as both a bioactive encapsulant and a therapeutic synergist offers a sustainable and effective alternative to synthetic drug delivery systems, highlighting its potential for clinical applications in liver diseases and cancer therapy.

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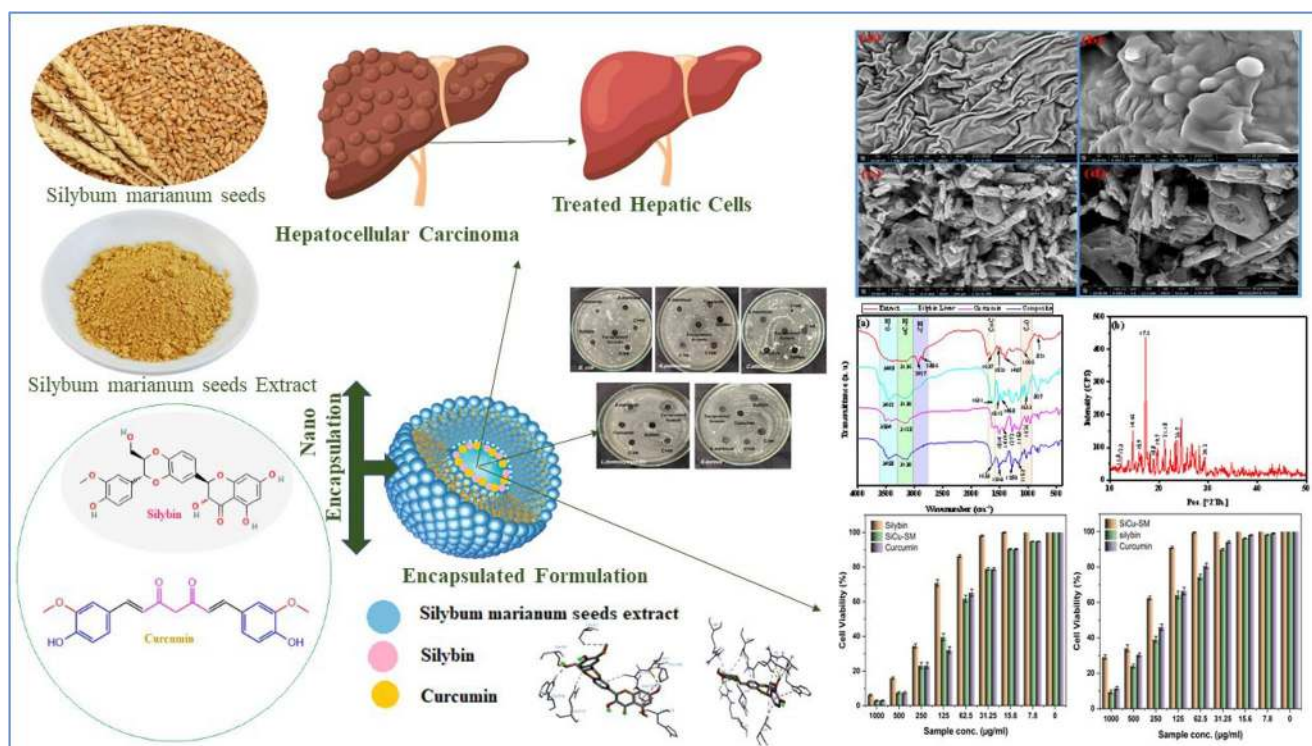
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Graphical Abstract



Keywords Nanoencapsulation · Hepatoprotection · Anticancer efficacy · Silybum marianum · Silybin-curcumin synergy · Bioavailability enhancement · Molecular docking · Sustained drug release

Abbreviations

SIF	Simulated Intestinal Fluid
SGF	Simulated Gastric Fluid
SM	Silybum marianum Seed Extract
Si	Silybin
Cu	Curcumin
SiCu-SM	Silybin–Curcumin Encapsulated in Silybum marianum Seed Extract
PBS	Phosphate-Buffered Saline
DLS	Dynamic Light Scattering
PDI	Polydispersity Index
FTIR	Fourier Transform Infrared Spectroscopy
XRD	X-ray Diffraction
SEM	Scanning Electron Microscopy
MIC	Minimum Inhibitory Concentration
IC ₅₀	Half Maximal Inhibitory Concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
DMEM	Dulbecco's Modified Eagle Medium
RPMI-1640	Roswell Park Memorial Institute Medium 1640

DMSO	Dimethyl Sulfoxide
UV–Vis	Ultraviolet–Visible Spectrophotometry
Nrf2	Nuclear Factor Erythroid 2–Related Factor 2
COX-2	Cyclooxygenase-2
TNF- α	Tumor Necrosis Factor Alpha
HCC	Hepatocellular Carcinoma
NAFLD	Non-Alcoholic Fatty Liver Disease

Introduction

The growing interest in advanced drug delivery systems has underscored their potential to overcome the limitations of conventional formulations, particularly for bioactive compounds with poor solubility, low bioavailability, and rapid degradation. Among these systems, nanoencapsulation has emerged as a transformative approach that enhances the stability, bioavailability, and controlled release of therapeutic agents. This study investigated the nanoencapsulation of two powerful bioactive compounds, silybin and curcumin,

using *Silybum marianum* seed extract as a natural encapsulant. This approach offers a promising solution to the limitations of traditional drug formulations, particularly in the areas of hepatoprotective and anticancer therapies. Silybin, the main active component of *Silybum marianum* (milk thistle), is well known for its liver-protective effects. It has been extensively researched for its ability to shield the liver from oxidative stress, toxins, and inflammation, showing considerable potential in the treatment of liver diseases such as hepatitis, cirrhosis, and nonalcoholic fatty liver disease (NAFLD) [1, 2]. It exerts its effects by reducing oxidative stress, promoting detoxification, and enhancing liver regeneration [3]. Curcumin, the active compound in *Curcuma longa* (turmeric), is widely recognized for its anticancer, anti-inflammatory, and antioxidant properties. It has demonstrated cytotoxic effects on various cancer cells by targeting and inhibiting crucial molecular pathways involved in cell proliferation, apoptosis, and metastasis [4, 5]. Furthermore, curcumin has demonstrated the ability to sensitize cancer cells to chemotherapy and radiotherapy, highlighting its potential as an adjunctive therapy in cancer treatment [6]. Despite their promising therapeutic potential, both silybin and curcumin are hindered by poor water solubility and low bioavailability, limiting their clinical effectiveness. Nanoencapsulation offers a solution by encapsulating these compounds in nanosized particles, which act as protective carriers. These nanoparticles safeguard the active compounds from degradation, oxidation, and metabolism, thereby improving their stability and increasing their bioavailability [7, 8]. The small particle size of the nanoparticles further enhances their ability to be efficiently taken up by cells, ensuring that the encapsulated compounds can reach their target sites with greater precision and effectiveness. A critical component of the encapsulation process is the use of natural extracts, such as *Silybum marianum* seed extract. This extract contains a range of bioactive molecules, including saponins, flavonoids, and glycosides, which possess natural nanocarrier properties, making it ideal for enhancing the delivery of therapeutic compounds [9–11]. Critically, beyond its role as a carrier, the extract itself possesses documented biological activities, suggesting its function in the final formulation is not merely structural but also therapeutic. The biocompatible and biodegradable components of *Silybum marianum* seed extract interact with silybin and curcumin to form stable nanoassemblies through hydrophobic, electrostatic, and hydrogen bonding. This natural encapsulating agent provides a sustainable alternative to synthetic polymers and may enhance therapeutic effects through synergistic interactions between the compounds and the bioactive molecules in the extract. Combining silybin and curcumin in a single formulation offers a synergistic approach for treating liver diseases and

cancer, with silybin providing hepatoprotection through antioxidant and anti-inflammatory activities, whereas curcumin targets cancer cells by inducing apoptosis and cell cycle arrest [12]. When encapsulated together, these two compounds can work in concert to enhance liver regeneration, reduce oxidative damage, and suppress tumor growth. Recent studies suggest that the combined use of silybin and curcumin can significantly increase their therapeutic potential, especially when these compounds are encapsulated in nanoparticles [13]. The nanoparticle formulation enhances the bioavailability of both silybin and curcumin while ensuring controlled release for prolonged therapeutic effects. This dual-target approach is particularly beneficial for conditions such as hepatocellular carcinoma (HCC), where liver damage and cancer progression are often linked. The novelty of this study lies in the integration of natural bioactive compounds (silybin, curcumin) with a multifunctional natural agent (*Silybum marianum* seed extract) that serves as both a bioactive encapsulating matrix and a source of synergistic therapeutic effects. While liposomal and polymeric nanoparticles have been widely explored for the delivery of these compounds, this approach offers a new, sustainable alternative [7, 14, 15]. Research on the use of *Silybum marianum* seed extract as a nanoencapsulation carrier is limited, yet it holds significant potential. This natural extract not only serves as a biodegradable and biocompatible carrier but also may enhance the therapeutic properties of encapsulated compounds through synergistic interactions with bioactive molecules. The combination of silybin and curcumin in a single formulation offers a synergistic approach to address both liver damage and cancer progression, potentially reducing the side effects associated with conventional treatments such as chemotherapy. This formulation ensures controlled release, promoting gradual delivery of bioactive compounds for improved therapeutic efficacy, fewer side effects, and better patient compliance. Such an approach could have a transformative impact on the treatment of liver cancer and other liver diseases, offering a more effective, sustainable, and natural alternative.

Experimental Section

Materials

Organic Milk Thistle Seeds (*Silybum Marianum* Seed) were purchased from Agro Food Processing Emporium (Rawalpura, India). Silybin with a purity of 99.35% was purchased from ©Tokyo Chemical Industry Pvt. Ltd. (India). Curcumin (98.53% purity) was obtained from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. Ethanol was purchased from Merck, Darmstadt, Germany, and sodium

chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide, and hydrochloric acid (37%) were obtained from Scharlau (Barcelona, Spain). Distilled water was used. Dimethyl sulfoxide (DMSO), MTT, and trypan blue dye were purchased from Sigma (St. Louis, MO, USA). Additionally, fetal bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin, and 0.25% trypsin-EDTA were purchased from Lonza (Belgium).

Preparation of Silybum Marianum Seed Extract (SM, Encapsulating Material)

The *Silybum marianum* seeds were cleaned and dried to remove any contaminants, and the dried seeds were ground into a fine powder via a Retsch ZM 200 Ultra-Centrifugal Mill (Haan, Germany). Then, 25 g of powdered seeds were soaked in 100 mL of 70% ethanol and stirred continuously for 6 h at 35 °C. The extract was filtered with Whatman filter paper to remove any insoluble residues, and the prepared extract was concentrated with a Henan Yuhua Instrument RE-2000 A rotary evaporator (Zhengzhou, China) at 40 °C.

Preparation of the Silybin-curcumin Encapsulation Mixture

One hundred milligrams of pure silybin (99.35% purity) and 100 mg of pure curcumin (98.53% purity) were accurately weighed. Both compounds were dissolved in 10 mL of *Silybum marianum* seed extract, which serves as the natural encapsulating matrix. The mixture was sonicated for 30 min to ensure complete dispersion and then stirred for 2 h at room temperature. It is important to note that the *Silybum marianum* seed extract itself contains a complex mixture of bioactive compounds, including silymarin flavonoids. The addition of pure silybin was to standardize and augment the total silybin content in the final nanoformulation, ensuring a precise and therapeutically relevant dose for our experimental studies. We refer to this as the organic phase.

Preparation of Encapsulated Silybin-curcumin (SiCu-SM)

The nanoprecipitation method was conducted by preparing 100 mL of phosphate-buffered saline (PBS) at pH 7.4 by dissolving 0.8 g of sodium chloride, 0.02 g of potassium chloride, 0.144 g of disodium hydrogen phosphate, and 0.024 g of potassium dihydrogen phosphate in 100 mL of distilled water and adjusting the pH via 1 M sodium hydroxide or 1 M hydrochloric acid at pH 7.4; we refer to this phase as the aqueous phase. Phosphate-buffered saline (PBS, pH 7.4) was specifically chosen as the aqueous phase because it closely

mimics physiological conditions, providing an isotonic and biocompatible environment that helps maintain the stability and structural integrity of both the nanoparticles and the encapsulated bioactive compounds during preparation. The use of PBS ensures that the nanoformulation process occurs under conditions similar to those encountered in biological systems, which is essential for the reproducibility, compatibility, and potential biomedical application of the resulting nanoparticles. Additionally, PBS maintains a stable pH during nanoprecipitation, which is critical for the encapsulation efficiency and overall stability of silybin and curcumin. The organic phase was slowly added dropwise into the aqueous phase with continuous stirring. Then, the prepared solution was centrifuged at 10,000 rpm for 20 min to separate the encapsulated particles. The precipitate was removed after the supernatant was transferred to a different flask to be used later in the encapsulation efficiency assessment. The precipitate was allowed to dry in a vacuum oven under a gentle oven at 38 °C.

Determination of the Encapsulation of Silybin (Si) and Curcumin (Cu) in *S. Marianum* Seed Extract (EE%)

The encapsulation efficiency of silybin and curcumin within the *Silybum marianum* seed extract matrix was determined using a validated two-step method designed to accurately separate and quantify both free and encapsulated drug fractions. Standard calibration curves were first established by preparing serial dilutions of pure silybin and curcumin in methanol. The absorbance of these standards was measured using an Evolution 350 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at the pre-determined maximum absorbance wavelengths of 288 nm for silybin and 421 nm for curcumin. Methanol was selected as the solvent due to its high efficiency in dissolving both compounds, favorable UV transparency at the analytical wavelengths, and established utility in extracting hydrophobic molecules from nanocarrier systems [16–19].

For the encapsulation efficiency analysis, a 1 mL aliquot of the nanoformulation suspension was centrifuged at 14,000 rpm for 30 min at 4 °C to separate the nanoparticles from the aqueous medium. The supernatant, containing the free (unencapsulated) drugs, was carefully collected and analyzed to determine the concentration of non-encapsulated silybin and curcumin. To quantify the encapsulated fraction, the nanoparticle pellet was resuspended in 2 mL of methanol and subjected to probe sonication (Q700 Sonicator®, Australia) in an ice bath for 15 min at 40% amplitude to ensure complete disruption of the nanocarrier matrix and release of the encapsulated compounds. The resulting suspension was centrifuged at 14,000 rpm for 10 min to remove

insoluble residues, and the supernatant was analyzed to determine the concentration of encapsulated silybin and curcumin.

The encapsulation efficiency (EE%) was calculated using Eqs. (1) and (2), where the encapsulated drug represents the amount quantified directly from the dissolved nanoparticle pellet, and the total drug added represents the mass of pure compound incorporated during formulation preparation:

$$\begin{aligned} & \text{Encapsulation efficiency for silybin\%} \\ &= \frac{\text{Amount of encapsulated silybin}}{\text{Total amount of silybin added}} \times 100 \end{aligned} \quad (1)$$

$$\begin{aligned} & \text{Encapsulation efficiency of curcumin\%} \\ &= \frac{\text{Amount of encapsulated curcumin}}{\text{Total amount of curcumin added}} \end{aligned} \quad (2)$$

It is important to note that this method specifically quantified the encapsulation of the weighed, pure silybin and curcumin standards added during formulation. The total silymarin content originating natively from the *Silybum marianum* seed extract was not separately analyzed in this study.

Characterization of the Encapsulated Particles

The physical and chemical properties of the encapsulated and nonencapsulated *Silybum marianum* seed extract formulations were thoroughly characterized via a range of analytical techniques. The particle size, polydispersity index (PDI), and zeta potential were measured with a Zetasizer Ultra (Malvern, USA) via dynamic light scattering (DLS) and electrophoretic light scattering. These measurements provided insights into the particle size distribution, surface charge, colloidal stability, and homogeneity of the nanoformulations [20]. The encapsulation efficiency of silybin and curcumin was determined via a Thermo Evolution 350 UV–Vis spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) as described in Sect. "Determination of the Encapsulation of Silybin (Si) and Curcumin (Cu) in *S. Marianum* Seed Extract (EE%)".

To further assess the structural and morphological characteristics of the nanoparticles, X-ray diffraction (XRD) analysis was performed via a Panalytical Empyrean diffractometer (Cu-K α radiation, $\lambda = 0.154$ nm, 35 mA, 40 kV) scanning from 5° to 80° (2 θ) at a rate of 8° min⁻¹.

Crystallite sizes were evaluated via Scherrer's Eq. (3):

$$D = \frac{K\lambda}{\beta \cos\theta} \quad (3)$$

where D is the crystallite size, λ is the X-ray wavelength (0.071069 nm), θ is the Bragg angle, and K is the Scherrer constant (0.85). Lattice constants were determined through Rietveld refinement via SmartLab Studio II software (Rigaku). The % crystallinity of the prepared composite was calculated via Eq. (4) [21, 22]:

$$\% \text{ Crystallinity} = \frac{\text{Area of crystalline peaks}}{\text{Total area under XRD pattern}} \times 100 \quad (4)$$

Fourier transform infrared (FTIR) spectroscopy was conducted via a Bruker-Vertex 70 spectrometer (Germany) in the range of 4000–400 cm⁻¹ to investigate potential interactions between the encapsulated compounds and the seed extract matrix. The surface morphology and particle shape were examined by scanning electron microscopy (SEM).

These complementary techniques enabled comprehensive evaluation of the size, surface charge, encapsulation efficiency, crystallinity, chemical interactions, and morphology of the encapsulated silybin-curcumin nanoformulation.

In Vitro Release Study

In this study, *Silybum marianum* seed extract was used as the natural encapsulating matrix for both silybin and curcumin. The extract, rich in bioactive molecules such as saponins, flavonoids, and glycosides, acts as a biocompatible and biodegradable carrier, forming stable nanoassemblies with the active compounds during the nanoprecipitation process. Thus, the term "silybin and curcumin encapsulated in *Silybum marianum* seed extract" refers to a nanoformulation in which the seed extract serves as the encapsulating material rather than merely as a co-ingredient or solvent.

The in vitro drug release of silybin and curcumin encapsulated in *Silybum marianum* seed extract was evaluated via the dialysis bag method to simulate controlled drug release. First, the dialysis bags were soaked in the dissolution (release) medium overnight. Then, 100 mg of encapsulated formulation (encapsulated silybin and curcumin) and corresponding amounts of free silybin and curcumin (as a control) were placed in dialysis bags made of cellulose membrane (molecular weight cutoff of 14,000 Da, SERAVA Electrophoresis) that were sealed at both ends with thermo-resistant thread.

These bags were immersed in a glass jar containing 900 mL of a phosphate buffer solution (PBS, 50 mM, pH 6.8) at 37 °C $\pm$ 0.5 °C. This enzyme-free medium was specifically selected to serve as a Simulated Intestinal Fluid (SIF) for the purpose of evaluating the intrinsic release kinetics of the nanoformulation. The use of this

controlled system allows for the precise assessment of drug release mechanisms, such as diffusion and matrix erosion, without the confounding variable of enzymatic degradation that is present in more complex biorelevant media. The PBS was prepared by dissolving 0.68 g of monobasic potassium phosphate (KH_2PO_4) and 7.7 mL of 0.2 N NaOH in distilled water, adjusting the final volume to 100 mL, and fine-tuning the pH to 6.8 using NaOH or HCl. The resulting buffer closely simulates the pH of the intestinal environment, which is the primary site for drug absorption.

Simulated gastric fluid (SGF, pH 1.2) was not used because curcumin is unstable under highly acidic conditions and may degrade. The jar was then placed in a shaker incubator (Labsol, India), and it was set at 50 rpm. At predefined time intervals (10 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, and 48 h), 5 mL of the medium surrounding the dialysis bag was withdrawn and replaced with an equal volume of fresh, pre-warmed PBS to maintain sink conditions. Each sample was filtered through a 0.22 μm nylon syringe filter to remove any nanoparticles that might have passed through the dialysis bag. The concentrations of silybin and curcumin released into the surrounding medium were measured via a UV–Vis spectrophotometer at 288 nm for silybin and at 421 nm for curcumin.

The cumulative drug release percentage was calculated by plotting the concentration of released silybin and curcumin against time, allowing for an evaluation of the release profile of the encapsulated silybin and curcumin from *Silybum marianum* seed extract compared with the free drug. This method reliably simulates the controlled release of drugs from nanoparticle-based formulations and is particularly useful for studying sustained-release profiles [23–25]. Owing to its efficient diffusion-based release mechanism, the dialysis bag technique is ideal for studying the release of drug-loaded nanoparticles and is particularly advantageous for examining formulations aimed at overcoming drug resistance [26, 27]. Finally, the cumulative drug release (y-axis) was plotted against time (x-axis) to obtain the release profiles of both silybin and curcumin. Drug release at time t and the cumulative drug release (%) were calculated via Eqs. (5) and (6):

$$\text{Released Drug at time } t = C_t * V \quad (5)$$

where C_t represents the drug concentration at time t (mg/mL) and V represents the volume of the dissolution medium (mL).

$$\begin{aligned} \text{Cumulative Drug release (\%)} \\ = \frac{\text{Released Drug at time}}{\text{initial amount of Drug loaded}} \times 100 \end{aligned} \quad (6)$$

In vitro Release Kinetics

Various release kinetics models have been applied, including zero-order kinetics (constant release rate), first-order kinetics (the release rate depends on the amount of drug remaining), the Korsmeyer–Peppas model (power law), and the Higuchi model (diffusion-controlled release), to elucidate the release mechanism through diffusion, swelling, or erosion of the encapsulated formulation via the following equations:

$$\text{Zero – order kinetics } M_t = M_0 + Kt \quad (7)$$

$$\text{First – order kinetics } \frac{M_t}{M_\infty} = 1 - e^{-k_1 t} \quad (8)$$

$$\text{Korsmeyer – Peppas Model } \frac{M_t}{M_\infty} = Kt^n \quad (9)$$

$$\text{Higuchi model } M_t = k_H \sqrt{t} \quad (10)$$

M_t = amount of drug released at time t , M_∞ = initial amount of drug, k_1 = first-order rate constant, n is the release exponent that indicates the release mechanism, t = time, k_H = Higuchi rate constant (depending on properties such as the diffusion coefficient, solubility, and surface area).

Molecular Docking

While the individual binding interactions of silybin and curcumin with various biological targets have been explored in prior literature, the primary objective of this docking study was not to replicate these established findings. Instead, it was conducted to build a coherent, multi-target theoretical model that provides a mechanistic rationale for the observed synergistic efficacy of our novel co-encapsulated nanoformulation. Silybin and curcumin are natural compounds with well-documented anti-inflammatory and anticancer properties. Extensive research has demonstrated their ability to modulate key signaling pathways involved in inflammation, oxidative stress, and cancer progression [28, 29]. This molecular docking study focused on the interactions of these proteins with five critical proteins: Nrf2, COX-2, TNF- α , P53, and AKT1. These proteins

were selected because of their central roles in disease pathogenesis, making them ideal targets for evaluating the therapeutic potential of silybin and curcumin. Nrf2 (nuclear factor erythroid 2-related factor 2) is a key regulator of antioxidant proteins that protects against oxidative damage, with dysregulation linked to inflammation and cancer [30]. COX-2 (cyclooxygenase-2) is an enzyme that promotes inflammation and tumor growth, and its inhibition helps prevent cancer progression [31, 32]. Tumor necrosis factor- α (TNF- α) is a cytokine involved in inflammation and the immune response, with high levels contributing to chronic diseases and cancer; targeting it can reduce tumor growth [33]. P53 (tumor suppressor protein 53) is a tumor suppressor that controls the cell cycle and apoptosis, and its loss or mutation is common in cancers, making its restoration a potential therapeutic approach [34]. Protein kinase B (AKT1) promotes cell survival and growth, and its overactivation is associated with cancer; thus, inhibiting AKT1 may suppress tumor development and enhance cancer treatments [35].

Ligand Preparation

The structures of silybin and curcumin were retrieved from the PubChem database in SDF format. The 3D structure was prepared for docking as a ligand via Chem3D 12.0 software, including energy minimization with the MMFF94 force field.

Protein Preparation

The structures of the Nrf2 (PDB ID: 7 $\times$ 5 F), COX-2 (PDB ID: 3MQE), TNF- α (PDB ID: 2AZ5), P53 (PDB ID: 1TUP) and AKT1 (PDB ID: 3O96) proteins were retrieved from the RCSB Protein Data Bank. The proteins were prepared for docking via AutoDock Tools 1.5.7 software [36], which involves removing water molecules, adding polar hydrogens, and assigning a Kollman charge.

Molecular Docking Methodology

Molecular docking studies were performed via PyRx 0.8 software, a virtual screening tool that integrates AutoDock and AutoDock Vina for predicting protein–ligand interactions. A blind docking approach was utilized to identify potential binding sites across the entire surface of the target protein. The docking grid was configured to encompass the entire protein structure, with grid box dimensions automatically calculated by PyRx to ensure comprehensive coverage. Docking simulations

were executed via the AutoDock Vina algorithm, which employs a stochastic search method to predict optimal ligand binding poses. The exhaustiveness parameter was set to 8.

Visualization and Analysis

The docked complexes were visualized and analyzed via the BIOVIA Discovery Studio Visualizer (v24.01.23298). Binding affinities (ΔG values) were assessed, and key intermolecular interactions, such as hydrogen bonds and hydrophobic interactions, were identified and reported to provide insights into ligand–protein binding.

Evaluation of the Antimicrobial Activities of the Tested Compounds against Mastitis-Related Microbial Strains

Preparation of the Microbial Strains

The in vitro antimicrobial activities of curcumin, *Silybum marianum* extract, silybin powder, and their encapsulated formulations were evaluated against five microbial species isolated from patients with clinical mastitis. These species included the yeast *Candida albicans* (*C. albicans*), the gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) and *Listeria monocytogenes* (*L. monocytogenes*), and the gram-negative bacteria *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*). Conventional biochemical methods were used to identify each isolate. The microbial strains were stored as frozen stocks at -80°C with 15% glycerol. Before testing, the strains were streaked onto the surface of Tryptic Soya Agar (TSA, Oxoid, Catalog number: CM0131B) plates and incubated at 37°C for 24 h. Additionally, the strains were regenerated in tryptone soya broth (TSB, Oxoid, Catalog number: R118881) for 24 h. After incubation, two to three colonies from each microbial species were suspended in 5 mL of phosphate-buffered saline (PBS, 0.9%) to achieve a final concentration of 1.5×10^8 colony-forming units per milliliter (CFU/mL), corresponding to 0.5 McFarland standards.

Preparation of Chemical Suspensions for Antimicrobial Testing

First, suspensions of the tested chemicals at a concentration of 1000 $\mu\text{g/mL}$ were prepared by dissolving them in 10.0% dimethyl sulfoxide (DMSO). The solutions were then sonicated for 5–10 min at a frequency of 20 kHz using a Q700 Sonicator[®] (Australia) to ensure thorough mixing and uniform dispersion.

Agar Well Diffusion Method

The 0.5 McFarland of the tested microbial inoculum was evenly cultured on the surface of Mueller–Hinton agar (MHA, Sigma–Aldrich, Dorset, UK). A sterile corker was used to generate wells with a diameter of 6 mm, and each well was loaded with the material at a concentration of 1000 µg/mL. After that, the agar plates were incubated for 24 h at 37 °C. The diameters were measured in millimeters (mm), and the zone of inhibition was recorded. For every sample, three replicates were performed, and the means were determined [37]. Furthermore, gentamicin (10 µg/mL) and amphotericin B (10 µg/mL) were used as positive controls, and 10.0% DMSO was used as a negative control in the study of bacterial pathogens and *C. albicans*., respectively.

Minimum Inhibitory Concentrations (MICs)

The broth macrodilution method was used to estimate the minimum inhibitory concentrations (MICs) of the investigated compounds against the strains mentioned above. First, 500 mL of Mueller–Hinton broth (MHB) was autoclaved and placed into each sterile glass test tube in a volume of one mL. Then, 1 millilitre of each chemical was diluted twice until it reached ten dilutions as follows: 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.9, and 0.97 µg/mL. Additionally, each dilution was injected with 100 µL of the previously prepared strain and incubated for 24 h at 37 °C. Following incubation, the absence of turbidity in the screened tubes, which indicated the suppression of microbial growth, was used to assess the antibacterial properties of the screened compounds, and the MIC results were the minimal dilution that resulted in no turbidity [38]. Additionally, to determine the minimum bactericidal concentrations (MBCs) and minimum fungicidal concentrations (MFCs), TSA plates were seeded with approximately 10 µL of inoculum from each tube that showed no evidence of growth, and the plates were then incubated at 37 °C overnight. Plates that exhibited >99.9% no apparent development of the tested pathogens at the lowest concentration of antimicrobial material were referred to as the MBC or MFC thresholds [39]. For every sample, three replicates were performed, and the means were investigated.

Statistical Analysis

All in vitro release experiments were performed in triplicate ($n=3$) under controlled conditions. The data are presented as the means $\pm$ standard deviations (SDs). Statistical analysis was performed via GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Two-way analysis of variance (ANOVA) was used to compare release profiles

between encapsulated and unencapsulated formulations across different time points, followed by Tukey's multiple comparison test for post hoc analysis. Kinetic model parameters were compared via unpaired t tests. Statistical significance was set at $p<0.05$. Confidence intervals (95% CI) were calculated for all kinetic parameters.

In Vitro Cytotoxicity and Cell Viability

Cell Lines and Culture Conditions

MCF-7 cells, a human breast cancer cell line, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cell lines were propagated and tested for cytotoxicity at the Tissue Culture Unit of the Regional Center for Mycology and Biotechnology, Al-Azhar University. These cells were propagated in RPMI-1640 medium (Lonza, Belgium) supplemented with 10% inactivated fetal bovine serum (FBS) and 50 µg/mL gentamycin (Lonza, Belgium). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and subcultured two to three times per week. HepG2 cells, a human hepatocellular carcinoma cell line, were also obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50 µg/ml gentamycin. All the cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ and subcultured two times a week.

Cytotoxicity Evaluation Via the MTT Assay

To evaluate the cytotoxicity of the tested compounds, MCF-7 cells were cultured in Corning® 96-well tissue culture plates at a density of 5×10^4 cells per well and incubated for 24 h. After this period, the cells were treated with various concentrations of the test compounds in triplicate for an additional 24 h. Six vehicle controls, consisting of media, were included on each plate. Following incubation, the media was removed and replaced with 100 µL of fresh RPMI-1640 medium (without phenol red). To assess cell viability, 10 µL of a 12 mM MTT stock solution (5 mg of MTT in 1 mL of PBS) was added to each well, and the plates were incubated at 37 °C with 5% CO₂ for 4 h. After incubation, 85 µL of the medium was removed, and 50 µL of DMSO was added to each well to dissolve the formazan crystals. The plates were then thoroughly mixed and incubated at 37 °C for an additional 10 min. The absorbance was measured at 590 nm via a microplate reader (SunRise, TECAN, Inc., USA). Cell viability was calculated as a percentage of the optical density (OD) of treated cells relative to the OD of untreated control cells via the following equation:

$$\text{Percentage of Viability} = \left[\frac{\text{ODt}}{\text{ODc}} \right] \times 100\% \quad (11)$$

Cytotoxicity Evaluation Via the MTT Assay

where ODt is the mean optical density of the treated wells and where ODc is the mean optical density of the untreated wells. Survival curves were constructed by plotting the percentage of viable cells against the drug concentration. The 50% inhibitory concentration (IC₅₀), defined as the concentration required to inhibit 50% of viable cells, was calculated from the dose–response curve via GraphPad Prism software (San Diego, CA, USA).

For the HepG2 cytotoxicity assay, the cells were seeded at a concentration of 1×10^4 cells per well in 100 μL of growth medium in a 96-well plate. After 24 h, 100 μL of the test sample was added to the confluent cell monolayers. Three wells were used for each test concentration, while control cells were incubated without the test sample. The microplates were then incubated at 37 °C with 5% CO₂ for 24 h. After 24 h of incubation, cell viability was determined via the MTT assay. The media was removed and replaced with 100 μL of fresh DMEM (without phenol red). Next, 10 μL of a 12 mM MTT stock solution (5 mg of MTT in 1 mL of PBS) was added to each well, including the untreated controls. The plates were incubated at 37 °C with 5% CO₂ for 4 h. After incubation, 85 μL of the media was removed, and 50 μL of DMSO was added to each well to dissolve the formazan crystals. The wells were mixed thoroughly and incubated at 37 °C for 10 min. The optical density was measured at 590 nm via a microplate reader (TS800, BioTek, Inc., USA). Cell viability was calculated via the

same formula as above, and survival curves were plotted by correlating surviving cells with drug concentration. The cytotoxic concentration (CC₅₀), which is the concentration required to induce toxic effects in 50% of intact cells, was determined from the dose–response curve via GraphPad Prism software (San Diego, CA, USA).

Results and Discussion

Characterization of the Encapsulated Formulation

To investigate the surface functional groups, FT-IR analysis was performed on the extract, silybin, curcumin, and composite over a spectral range of 400–4000 cm^{-1} , as illustrated in Fig. 1(a). The FT-IR spectrum of the extract exhibited a broad absorption peak at approximately 3445 cm^{-1} , corresponding to O–H stretching vibrations of alcoholic phytoconstituents. The 3182 cm^{-1} band is attributed to =C–H stretching, confirming the presence of hydroxyl functional groups. The peak at 2924 cm^{-1} corresponds to C–H stretching vibrations, whereas the absorption at 2850 cm^{-1} indicates significant variations in –CH₂– bending. Additionally, the 1644 cm^{-1} band is linked to combined amide (C=O) stretching and alkene (C=C) vibrational modes, whereas the two distinct infrared bands at 1510 cm^{-1} and 1469 cm^{-1} correspond to C–C stretching modes in the phenyl ring structure. The absorption peak at 1084 cm^{-1} is attributed to benzopyran ring vibrations, whereas the band at 821 cm^{-1} represents C–H bending motions [40, 41].

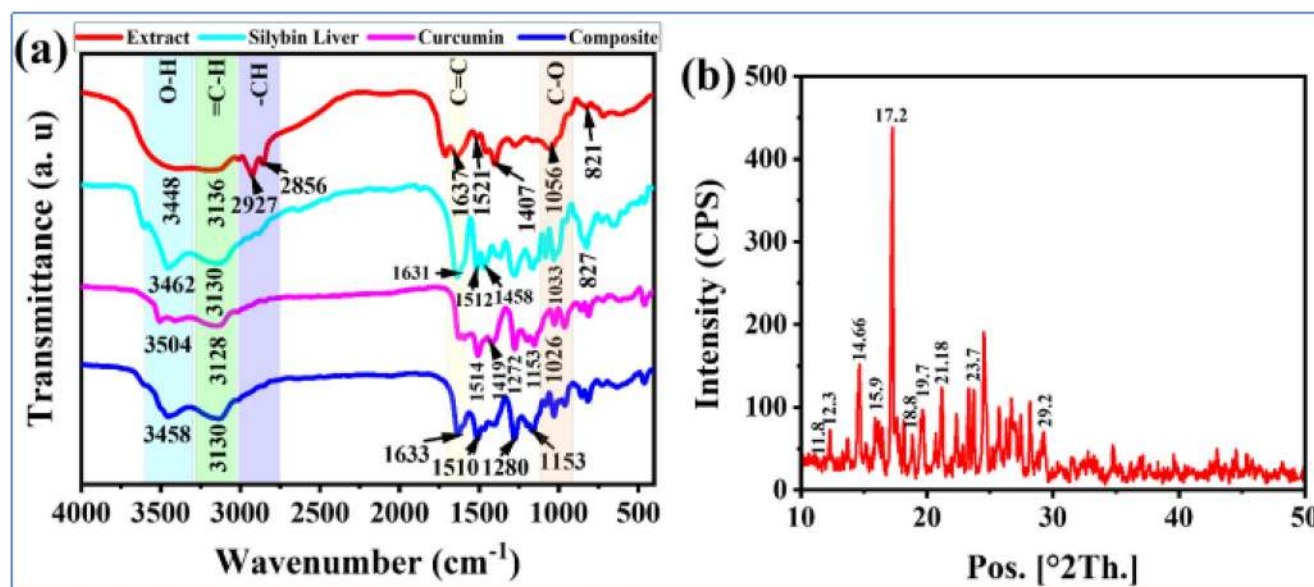
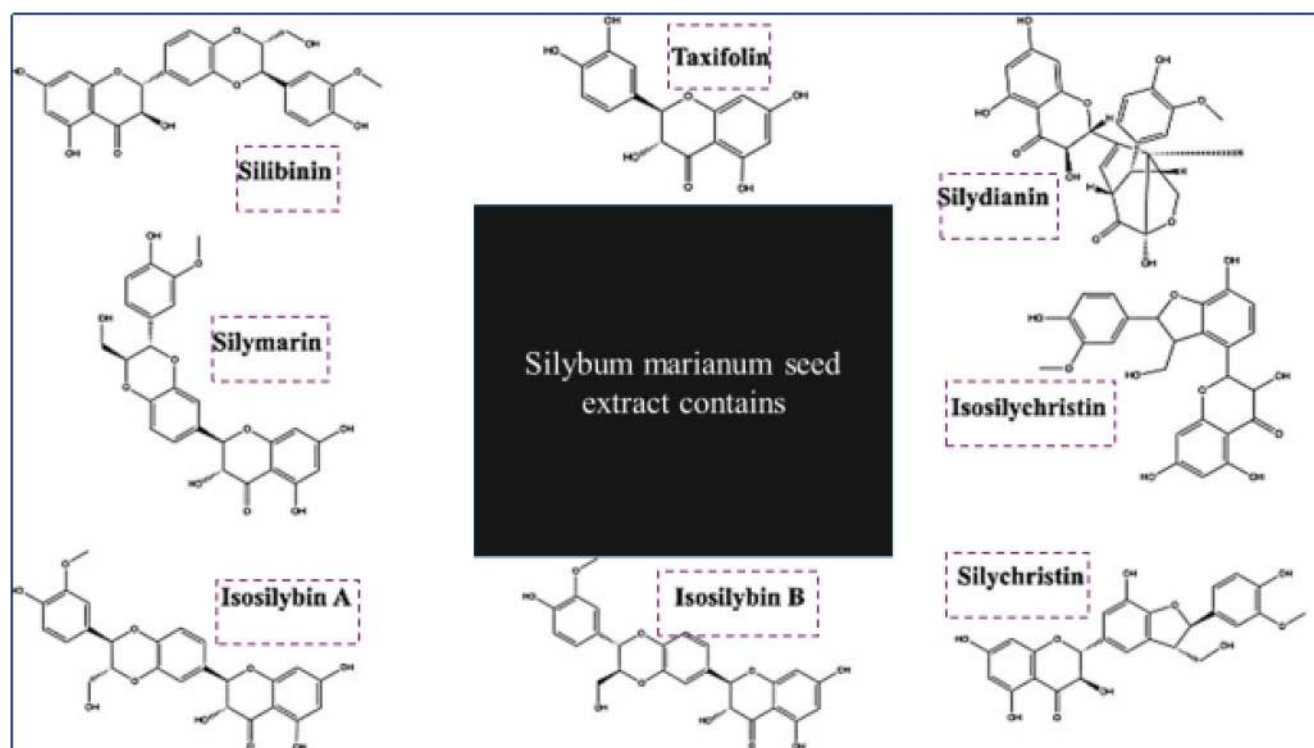


Fig. 1 (a) FT-IR spectra of the extract, silybin, curcumin, and composite (b) XRD pattern of the composite, revealing its crystalline nature with characteristic diffraction peaks



Scheme 1 Important compound present in the *Silybum marianum* seed extract contains

In the FT-IR spectrum of extract (, distinct peaks characteristic of silybin were observed, including a 3418 cm^{-1} band corresponding to O–H stretching vibrations and a peak at 3130 cm^{-1} . Characteristic peaks for pure silymarin (the primary active flavonolignan mixture in the seed extract as shown in Figure S1(a)) (Scheme 1). Other peaks like appearing at 1631 cm^{-1} correspond to functional groups such as C = O, C–O, aromatic and aliphatic C–H stretches, representing the flavonolignans and other phenolic compounds

[38]. Additionally, a peak at 1512 cm^{-1} corresponds to C – C stretching modes, whereas the bands at 1458 cm^{-1} and 1641 cm^{-1} are associated with benzene ring skeletal stretching vibrations in silybin [37]. FTIR is useful for confirming the presence of phenolics, flavonolignan and other silymarin components in the extract also its role in the encapsulation the component involving the material under study (Figure S1(b)), by comparing the FTIR of the extract and the composite sited in Table 1.

Table 1 FTIR peaks assignments for functional groups found in figure (a-b) of extract and composite sample

wavenumber (cm^{-1}) for the extract sample	wavenumber (cm^{-1}) for the Composite sample	Function group	Assignments	Ref.
3407	3418	$\delta(\text{O–H})$	Intra molecular hydrogen bond between water molecules	[42, 43]
3281.90	3232.55	$\nu(\text{O–H})$	Of phenol, Alcohol group	[44]
2922.20	2527.49	$\delta(\text{C–H}), \delta(\text{–CH}_2\text{–})$	Methylene, aldehyde	[45, 46]
2850			And methyl groups	
1746	-	$\delta(\text{C = O–H}), \delta(\text{O = C–H})$	Ester carbonyl functional group of the triglycerides and fatty acids	[47]
1632.01	1632.01	$\delta(\text{C = C})$ and O–H	Saturated fatty acids	[48, 49]
1509	1507.87	$\delta(\text{C = C})$	Aromatic rings from minor bio active compounds (i.e., phenols)	[50]
1398.90	1388.22	$\nu(\text{C–H}), \delta(\text{C–O})$	Esters	[44–46, 48, 51–55]
1276.61				
1054.17	1048.23	$\delta(\text{C–O})$	Unsaturated esters and esters derived from second ARY alcohols	[48, 54, 55]
852 – 730	958–831.73.73.73	$-(\text{CH}_2)_n-$	Long chain fatty acids	[54, 55]
608	471.41	$\nu(\text{–CH}_2)_n\text{–}, \delta(\text{–HC = CH–(cis–)})$	Di-substituted olefinic alkenes	[56]

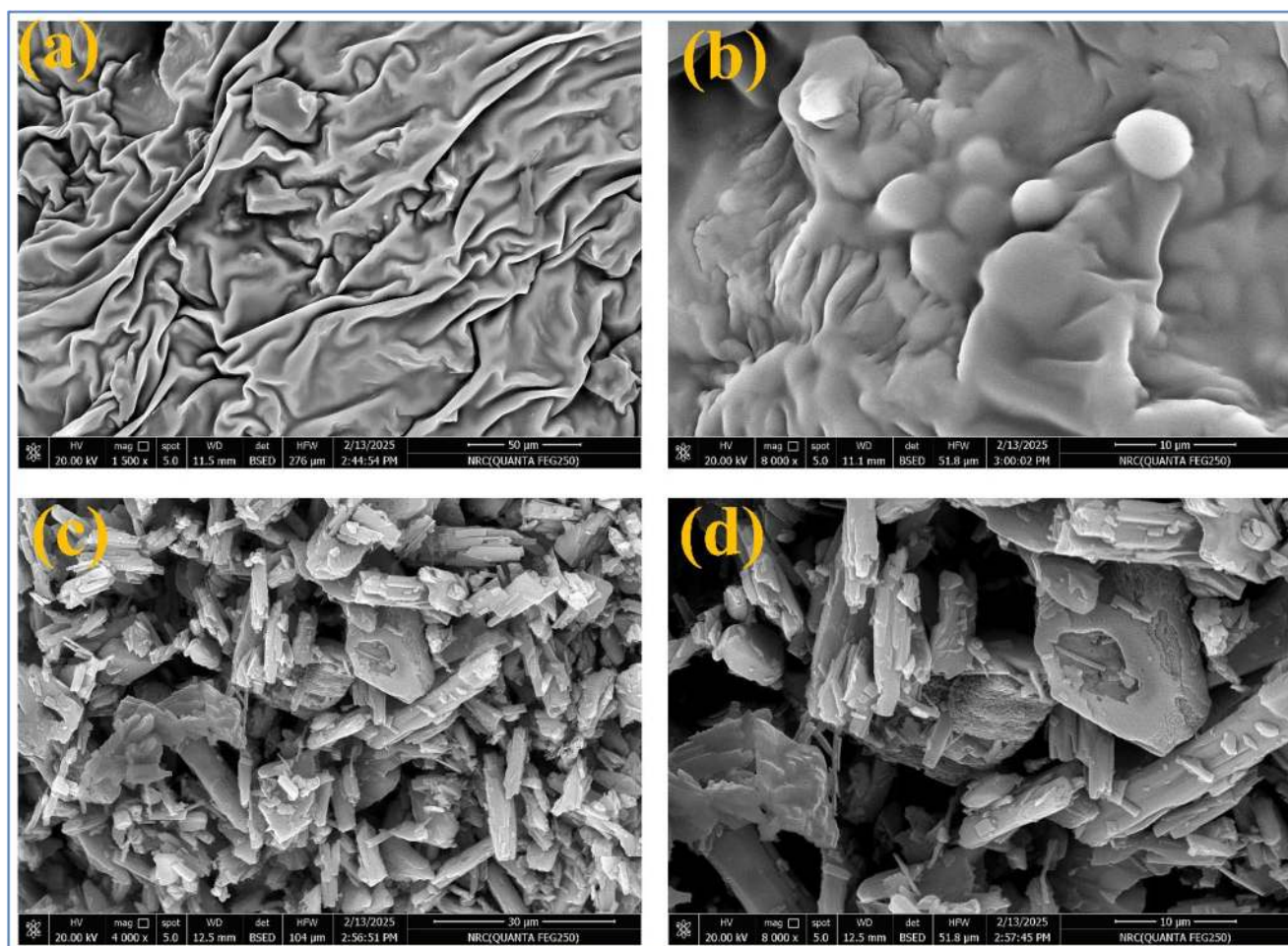


Fig. 2 FE-SEM images of (a, b) encapsulated *Silybum marianum* seed extract showing an irregular and rough surface with folds, wrinkles, and spherical structures and (c, d) the composite material exhibiting a

flaky, plate-like crystalline structure with good particle dispersion and sharp-edged, needle-like morphologies

In the FT-IR spectrum of curcumin, a broad, intense vibrational band at 3501 cm^{-1} represents hydrogen-bonded O–H stretching vibrations present in nanocurcumin. The peak at 1412 cm^{-1} indicates aromatic C–C stretching in the benzene ring. The band near 1419 cm^{-1} signifies conjugated C=O stretching vibrations. Furthermore, double-bonded carbon stretching vibrations appear, with additional peaks at 1272 cm^{-1} and 1153 cm^{-1} attributed to $\text{Csp}^2\text{--O}$ and $\text{Csp}^3\text{--O}$ stretching vibrations, respectively. In the IR spectrum of the composite (Figure S1(b)), the signature peaks of extract, silybin, and curcumin remained evident, although slight peak shifts were observed. These shifts may be attributed to electrostatic interactions between the composite's constituents. Furthermore, a notable difference in absorption intensity was detected between the composite and its precursor materials, which could be associated with excipients modifying the absorption of key functional groups.

The crystalline state is one of the important factors affecting drug stability, solubility, and efficacy. The XRD pattern of the composite is shown in Fig. 1(b), and the composite displayed multiple sharp diffraction peaks reflecting its highly crystalline structure. The figure shows that the characteristic diffraction peaks at $2\theta = 11.8^\circ, 12.3^\circ, 18.8^\circ, 23.80^\circ, 26.25^\circ, 26.85^\circ$ and 28.28° are characteristic of the crystal structure of silybin [57]. The high-intensity crystalline peak at approximately $2\theta = 14.7^\circ, 15.9^\circ, 17.2^\circ, 19.7^\circ, 23.7^\circ$, and 29.2° corresponds to curcumin (ICDD Card No. 00–066–1420).

The final product has low crystallinity (13.20% crystallinity), the crystallite size of the composite is 63.13 nm, d spacing was 0.514 nm and the micro strain is 0.407%. This specific interlamellar distance provides sufficient space for the successful accommodation of the pharmaceutical agent within the layered structure [58, 59].

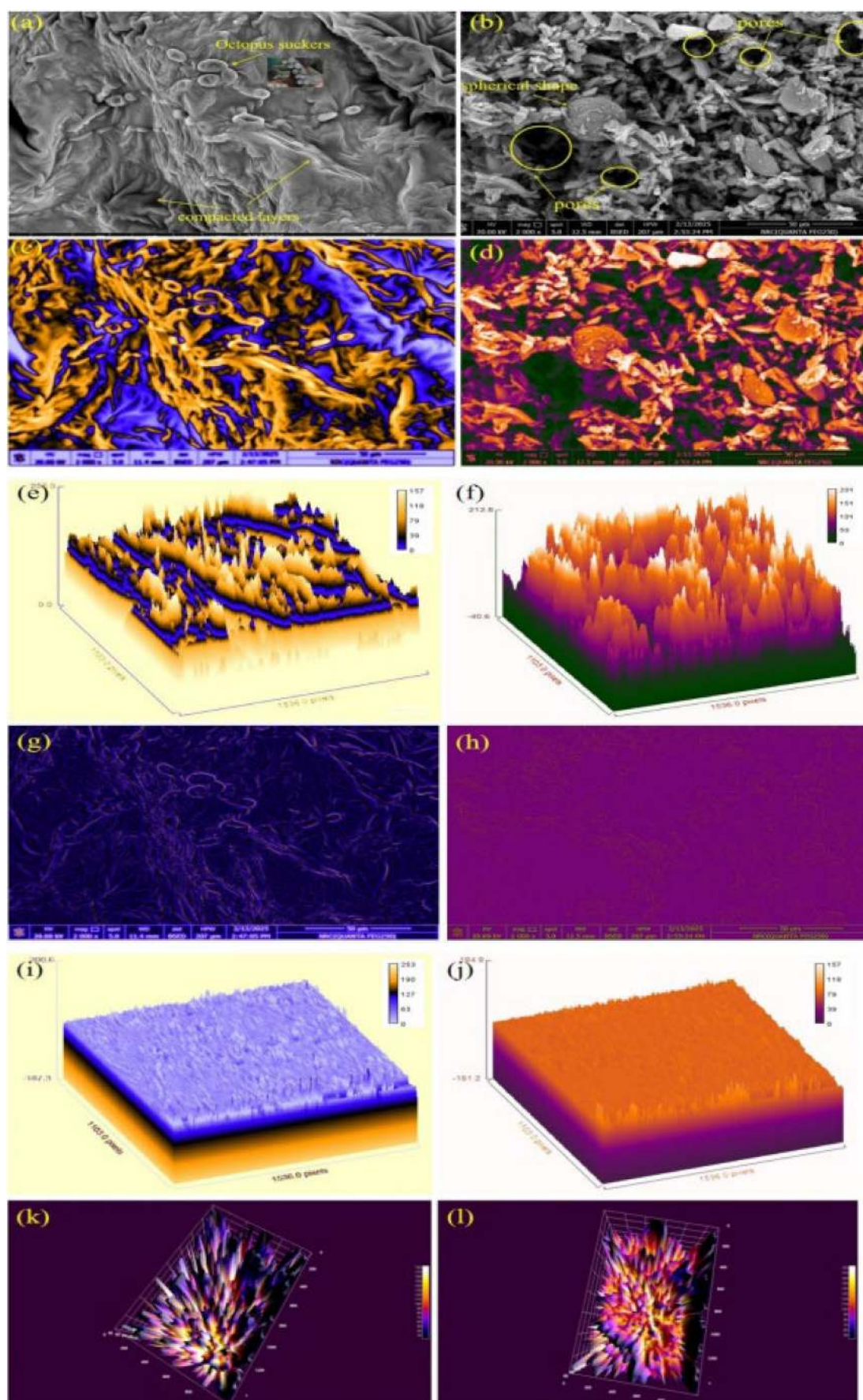


Fig. 3 SEM images (a) for extract and (b) for Composite; Waviness of SEM images (c) for extract and (d) for composite; Surface plot of waviness of SEM images (e) for extract and (f) for composite. Roughness of SEM images of (g) for extract and (h) for composite and 3D surface plot of SEM images (i) for extract and (k) for composite

The morphology of the encapsulated *Silybum marianum* seed extract and the composite materials was characterized via FE-SEM. The FE-SEM image of the encapsulated *Silybum marianum* seed extract revealed an irregular and rough surface with noticeable folds and wrinkles (Fig. 2(a, b)). In addition, some spherical or oval-shaped structures are visible, which might represent encapsulated extract droplets or surface irregularities. After compositing, the composite (Fig. 2(c, d)) has approximately the same morphology; however, the composite has a more detailed, flaky, and plate-like crystalline structure. In addition, the dispersion of the particles suggested good intercalation between the *Silybum marianum* seed extract and curcumin. Moreover, the crystalline structures become more apparent, showing sharp-edged and needle-like morphologies.

(FE-SEM) Field Emission Scanning Electron Microscope was used to investigate the configuration of the synthetic materials. As presented in Fig. 3(a), the prepared extract shows a homogenous compacted layer which arranged one of one another and Octopus suckers-like structure. However, in Figure (b) shows more porosity, fibrous and spherical shape-like structure in accordance to the results in the drug action with the targeted cell. By using image J, Surface texture and morphology were investigated and analyzed to prepare the roughness, waviness and 3d surface as shown in Fig. 3 (c-l). The roughness is characterized by parameters such as Arithmetic average of absolute surface deviations(Ra), Average of 5 highest peaks to 5 deepest valleys(Rz), and Root mean square of deviations from mean line(Rq), which describe the average surface roughness, the peak-to-valley height (Fig. 4), and the root-mean-square roughness, respectively. Whereas, waviness refers to the larger-scale variations in a surface that occur over longer wavelengths. It is a measure of the deviations from the nominal surface that occur at a slower rate than the roughness. Waviness can result from various factors such as machining, casting, and warping of the material (Table S1). The waviness of a surface is often measured using a form tester, which uses a sensor to measure the surface profile over a larger, this improvement is expected to favorably impact both the total surface area and the biochemical interactions of the loaded one, consequently enhancing its effectiveness for medical applications.

Compared with the blank formulation, the encapsulated formulation of *Silybum marianum* seed extract resulted in a significantly smaller particle size (191.00 ± 2.25 nm)

(10.20 ± 2.05 μ m), indicating successful nanoscale formulation, which can enhance bioavailability and cellular uptake. The polydispersity index (PDI) decreases from 0.68 to 0.25, suggesting improved uniformity and stability. Additionally, the zeta potential shifts from -17.50 ± 1.21 mV to -32.03 ± 1.63 mV, indicating greater electrostatic repulsion, which enhances colloidal stability and reduces aggregation, as illustrated in Table 2; Fig. 5(A, B). The formulation also demonstrated high encapsulation efficiency for silybin ($85.5 \pm 2.1\%$) and curcumin ($82.3 \pm 1.8\%$), highlighting the efficiency of the encapsulation process in incorporating the pure compounds within the extract matrix. The strategic addition of pure compounds was designed to standardize the total content of these specific bioactives for reproducible efficacy testing, while acknowledging the concurrent but unquantified presence of native phytoconstituents from the extract. The strategic addition of pure compounds was designed to standardize the total content of these specific bioactives for reproducible efficacy testing, while acknowledging the concurrent but unquantified presence of native phytoconstituents from the extract. This high encapsulation efficiency, combined with the native bioactive components of the extract, helps to offer enhanced stability, controlled release, and improved therapeutic potential for the nanoformulation.

In Vitro Release Study

The cumulative drug release profiles of silybin and curcumin were evaluated under controlled conditions in a phosphate buffer (pH 6.8) to elucidate the intrinsic release kinetics of the nanoformulation. The results demonstrated statistically significant differences between encapsulated and unencapsulated formulations for both bioactive compounds (Fig. 6). Two-way ANOVA revealed significant main effects of encapsulation ($p < 0.0001$), time ($p < 0.0001$), and their interaction ($p < 0.0001$) for both compounds (Table S2). The encapsulated silybin exhibited a biphasic release pattern characterized by an initial moderate release of $30.08 \pm 2.5\%$ within the first 2 h, followed by sustained release, reaching $98.1 \pm 1.0\%$ after 48 h. This release profile was significantly different from that of unencapsulated silybin at multiple time points: 30 min ($7.2 \pm 1.1\%$ vs. $23.4 \pm 2.0\%$, $p < 0.001$), 2 h ($30.08 \pm 2.5\%$ vs. $57.6 \pm 2.8\%$, $p < 0.001$), and 6 h ($45.3 \pm 3.2\%$ vs. $78.9 \pm 3.5\%$, $p < 0.001$). Tukey's post hoc analysis confirmed significant differences at all measured time points, with the largest difference observed at 6 h (mean difference: -33.60% , 95% CI: -39.38 to -27.82% , $p < 0.001$) (Table S3). Similarly, the controlled release characteristics of encapsulated curcumin were superior to those of its unencapsulated counterpart. The substantial cumulative release of encapsulated curcumin ($89.1 \pm 1.5\%$ over 48

Fig. 4 Roughness characterization of (a) extract and (b) of composite

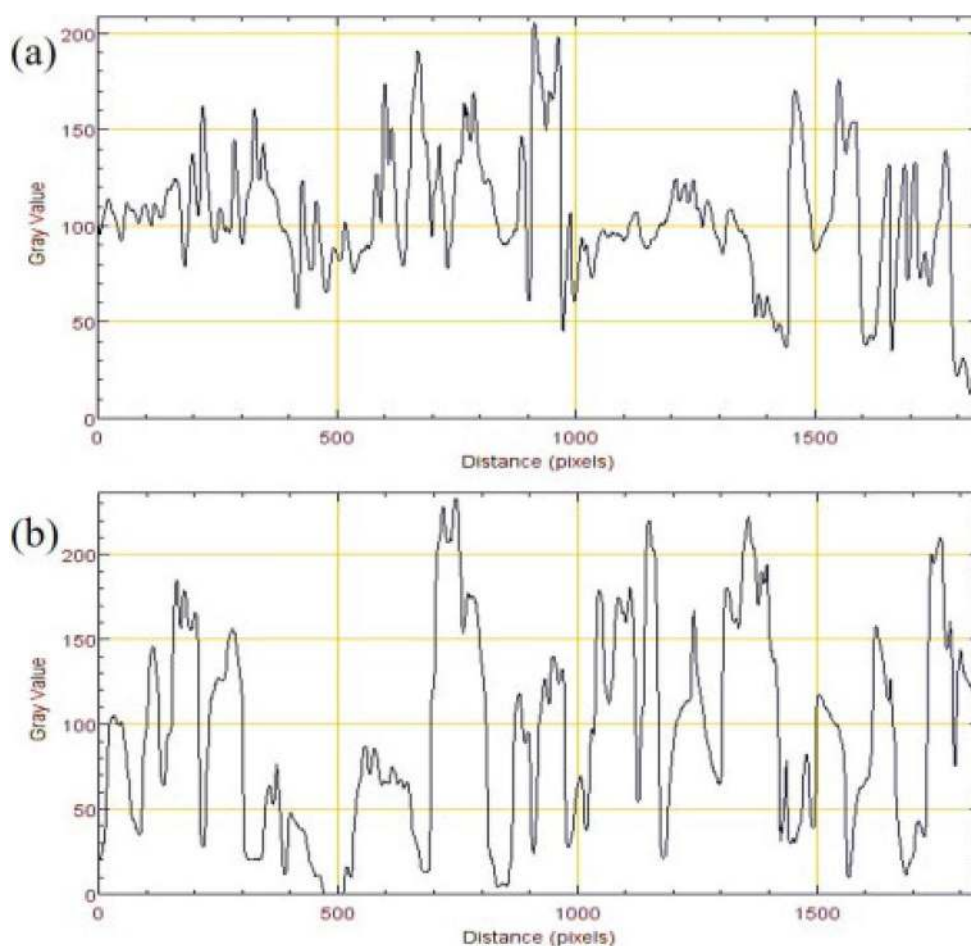


Table 2 Characterization of the encapsulated formulation (SiCu-SM): particle size, zeta potential, and encapsulation efficiency of the Silybin and Curcumin

Formulation	Mean particle size	Dispersity (PDI)*	Zeta potential (mV)	Encapsulation Efficiency (EE%)	
				Added Silybin	Added Curcumin
Blank (SM)	10.20±2.05 µm	0.68	-17.50±1.21	-	-
Encapsulated Formulation	191.00±2.25 nm	0.25	-32.03±1.63	85.5±2.1%	82.3±1.8%

*Polymodal refers to PDI values that exceed 0.3, whereas monomodal refers to PDI values below 0.3.

h) represents a remarkable improvement over conventional curcumin formulations, which aligns with numerous literature reports demonstrating that nanoencapsulation can enhance curcumin release by 5–10-fold compared to unformulated curcumin. The encapsulated formulation showed minimal burst release ($3.96 \pm 0.3\%$ at 10 min) and maintained sustained release kinetics, achieving $89.1 \pm 1.5\%$ release at 48 h. Statistical analysis revealed significant differences at key time points: 30 min ($8.1 \pm 0.8\%$ vs. $21.6 \pm 1.8\%$, $p < 0.001$), 2 h ($18.5 \pm 1.4\%$ vs. $50.4 \pm 2.5\%$, $p < 0.001$), and 6 h ($32.7 \pm 2.1\%$ vs. $71.2 \pm 3.1\%$, $p < 0.001$). Post hoc analysis demonstrated consistent statistical significance across all time points, with the maximum difference occurring at

6 h (mean difference: -38.50% , 95% CI: -44.47 – 32.53% , $p < 0.001$) (Table S3).

The sustained and synchronized release profile observed under these controlled conditions provides clear mechanistic insight into the release behavior of the nanoformulation, demonstrating that the Silybum marianum seed extract matrix effectively modulates drug release through diffusion-controlled mechanisms.

The enhanced release profile observed for our nanoformulation can be attributed to several factors: (1) the nanoscale particle size (191.00 ± 2.25 nm) providing dramatically increased surface area for dissolution; (2) the natural surfactant properties of Silybum marianum seed extract

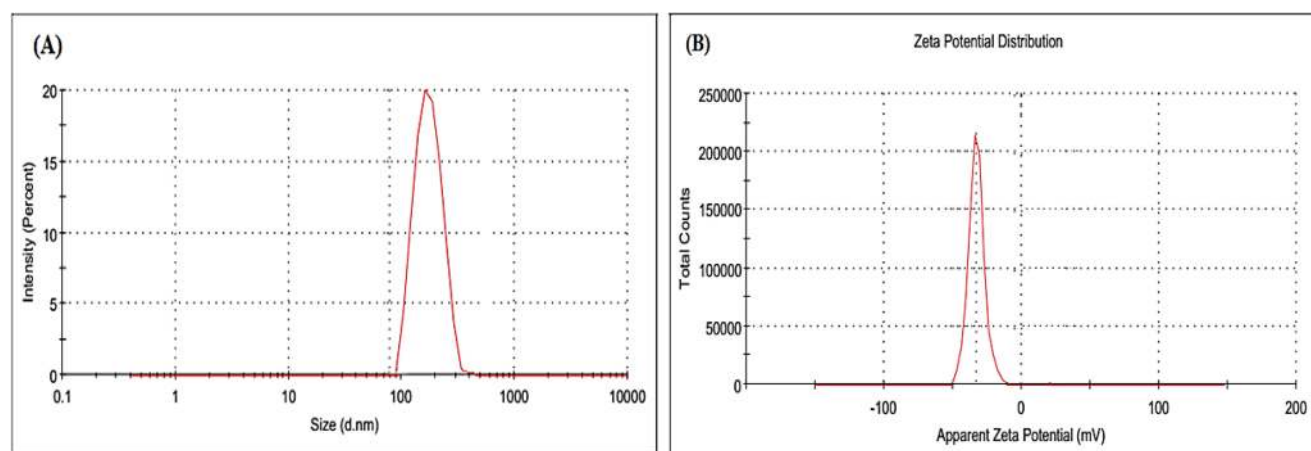


Fig. 5 (A) Dynamic light scattering (DLS) analysis showing the particle size distribution of the encapsulated formulation, indicating a significant reduction in size compared with that of the blank formula-

tion. **B** Zeta potential distribution demonstrating an increase in surface charge, increasing colloidal stability and reducing aggregation

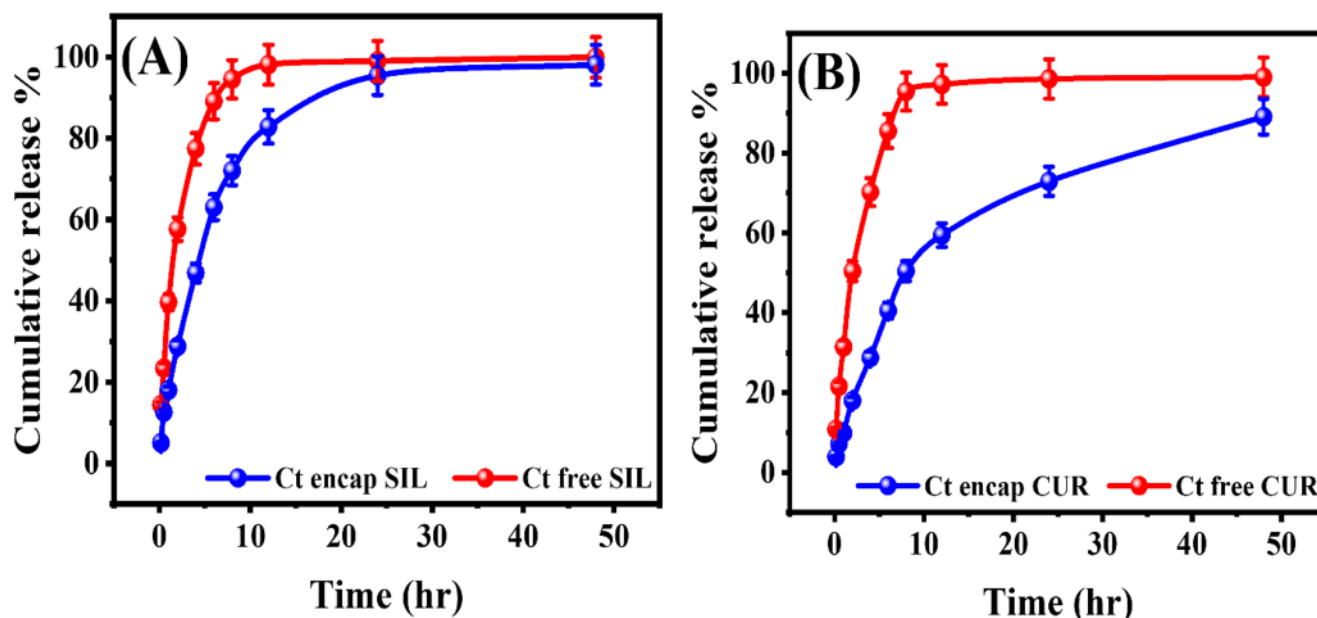


Fig. 6 Cumulative drug release (%) vs. time intervals for (A) encapsulated and free formulations of silybin (SIL) and (B) encapsulated and free formulations of curcumin (CUR) in stimulated intestinal fluid

components facilitating improved solubilization; and (3) the matrix-controlled release mechanism preventing the rapid precipitation and degradation typically observed with pure curcumin. These findings are consistent with previous studies reporting that nanoencapsulation strategies can significantly improve curcumin release profiles and bioavailability [60–65].

In Vitro Release Kinetics

Mathematical modeling of release kinetics revealed distinct mechanisms governing drug release from encapsulated versus

unencapsulated formulations (Fig. 7; Table 3). The coefficient of determination (R^2) values for all the models exceeded 0.950, indicating excellent mathematical correlation with the experimental data. Zero-order kinetics analysis demonstrated that the encapsulated formulations presented more consistent release rates. Compared with unencapsulated silybin, encapsulated silybin presented a significantly greater zero-order rate constant ($K_0 = 1.8211 \pm 0.15$ mg/h, 95% CI: 1.67–1.97) ($K_0 = 1.3777 \pm 0.10$ mg/h, 95% CI: 1.28–1.48, $p < 0.01$). Unpaired t test analysis confirmed that this difference was statistically significant ($t = 4.89$, $df = 40$, $p = 0.0078$) (Table S4). Similarly, encapsulated curcumin resulted in increased zero-order

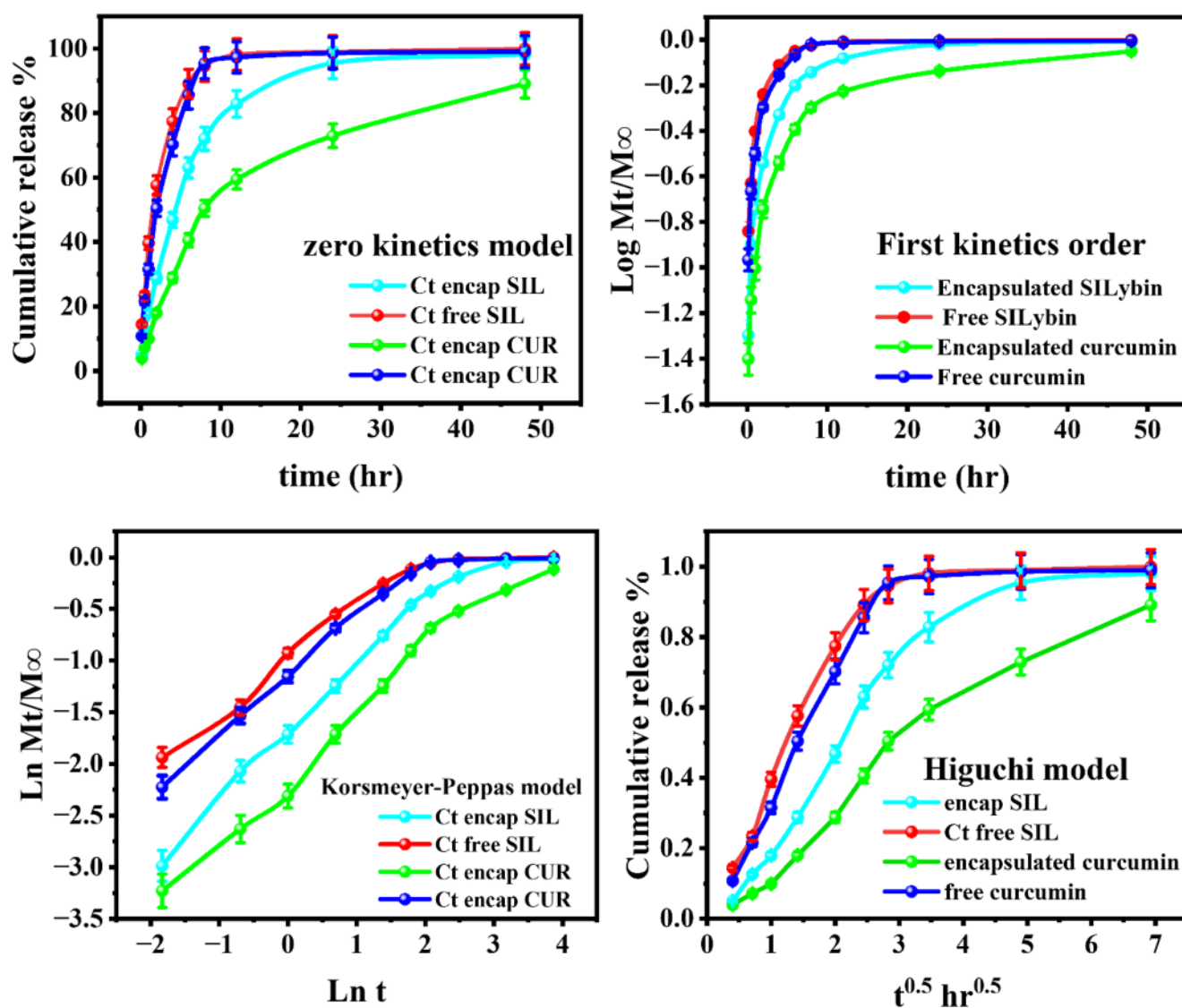


Fig. 7 In vitro release kinetics of encapsulated and unencapsulated silybin and curcumin formulations analyzed via different kinetic models: zero-order kinetics, first-order kinetics, the Korsmeyer–Peppas model, and the Higuchi model. The data are presented as the means $\pm$ SDs ($n=3$)

Table 3 Kinetic parameters and statistical analysis of the encapsulated and unencapsulated Silybin and Curcumin formulations

Release Kinetic Model	Equation	Parameter	Encapsulated Silybin	Unencapsulated Silybin	<i>p</i> value	Encapsulated Curcumin	Unencapsulated Curcumin	<i>p</i> value
Zero-order	$M_t = M_0 + K_0 t$	K_0 (mg/h)	1.8211 ± 0.15	1.3777 ± 0.10	<0.01 **	1.7418 ± 0.12	1.437 ± 0.11	<0.05 *
		R^2	0.9645 ± 0.0234	0.9523 ± 0.0287		0.9598 ± 0.0198	0.9534 ± 0.0245	
First-order	$\log (M_t/M_\infty) = -(Kt)/2.303$	K (h^{-1})	0.0059 ± 0.0003	0.0184 ± 0.0015	<0.001 ***	0.0135 ± 0.0010	0.0023 ± 0.0003	<0.001 ***
		R^2	0.9734 ± 0.0156	0.9812 ± 0.0134		0.9756 ± 0.0178	0.9689 ± 0.0203	
Korsmeyer–Peppas	$M_t/M_\infty = Kt^n$	K	0.1837 ± 0.015	0.3657 ± 0.025	<0.001 ***	0.1181 ± 0.010	0.3143 ± 0.020	<0.001 ***
		n	0.5496 ± 0.03	0.3653 ± 0.02				

*Data are presented as the means $\pm$ standard deviations ($n=3$). Statistical significance was determined by an unpaired *t* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

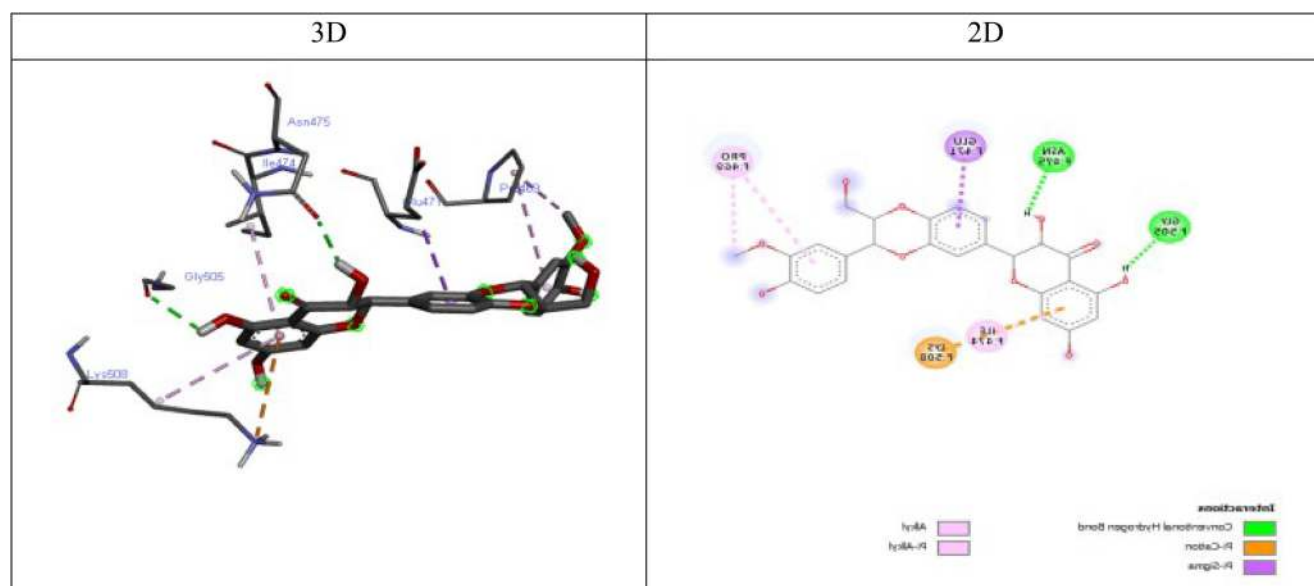


Fig. 8 3D and 2D visualizations of the interactions between NRF2 and Silybin

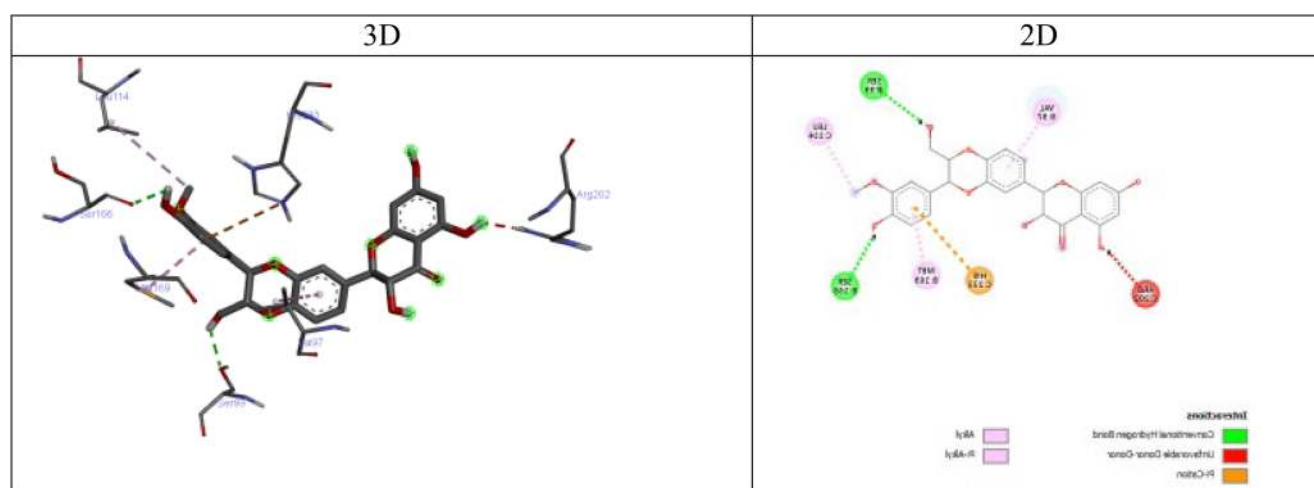


Fig. 9 3D and 2D visualizations of the interactions between P53 and Silybin

release ($K_0 = 1.7418 \pm 0.12$ mg/h, 95% CI: 1.62–1.86) compared with unencapsulated curcumin ($K_0 = 1.437 \pm 0.11$ mg/h, 95% CI: 1.33–1.54, $p < 0.05$). First-order kinetics revealed compound-specific responses to encapsulation. The first-order release of encapsulated silybin was significantly slower ($K = 0.0059 \pm 0.0003$ h⁻¹, 95% CI: 0.0056–0.0062) than that of unencapsulated silybin ($K = 0.0184 \pm 0.0015$ h⁻¹, 95% CI: 0.0169–0.0199, $p < 0.001$). Conversely, encapsulated curcumin had faster first-order kinetics ($K = 0.0135 \pm 0.0010$ h⁻¹, 95% CI: 0.0125–0.0145) than unencapsulated curcumin did ($K = 0.0023 \pm 0.0003$ h⁻¹, 95% CI: 0.0020–0.0026, $p < 0.001$).

Statistical validation of these kinetic parameters through unpaired *t* tests revealed highly significant differences ($p < 0.001$ for both compounds) (Table S4).

Korsmeyer–Peppas modeling provided insights into release mechanisms. The release exponent (*n*) values for encapsulated silybin ($n = 0.5496 \pm 0.03$, 95% CI: 0.52–0.58) and curcumin ($n = 0.5933 \pm 0.04$, 95% CI: 0.55–0.63) were significantly closer to 0.5 than their unencapsulated counterparts (silybin: $n = 0.3653 \pm 0.02$, $p < 0.001$; curcumin: $n = 0.4138 \pm 0.03$, $p < 0.01$), indicating anomalous transport involving both Fickian diffusion and Case-II transport mechanisms for the encapsulated formulations, which explains their superior release profiles compared to the predominantly diffusion-controlled release of unencapsulated compounds. Detailed statistical comparisons of all the Korsmeyer–Peppas parameters are provided in Table S4. Higuchi model analysis confirmed matrix-controlled

diffusion in the encapsulated systems. Encapsulated silybin ($K_H = 18.503 \pm 1.2 \text{ mg/h}^{0.5}$, 95% CI: 17.3–19.7) and curcumin ($K_H = 14.401 \pm 1.0 \text{ mg/h}^{0.5}$, 95% CI: 13.4–15.4) presented significantly lower diffusion constants than unencapsulated forms did (silybin: $K_H = 21.774 \pm 1.5 \text{ mg/h}^{0.5}$, $p < 0.05$; curcumin: $K_H = 21.27 \pm 1.4 \text{ mg/h}^{0.5}$, $p < 0.01$), confirming controlled matrix diffusion. The results revealed that the Korsmeyer–Peppas model best described the encapsulated formulations ($R^2 = 0.9891 \pm 0.0045$ for silybin, $R^2 = 0.9876 \pm 0.0052$ for curcumin), whereas the Higuchi model provided an optimal fit for the unencapsulated formulations ($R^2 = 0.9834 \pm 0.0067$ for silybin, $R^2 = 0.9798 \pm 0.0078$ for curcumin). A comprehensive statistical analysis of all kinetic parameters, including *t* statistics, degrees of freedom, and confidence intervals, is detailed in Table S4.

The kinetic analysis substantiates our experimental release data, demonstrating that the encapsulation process fundamentally alters the release mechanism of both silybin and curcumin, resulting in more controlled and sustained release profiles that are consistent with successful nanof ormulation strategies reported in different studies [60, 62, 65].

Molecular Docking Analysis

Silybin-Binding Pose and Molecular Interactions with NRF2, P53, COX-2 and TNF- α

Figure 8 illustrates Silybin's binding pose and molecular interactions with NRF2, which is a transcription factor that regulates the expression of genes involved in antioxidant defense and detoxification, helping protect cells from oxidative stress and damage. The 3D visualization (left) shows the spatial orientation of the ligand, whereas the 2D diagram (right) details specific molecular interactions. The key interactions include conventional hydrogen bonds with GLY F:505 and ASN F:475, as well as multiple hydrophobic interactions with ILE F:474, LYS F:508, and PRO F:469. These hydrophobic interactions reinforce binding by creating van der Waals forces between the hydrophobic rings of silybin and the protein's aliphatic residues. Figure 9 illustrates Silybin's binding pose and molecular interactions with P53, which is a tumor suppressor protein that prevents cancer by regulating the cell cycle and initiating DNA repair or cell death in response to damage. Silybin forms multiple stabilizing interactions, including conventional hydrogen bonds with SER B:99 and SER B:166, which are critical for anchoring the ligand in the binding site. Additionally, Pi-alkyl interactions with residues such as LEU C:114 and MET B:169 provide hydrophobic stability. A notable Pi-Cation interaction with HIS C:233 suggests a strong electrostatic contribution. Figure 10 shows the Silybin binding pose and molecular interactions within cyclooxygenase-2 (COX-2), highlighting

key interactions that contribute to its stability and affinity for the enzyme. Several key amino acid residues are involved in binding where conventional hydrogen bonds are formed with serine (SER B:34), tryptophan (TRP A:309), histidine (HIS B:119), and valine (VAL B:141); carbon–hydrogen bonds are formed with cysteine (CYS B:32) and glutamine (GLN A:313); pi–anion interactions occur with aspartic acid (ASP B:143); and pi–alkyl interactions are observed with proline (PRO B:140) and alanine (ALA B:142). The presence of multiple hydrogen bonds suggests a strong binding affinity between silybin and COX-2, potentially enhancing its inhibitory effects on the enzyme. Additionally, the pi-anion and pi-alkyl interactions contribute to the stability of the Silybin-COX-2 complex, reinforcing Silybin's potential as an anti-inflammatory and anticancer agent by targeting COX-2, an enzyme critical in both inflammation and carcinogenesis. Figure 11 shows the Silybin binding pose and molecular interactions within tumor necrosis factor- α (TNF- α), highlighting the various binding interactions that contribute to the stability and binding affinity of Silybin for TNF- α . The key amino acid residues involved in binding include conventional hydrogen bonds with leucine (LEU D:55), glycine (GLY A:121), tyrosine (TYR B:151), serine (SER B:95), and glutamine (GLN D:125); a Pi–sigma interaction with leucine (LEU D:55); a Pi–Pi stacked interaction with tyrosine (TYR B:59); and Pi–alkyl interactions with alanine (ALA B:96). The presence of multiple hydrogen bonds indicates a strong binding affinity between silybin and TNF- α , enhancing its potential to modulate the inflammatory response. The Pi–Pi stacking and Pi–sigma interactions further stabilize the complex, whereas the unfavorable donor–donor interaction with glutamine (GLN D:125) may slightly reduce the binding efficiency. These interactions support the potential of silybin as an anti-inflammatory agent that targets TNF- α , a key cytokine involved in inflammation-related diseases, including cancer. Figure 12 illustrates the molecular interactions between AKT1 and silybin, highlighting various binding interactions that contribute to the stability and binding affinity of silybin for AKT1.

The key amino acid residues involved include the following: conventional hydrogen bonds with arginine (ARG A:273), asparagine (ASN A:54), glutamine (GLN A:79), threonine (THR A:82), and tryptophan (TRP A:80); a carbon–hydrogen bond with glutamine (GLN A:79); and pi–sigma interactions with valine (VAL A:270). Pi-alkyl interactions occur with arginine (ARG A:86), valine (VAL A:270), and isoleucine (ILE A:84). The presence of multiple hydrogen bonds suggests a strong binding affinity, enhancing Silybin's potential to modulate AKT1 signaling pathways. The Pi-sigma and Pi-alkyl interactions with hydrophobic residues such as valine and isoleucine further stabilize the complex. However, the unfavorable donor–donor interaction

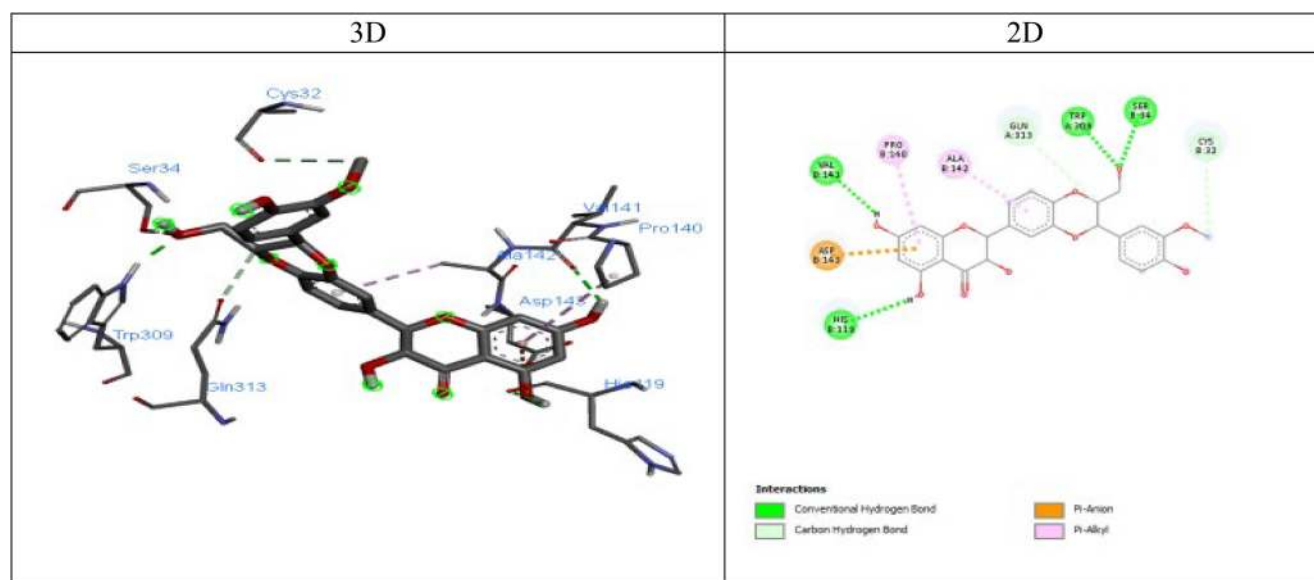


Fig. 10 3D and 2D visualizations of the interactions between COX-2 and silybin

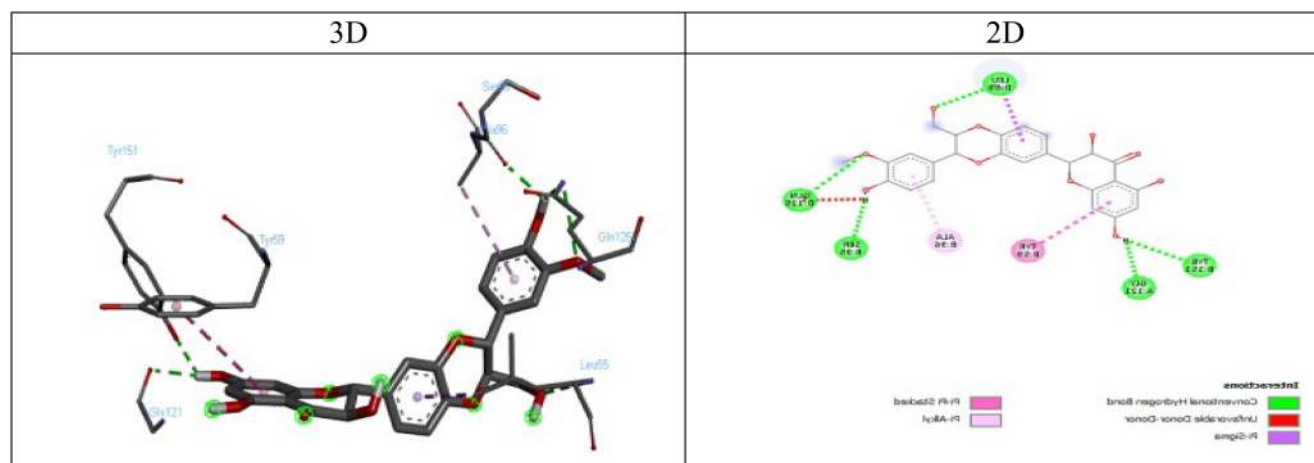


Fig. 11 3D and 2D visualizations of the interactions between TNF- α and silybin

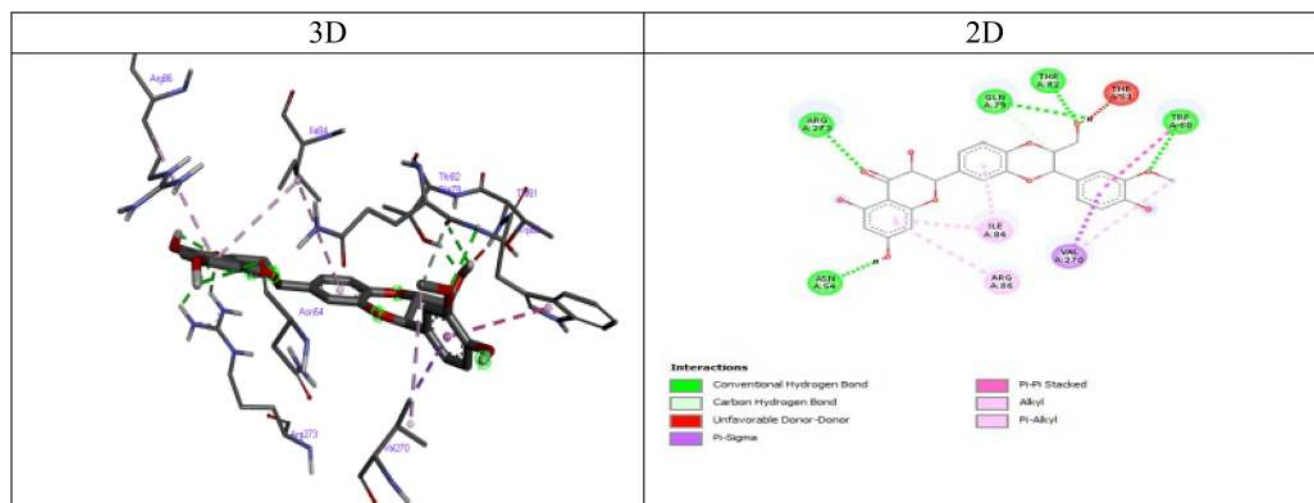


Fig. 12 3D and 2D visualizations of the interactions between AKT-1 and silybin

with threonine (THR A:81) may slightly reduce the binding efficiency. Overall, these interactions support the potential of Silybin as a therapeutic agent that targets AKT1, a key kinase involved in cell survival and proliferation.

Curcumin Binding Pose and Molecular Interactions with NRF2, P53, COX-2 and TNF- α

Figure 13 shows the binding pose and molecular interactions of curcumin within NRF2. Curcumin forms several significant hydrophobic interactions, including Pi-alkyl interactions with LYS B:462, ALA B:461, and PRO B:467,

which stabilize the ligand within the binding pocket. Notably, unfavorable donor–donor and acceptor–acceptor interactions involving GLU B:458 and VAL B:470 indicate steric or electronic clashes that could hinder binding efficiency. However, hydrophobic interactions with residues such as PRO B:469 likely compensate for stability. Figure 14 shows the binding pose of curcumin and the molecular interactions within P53. Key interactions include conventional hydrogen bonds with the residues ARG A:174 and GLU B:180, which contribute to its stability and specificity. Carbon–hydrogen bonds are observed with VAL A:172 and HIS A:214. Pi–Pi interactions, such as stacked and

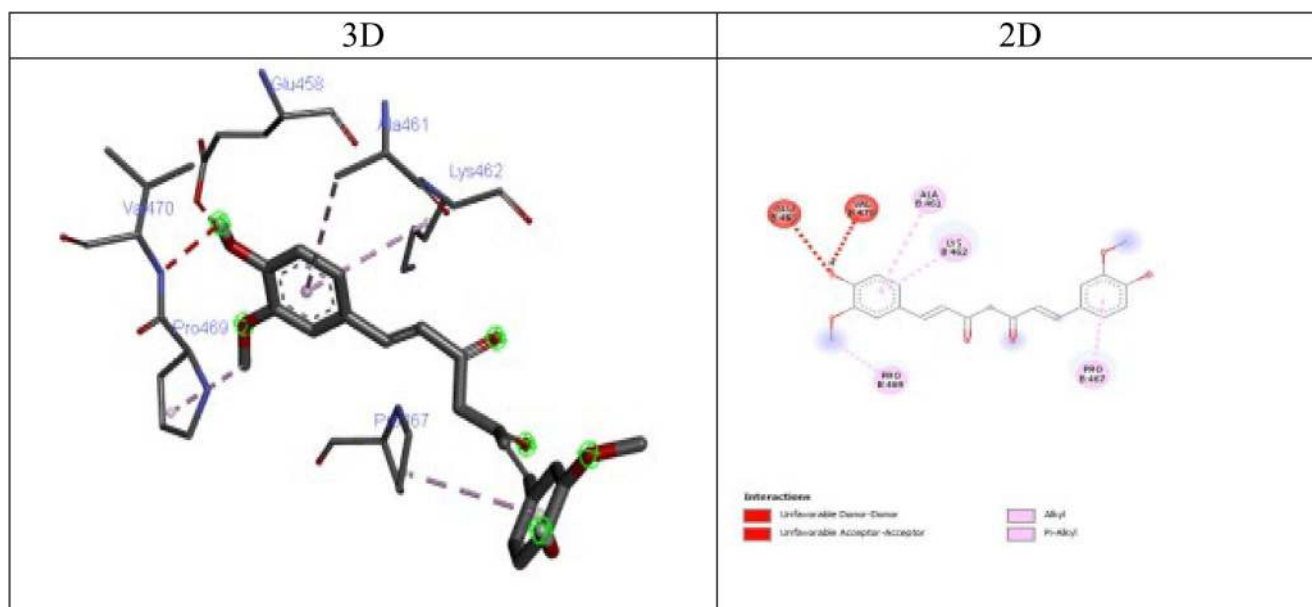


Fig. 13 3D and 2D visualizations of the interactions between NRF2 and curcumin

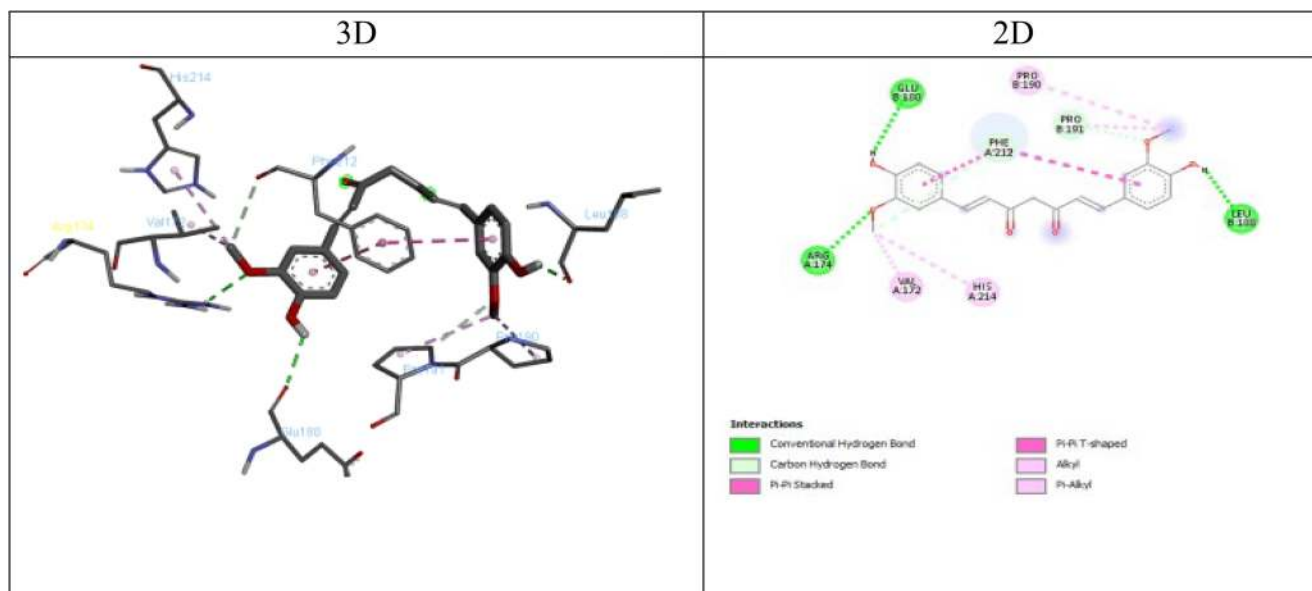


Fig. 14 3D and 2D visualizations of the interactions between P53 and curcumin

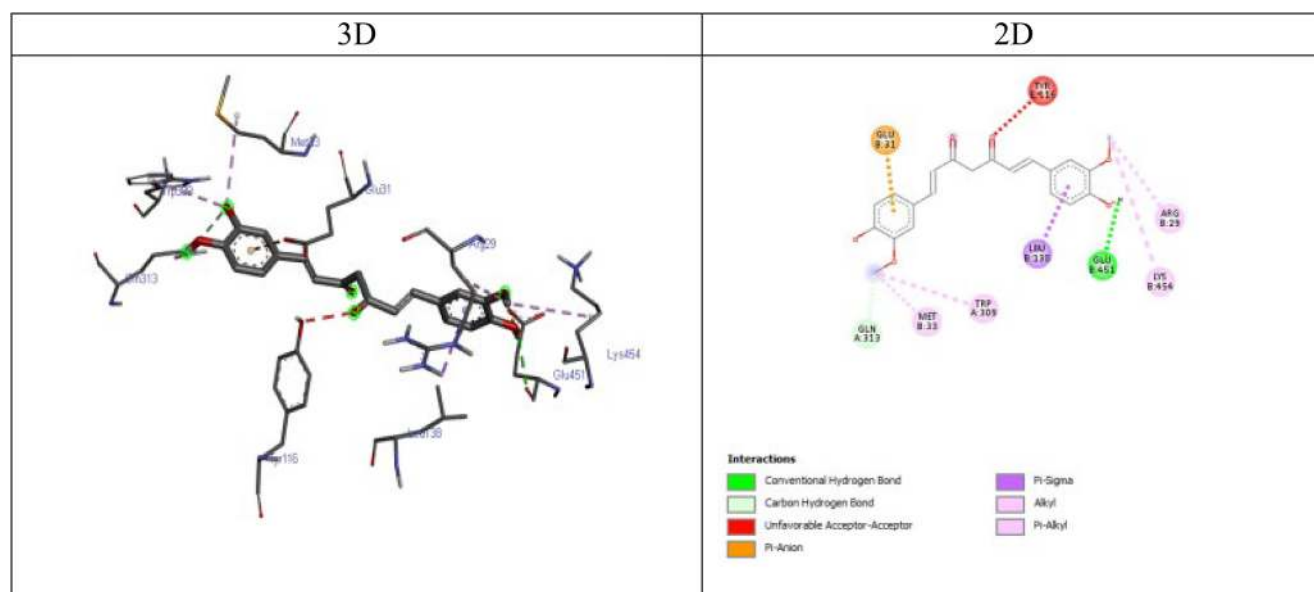


Fig. 15 3D and 2D visualizations of the interactions between COX-2 and curcumin

T-shaped arrangements, involve aromatic residues such as PHE A:212, increasing binding affinity. Hydrophobic interactions, including alkyl and Pi-alkyl contacts, involve residues such as LEU B:188 and PRO B:190/B:191. These combined interactions suggest strong binding of curcumin to the p53 protein, potentially influencing its activity. Figure 15 shows the 2D and 3D interaction diagram of the molecular interactions between cyclooxygenase-2 (COX-2) and curcumin, highlighting various binding interactions that contribute to the stability of their complex. Several key amino acid residues are involved in binding, including glutamate (GLU B:451), which forms a conventional hydrogen bond, and glutamine (GLN A:313), which forms a carbon–hydrogen bond. Glutamate (GLU B:31) participates in a Pi-anion interaction, whereas leucine (LEU B:138) is involved in a Pi-sigma interaction. Additionally, Pi-alkyl interactions occur with methionine (MET B:33), tryptophan (TRP A:309), arginine (ARG B:29), and lysine (LYS B:454). These interactions indicate a strong binding affinity between curcumin and COX-2, enhancing its inhibitory effects. However, the unfavorable acceptor–acceptor interaction with tyrosine (TYR B:116) may slightly decrease the binding efficiency. Overall, these interactions support the potential of Curcumin as an anti-inflammatory and anticancer agent by targeting COX-2, a key enzyme in inflammation and cancer development. Figure 16 shows the molecular interactions between tumor necrosis factor-alpha (TNF- α) and curcumin, highlighting various binding interactions that contribute to the stability and binding affinity of curcumin for TNF- α . The key amino acid residues involved in binding include conventional hydrogen bonds with glycine (GLY A:121) and

tyrosine (TYR B:151); Pi-Pi stacked and T-shaped interactions with tyrosine (TYR A:119, TYR B:119, TYR A:59); and a carbon–hydrogen bond with glutamine (GLN A:61). Multiple hydrogen bonds significantly increase the binding affinity of curcumin, making it a strong candidate for modulating TNF- α activity. The Pi–Pi stacking and T-shaped interactions with the aromatic rings of the tyrosine residues further stabilize the complex, whereas the carbon–hydrogen bond offers additional, although weaker, support. These interactions collectively underscore the potential of curcumin as an anti-inflammatory agent that targets TNF- α , a key cytokine involved in inflammation and related diseases. Figure 17 illustrates the molecular interactions between AKT1 and Curcumin, highlighting various binding interactions that contribute to the stability and binding affinity of Curcumin for AKT1. The key residues involved in binding include a conventional hydrogen bond with tryptophan (TRP A-80), a Pi–Pi stacked interaction with tryptophan (TRP A-80), carbon–hydrogen bonds with lysine (LYS A-268) and serine (SER A-205), and alkyl and pi-alkyl interactions with valine (VAL A-270), leucine (LEU A-264), and leucine (LEU A-210). Additionally, a Pi-anion interaction with aspartate (ASP A-292) contributes to binding stability. The hydrogen bonds and Pi–Pi stacking with tryptophan (TRP A-80) suggest a strong binding affinity, whereas the hydrophobic interactions with leucine further stabilize the complex. The Pi-anion and Pi-alkyl interactions also play key roles in reinforcing binding stability. Together, these interactions support the potential of Curcumin as a therapeutic agent that targets AKT1, a crucial kinase involved in cell survival and proliferation pathways.

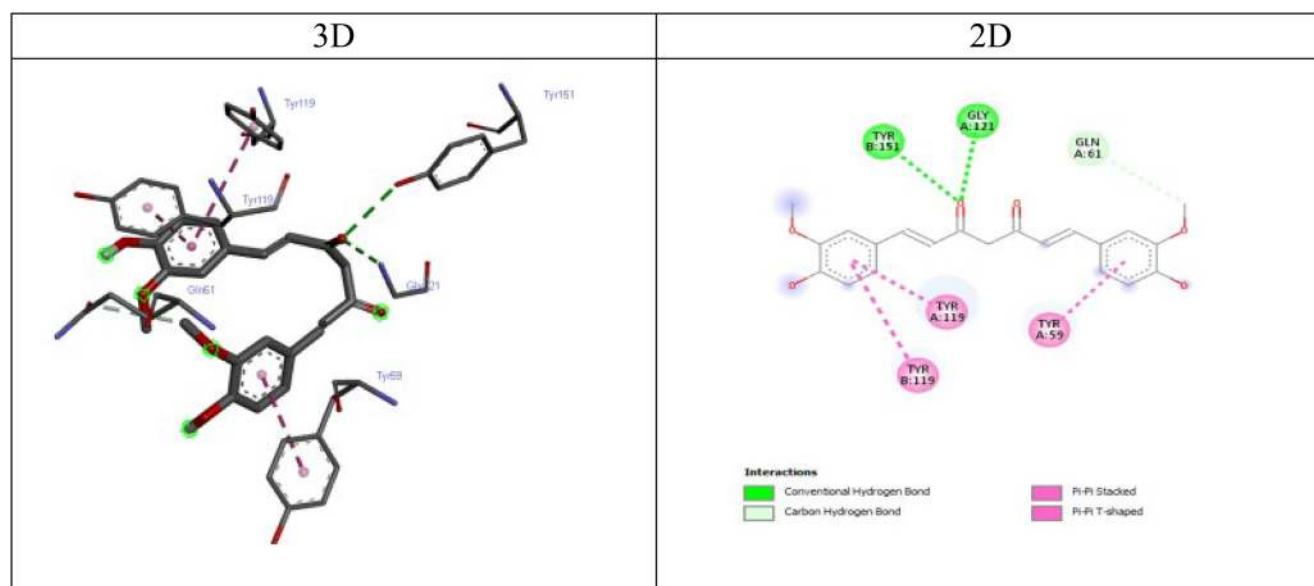


Fig. 16 3D and 2D visualizations of the interactions between TNF- α and curcumin

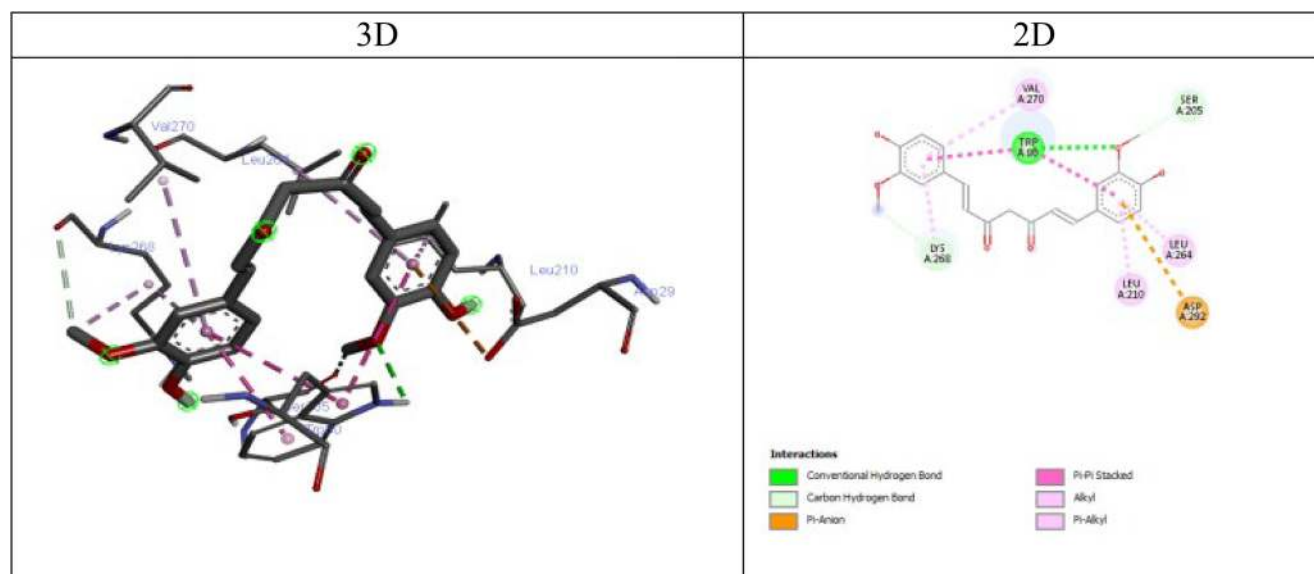


Fig. 17 3D and 2D visualizations of the interactions between AKT-1 and curcumin

Binding Affinities of Silybin and Curcumin Across Target Proteins

Molecular docking studies provide insights into the binding interactions between silybin and curcumin and different key proteins involved in regulating critical cellular processes, such as Nrf2, COX-2, TNF- α , P53, and AKT1, which play central roles in diseases such as cancer, inflammation, and oxidative stress, making them important therapeutic targets. Table 4 reveals a pattern of binding capabilities of silybin

and curcumin toward all target proteins. Silybin has a strong binding affinity for AKT1 (−11.9 kcal/mol), followed by COX-2, which has a binding affinity (−10.2 kcal/mol), TNF- α (−9 kcal/mol), and p53 (−8.7 kcal/mol), and it has a moderate binding affinity for Nrf2 (−7 kcal/mol). However, curcumin has shown strong binding affinities with AKT1 (−9.4 kcal/mol) and COX-2 (−8.4 kcal/mol), followed by moderately strong interactions with TNF- α (−7.4 kcal/mol) and p53 (−7.3 kcal/mol), and relatively weak but still significant binding with Nrf2 (−6.5 kcal/mol). This consistent pattern of high binding affinities suggests the potential of

Table 4 Molecular Docking binding affinity (Kcal/mol) for each target protein

Receptor	Ligand	binding affinity (kcal/mol)
Nrf2	Silybin	-7
	Curcumin	-6.5
COX-2	Silybin	-10.2
	Curcumin	-8.4
TNF- α	Silybin	-9
	Curcumin	-7.4
P53	Silybin	-8.7
	Curcumin	-7.3
AKT1	Silybin	-11.9
	Curcumin	-9.4

both silybin and curcumin to modulate oxidative stress, inflammation, and tumor suppression, supporting their roles as multitherapeutic agents for liver protection and cancer treatment.

Molecular Docking Implications

The docking results revealed that both silybin and curcumin strongly interact with several key proteins involved in inflammation, oxidative stress, and cancer progression. This multi-target binding profile provides a plausible mechanistic hypothesis for the enhanced efficacy observed in our biological assays. The strong, complementary binding affinities, particularly for AKT1 and COX-2, suggest that the co-encapsulated formulation can concurrently modulate multiple signaling pathways, offering a theoretical foundation for the synergistic effects seen in our nanoformulation.

The stability of these ligand–protein complexes is reinforced by a range of interactions, including hydrogen bonds, π – π stacking, and hydrophobic forces, all of which increase binding strength and specificity. These findings highlight the potential of both compounds to target multiple pathways simultaneously, suggesting that silybin and curcumin may offer a more comprehensive approach to modulating oxidative stress, inflammation, and tumor suppression. This

multitarget mechanism makes them promising candidates for therapeutic use in liver diseases, cancer, and inflammatory conditions.

Antimicrobial Efficacy of the Tested Compounds

Zone of Inhibition of Tested Compounds against Bacterial and *C. Albicans* Isolates

Our results revealed varying antimicrobial effectiveness of silybin, curcumin, *Silybum marianum* extract, and the encapsulated formula against different microbial species. As shown in Table 5; Fig. 18, the inhibition zones measured via the well diffusion method differed between the tested isolates. The encapsulated formula at a concentration of 1000 μ g/mL significantly inhibited all pathogens, with the largest zone observed for *E. coli* (14.00 ± 1.15 mm). The encapsulated formula demonstrated greater effectiveness against gram-negative bacteria than against gram-positive bacteria. Specifically, *E. coli* had the lowest concentration required to inhibit bacterial growth, with a minimum inhibitory concentration (MIC) of 20.90 ± 5.20 μ g/mL. Additionally, the combination of curcumin, silybin, and *Silybum marianum* extract, along with encapsulation, resulted in a synergistic effect. Compared with individual compounds, this combination reduced both the MICs and minimum bactericidal concentrations (MBCs) and increased the zone of inhibition.

Minimum Inhibitory Concentrations (MICs) of the Tested Compounds Against the Screened Bacterial and *C. albicans* Isolates

The antimicrobial activities of silybin, curcumin, and *Silybum marianum* extract, both in their free forms and as encapsulated formulas, were assessed through minimum inhibitory concentration (MIC) assays against a range of pathogenic microorganisms, including bacterial isolates and *Candida albicans*, as illustrated in Table 6. Silybin showed the best activity against *E. coli*, with weaker inhibition

Table 5 Zone of Inhibition of silybin, curcumin, *Silybum Marianum* and the encapsulated formula against the screened bacterial and *C. albicans* isolates

Tested organisms	Zone of inhibition (mm)			
	Silybin	Curcumin	Silybum marianum	Encapsulated formula
<i>E. coli</i>	8.00 ± 1.00	9.33 ± 1.45	9.67 ± 1.45	14.00 ± 1.15^a
<i>K. puenomiae</i>	8.00 ± 1.53	10.33 ± 0.67	11.33 ± 0.67	13.00 ± 1.73
<i>L.monocytogenes</i>	8.67 ± 1.45	8.67 ± 1.33	10.00 ± 1.15	10.00 ± 1.15
<i>S. aureus</i>	9.33 ± 0.67	8.33 ± 1.76	10.33 ± 0.88	11.67 ± 1.20
<i>C.albicans</i>	10.00 ± 2.08	8.67 ± 1.45	10.67 ± 0.88	11.67 ± 0.67

Data are expressed as the means $\pm$ standard errors (SEs) with dissimilar superscript letters within rows (significant differences at $P < 0.05$).

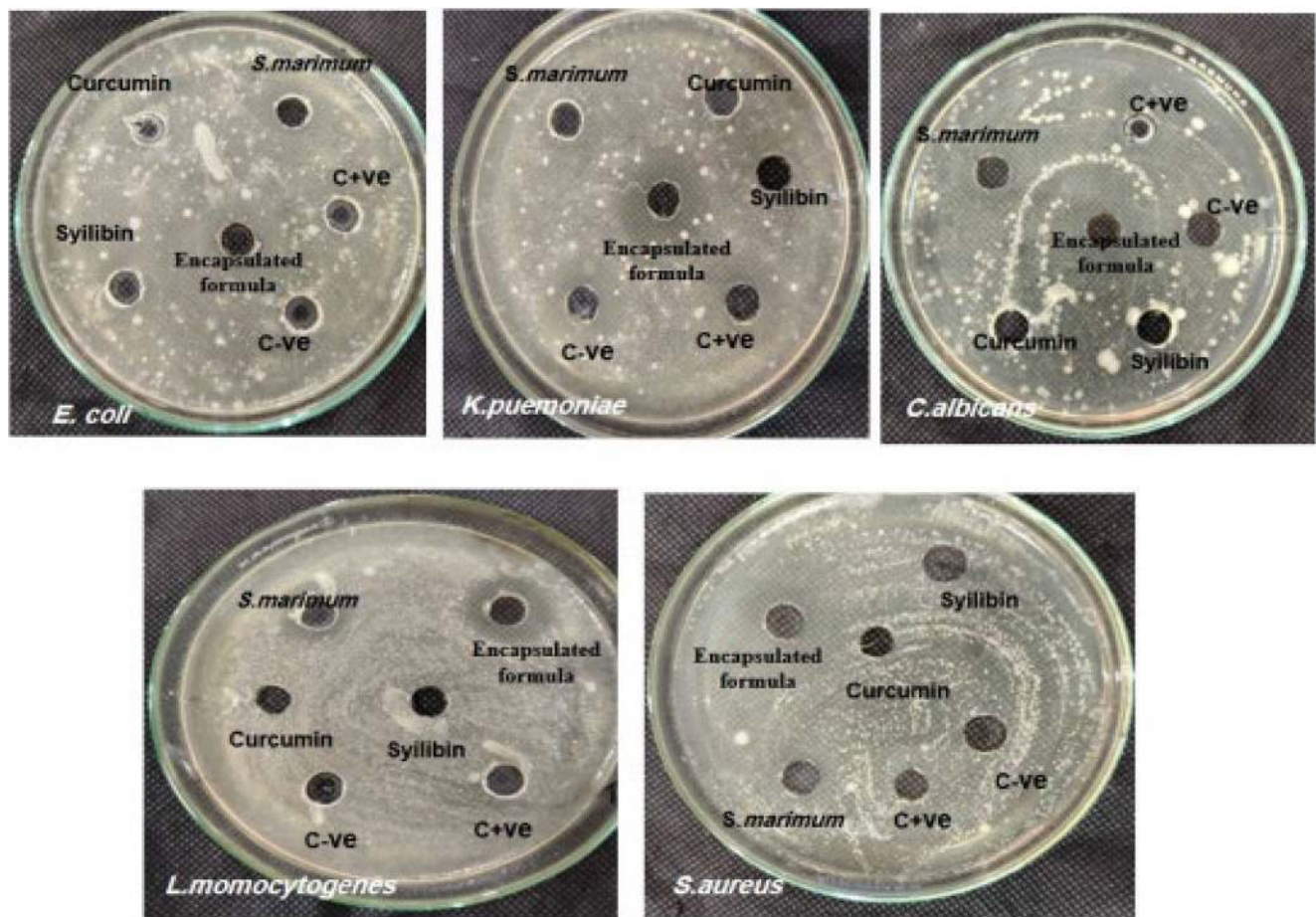


Fig. 18 Inhibition zones of silybin, curcumin, *S. maritimum*, and the encapsulated formula against the screened bacterial and *C. albicans* isolates. The controls used were DMSO (10.0%) for the negative con-

trol (C-ve) and gentamicin (10 µg/mL) and amphotericin B (10 µg/mL) for the positive control (C+ve)

Table 6 Minimal inhibitory concentrations of silybin, curcumin, Silybum Marianum and the encapsulated formula against the screened bacterial and *C. albicans* isolates

Tested organisms	MIC (µg/mL)			
	Silybin	Curcumin	Silybum marianum	Encapsulated formula
<i>E. coli</i>	166.67±41.67	83.33±20.83	41.70±10.40 ^a	20.90±5.20 ^a
<i>K. pneumoniae</i>	145.83±55.12	104.17±20.83	52.10±10.40	41.60±10.30
<i>L. monocytogenes</i>	291.67±110.24	72.93±27.55	83.33±20.83	26.07±5.23 ^a
<i>S. aureus</i>	208.33±41.67	104.17±20.83	83.33±20.83 ^a	52.10±10.40 ^a
<i>C. albicans</i>	104.17±20.83	104.17±20.83	145.83±55.12	72.93±27.55

Data are expressed as the means±standard errors (SEs) with dissimilar superscript letters within rows (significant differences at $P < 0.05$).

against *K. pneumoniae*, *S. aureus*, and *C. albicans* and the lowest activity against *L. monocytogenes*. Curcumin demonstrated consistent activity across most of the tested organisms, with slightly reduced activity against *S. aureus*. Silybum marianum extract was most effective against *K. pneumoniae* and *L. monocytogenes* but showed weaker activity against *C. albicans*. In contrast, the encapsulated formula combining silybin and curcumin within the Silybum marianum extract matrix significantly improved the

antimicrobial activity across all the organisms. The MIC values for the encapsulated formula were considerably lower, with the greatest reductions observed for *E. coli* and *S. aureus*, both of which were statistically significant. These findings suggest that encapsulation enhances the bioavailability and stability of the active compounds, improving their cellular uptake and overall antimicrobial effectiveness.

The observed improvements, especially against *E. coli* and *S. aureus*, point to a potential synergistic or additive

effect between silybin and curcumin, amplified by the encapsulation process. These results highlight the potential of the encapsulated formula as a more effective antimicrobial agent, particularly in combating drug-resistant pathogens.

Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) against Bacterial and *C. Albicans* Isolates

This study investigated the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of Silybin, curcumin, Silybum marianum extract, and an encapsulated formula combining Silybin and curcumin within a Silybum marianum extract carrier. The MBC and MFC values reflect the lowest concentrations required to kill bacteria and *Candida albicans* isolates, respectively, providing insight into the compounds' potency beyond just inhibition (MIC). Silybin had the lowest bactericidal activity against *E. coli* but had relatively high MBC/MFC values against *K. pneumoniae*, *S. aureus*, *L. monocytogenes*, and *C. albicans*, indicating varying levels of susceptibility. Curcumin exhibited consistent bactericidal activity across all bacterial species, with similar MBC and MFC values, and showed slightly reduced activity against *S. aureus*. Silybum marianum extract exhibited stronger activity against *K. pneumoniae* and *L. monocytogenes* but was less effective against *S. aureus* and *C. albicans*. The encapsulated formula significantly enhanced both the bactericidal and fungicidal activities, with substantial reductions in the MBC and MFC values across all the tested organisms, especially against *E. coli*, *S. aureus*, and *C. albicans*. These results suggest that encapsulation improves the antimicrobial efficacy of the combined compounds, likely through enhanced bioavailability, stability, and cellular uptake, as shown in Table 7. The observed synergistic or additive effects between silybin and curcumin, coupled with the role of Silybum marianum extract as a carrier, underscore the potential of the encapsulated formula as a more effective antimicrobial agent. The statistical significance of these improvements, particularly against *E. coli* and *S. aureus*, further supports the efficacy of the encapsulation strategy in optimizing antimicrobial activity.

Comparison with Previous Studies

Our results align with those of studies conducted by Górski [66], who reported that the MIC values for *E. coli* ranged from 0.312 to 1.250 mg/mL, with a mean of 0.885 mg/mL, and the MBCs ranged from 1.25 to 5 mg/mL. Similarly, curcumin exhibited significant antibacterial activity in our study, with MIC values ranging from 72.93 µg/mL to 145.83 µg/mL. In addition, the antibacterial and antifungal effects of curcumin, as well as its synergy with *S. marianum*, are consistent with previous findings. Al-Thubaiti demonstrated the effectiveness of curcumin against *E. coli*, reporting an MIC of 2.5 ± 0.31 mg/mL [67]. Sehim also reported that curcumin nanoparticles had enhanced antimicrobial effects with large inhibition zones against *S. aureus*, *E. coli*, *S. Typhimurium*, and *C. albicans* [68]. Silymarin and silybin, which have demonstrated antibacterial and antifungal activities in other studies [69], showed similar patterns of efficacy in our work. Silybin exhibited an MIC of 1024 µg/mL for *S. aureus*, while both silymarin and silybin demonstrated antifungal activity against *C. albicans*.

Mechanisms of Antimicrobial Action

The antimicrobial effects of curcumin and silybin are attributed to their ability to inhibit DNA replication, alter gene expression, disrupt cell membranes, and reduce the mobility of pathogens. These compounds also generate reactive oxygen species (ROS) and interfere with bacterial quorum sensing mechanisms, which play critical roles in microbial communication [70–72]. Additionally, curcumin and its derivatives have shown antiviral properties, particularly against herpes simplex virus-1 (HSV-1), and can induce apoptosis in *E. coli* [73–75]. A key finding of this study is the dual functional role of the *Silybum marianum* seed extract. Our results clearly demonstrate that the pure extract possesses significant intrinsic antimicrobial activity (Tables 4 and 5), which contradicts a simplistic view of it as merely an inert encapsulant. Therefore, the enhanced efficacy of the final encapsulated formula (SiCu-SM) arises from a multi-faceted synergy: the nanoencapsulation improves the delivery and bioavailability of silybin and curcumin, while the

Table 7 MBCs and MFCs of silybin, curcumin, Silybum Marianum and the encapsulated formula against the screened bacterial and *C. albicans* isolates

Tested organisms	MBCs & MFCs (µg/mL)			
	Silybin	Curcumin	Silybum marianum	Encapsulated formula
<i>E. coli</i>	166.67±41.67	104.17±20.83	104.17±20.83	41.70±10.40 ^a
<i>K. pneumoniae</i>	250.00±125.00	104.17±20.83	83.33±20.83	52.10±10.40
<i>L. monocytogenes</i>	291.67±110.24	104.17±20.83	83.33±20.83	52.10±10.40
<i>S. aureus</i>	250.00±00.00	125.00±00.00	145.83±55.12	83.33±20.83 ^a
<i>C. albicans</i>	250.00±125.00	104.17±20.83	208.33±41.67	83.33±20.83

Data are expressed as the means±standard errors (SEs) with dissimilar superscript letters within rows (significant differences at $P < 0.05$).

extract itself contributes its own bioactive properties to the overall therapeutic effect. This creates a combined antimicrobial action that is greater than the sum of the individual components.

In the developed nanoformulation, the combination of silybin and curcumin within the *Silybum marianum* extract matrix is proposed to create a synergistic antimicrobial effect, where the multi-target mechanisms of the individual compounds are complemented by the inherent bioactive profile of the extract, potentially leading to enhanced disruption of microbial cells.

Enhancing Bioavailability with Encapsulation

Research by Deljoo et al. (2019) has demonstrated that the encapsulation technique not only enhances the stability of these compounds but also improves their therapeutic effectiveness [76]. Encapsulation significantly improves the bioavailability of curcumin and silybin by protecting them from rapid breakdown and enhancing their absorption in the digestive system. While both curcumin and silybin are considered very safe, with no evidence of toxicity even at high doses in multiple animal and human studies [77, 78], they face some limitations, including low solubility in water, limited absorption, and fast elimination from the body. The use of *Silybum marianum* extract as an encapsulating agent further stabilizes these compounds, ensuring their sustained release at target sites. This, combined with the extract's own bioactivity, creates a synergistic system that enhances antimicrobial activity and overall effectiveness, making them safe and reliable options for drug delivery [79, 80].

In Vitro Cytotoxicity and Cell Viability

Cytotoxicity and Anti-Cancer Activity in MCF-7 Cells (Breast Cancer Cells)

The MTT assay is utilized to evaluate the cytotoxic effects of encapsulated silybin and curcumin on MCF-7 cells (breast cancer cells) in comparison to their free forms. After treating MCF-7 cells with different concentrations of the encapsulated formulation, cell viability was quantified, and the potency of the treatment was determined by calculating the IC₅₀ value. The IC₅₀ value represents the concentration of a compound required to reduce cell viability by 50%, providing a measure of its effectiveness in inhibiting cell growth. In this study, the encapsulated silybin and curcumin formulation had an IC₅₀ value of 81.11 ± 1.95 µg/mL, which was lower than the

IC₅₀ values for the free forms of silybin (120.44 ± 2.56 µg/mL) and curcumin (110.21 ± 2.16 µg/mL). Notably, the IC₅₀ of *Silybum marianum* seed extract alone was 165.25 ± 2.56 µg/mL, further emphasizing the enhanced cytotoxicity achieved through encapsulation. These findings indicate that the encapsulated combination is more effective at reducing MCF-7 cell viability than the individual free drugs or the plant extract itself. A lower IC₅₀ value for the encapsulated formulation suggests that a smaller concentration of the combination is required to achieve 50% inhibition of cell viability, highlighting its increased potency. The encapsulation of silybin and curcumin in *Silybum marianum* extract likely enhances their delivery and bioavailability, allowing greater therapeutic efficacy at lower doses. On the other hand, the free forms of silybin and curcumin require higher concentrations to achieve the same level of inhibition, which may lead to limitations in their clinical effectiveness and a greater likelihood of side effects. The results demonstrate that *Silybum marianum* extract-based delivery enhances the stability and targeting of silybin and curcumin, offering more effective breast cancer treatment at lower dosages, as shown in Fig. 19. These findings support the further development of *Silybum marianum* extract-based formulations for cancer therapy.

Cytotoxicity Evaluation of the Encapsulated Formulation in HepG2 Cells

The MTT assay was used to assess the cytotoxicity of encapsulated silybin and curcumin by measuring the viability of HepG2 cells after 1 day of treatment. The primary goal is to ensure that the encapsulated formulation does not significantly harm HepG2 cells while evaluating its potential to support cell viability compared with the free forms of silybin and curcumin. The results demonstrated that the encapsulated silybin and curcumin formulation had minimal cytotoxicity, with CC₅₀ values of 256.30 ± 5.26 µg/mL. In contrast, the free forms of silybin and curcumin exhibited greater cytotoxicity, with CC₅₀ values of 204.12 ± 3.65 µg/mL for free silybin and 225.8 ± 3.12 µg/mL for free curcumin. Additionally, the *Silybum marianum* seed extract alone had a CC₅₀ value of 375.30 ± 3.38 µg/mL, indicating its relatively low cytotoxicity and further supporting the safety profile of the encapsulated formulation. These results indicate that the encapsulated formulation is less cytotoxic and better supports cell viability than the free forms of silybin and curcumin are, making it a more promising candidate for hepatoprotective studies, as shown in Fig. 20.

Fig. 19 Viability of MCF-7 breast cancer cells treated with various concentrations of free silybin, free curcumin, Silybum marianum extract and Silybin-Curcumin encapsulated in Silybum marianum extract (SiCu-SM)

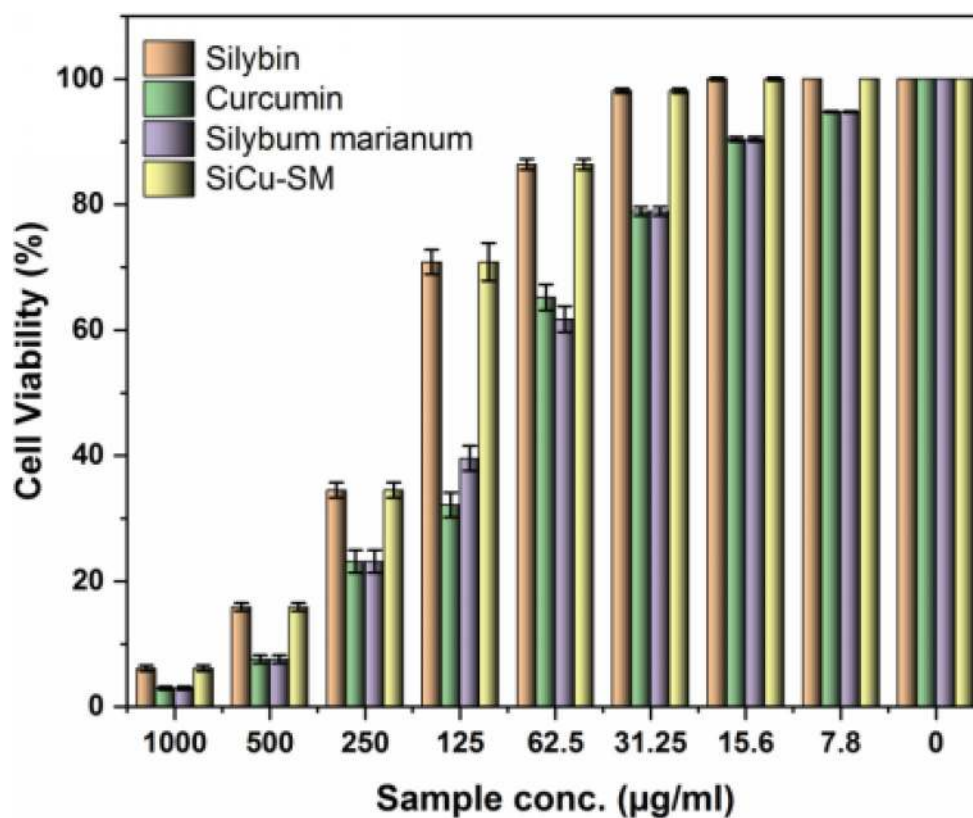
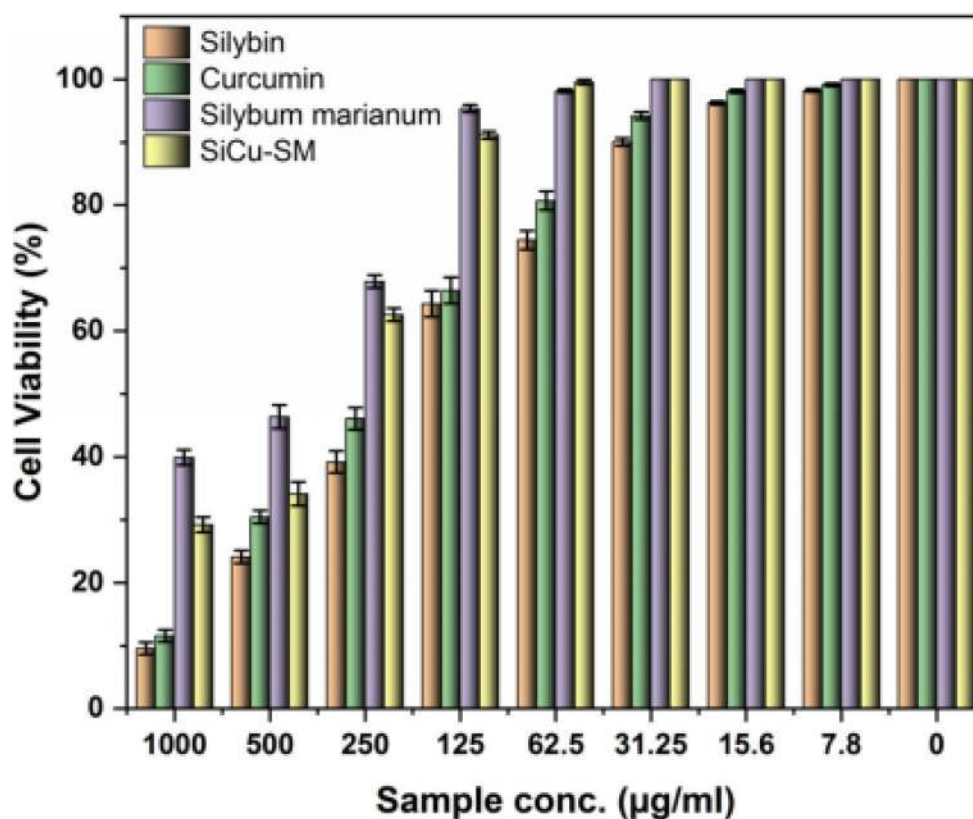


Fig. 20 Cytotoxicity evaluation of encapsulated silybin and curcumin (SiCu-SM), free silybin, free curcumin and Silybum marianum extract in HepG2 cells via the MTT assay after 24 h of treatment



Study Limitations and Future Prospects

While this study demonstrates the successful development and comprehensive characterization of a novel *Silybum marianum* seed extract-based nanoformulation for the code-livery of silybin and curcumin, several limitations should be acknowledged. Furthermore, while the encapsulation efficiency of the added pure silybin and curcumin was precisely determined, the total drug content of native silybin and other silymarin flavonoids originating from the *Silybum marianum* seed extract within the final nanoformulation was not separately quantified. This work is limited to in vitro assessments; while these include drug release kinetics, cytotoxicity, antimicrobial activity, and molecular docking, they lack in vivo validation of pharmacokinetics, biodistribution, therapeutic efficacy, or safety, which are crucial for clinical translation. Additionally, although comparative studies with free drug formulations were performed, direct comparisons with marketed or clinically used nano-formulations were not included. The mechanistic insights into cellular uptake and intracellular signaling pathways also remain limited. The antimicrobial evaluation, while informative, was restricted to a select panel of pathogens. Finally, the long-term stability under various storage conditions and the scalability of the formulation for industrial production were not assessed.

Future research should therefore focus on in vivo pharmacokinetic and pharmacodynamic studies to establish the formulation's bioavailability, tissue distribution, and pharmacological efficacy in relevant disease models. Additionally, future studies should employ advanced analytical techniques, such as High-Performance Liquid Chromatography (HPLC), to fully characterize the complete phytochemical profile, loading capacity, and fate of the extract's native compounds within the nanoformulation. Broader antimicrobial testing against a wider range of clinical isolates, including multidrug-resistant strains, is warranted. Detailed mechanistic investigations into the cellular uptake, subcellular localization, and effects on specific oncogenic or inflammatory pathways would provide deeper insight into the mode of action. Furthermore, comprehensive stability studies under ICH guidelines and the development of a scalable manufacturing process are essential next steps to advance this natural nanoencapsulation strategy toward clinical application in hepatoprotection and cancer therapy.

Conclusion

This study successfully developed an advanced nanoformulation by coencapsulating pure silybin and curcumin within a *Silybum marianum* seed extract matrix, creating a system with significant potential for hepatoprotection and

anticancer applications. A crucial finding was the dual role of the *Silybum marianum* extract, which functioned not only as an effective natural encapsulant but also as a source of intrinsic antimicrobial activity, contributing to a synergistic therapeutic effect. The nanoformulation resulted in a reduced particle size, improved uniformity, and enhanced colloidal stability, ensuring better bioavailability and cellular uptake. The high encapsulation efficiencies of the added silybin and curcumin, along with their sustained in vitro release profiles, further validated the formulation's stability and controlled release capabilities. Molecular docking studies revealed strong binding affinities of both compounds with key proteins involved in oxidative stress, inflammation, and cancer, suggesting a potent multi-target mechanism of action. The formulation demonstrated enhanced antimicrobial activity and superior anticancer efficacy in MCF-7 breast cancer cells while exhibiting minimal cytotoxicity in HepG2 liver cells. By leveraging the *Silybum marianum* extract as both a natural encapsulant and a synergistic bioactive, this work highlights a novel, effective, and stable therapeutic strategy with dual potential to address liver diseases and cancer.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12247-025-10280-5>.

Author Contributions Samar M. Mahgoub and Rehab Mahmoud were responsible for the conceptualization and experimental part. Abdullah S. Alawam was responsible for project funding and supervision. Rehab Mahmoud, Ahmed A. Allam, was responsible for material preparation, characterization, and adsorption study data analysis and interpretation, methodology, writing, and project administration. Sahar Abdel Aleem Abdel Aziz, Esraa Khaled, for editing and revising. Aya Shaban, was responsible for the calculation of the statistical parameters.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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