

Exploring the complexation by β -cyclodextrin for improving the anticancer potential of leelamine: Experimental and computational approach

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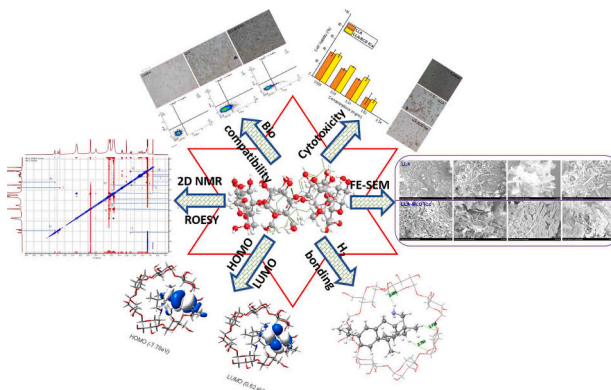
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HIGHLIGHTS

- The increasing demand for easily soluble & biocompatible pharmaceuticals.
- In this study, inclusion complexes of the drug leelamine with BCD are synthesized.
- ¹H NMR & ROESY revealed LLA is partially encapsulated in the BCD.
- The ICs significantly enhanced the biocompatibility with MRC-5 cell lines.
- The ICs showed an improved anti-cancer activity (MDA-MD-231) than the free LLA.

GRAPHICAL ABSTRACT



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ABSTRACT

Leelamine (LLA) is a natural diterpene alkaloid with promising anticancer activity, particularly against aggressive cancers such as triple-negative breast cancer. However, its therapeutic potential is limited by poor aqueous solubility, instability, and reduced bioavailability, which hinder its clinical translation. Complexation of LLA with β -Cyclodextrin (BCD) is therefore a necessary strategy to overcome these limitations, enhance its biocompatibility, and potentially augment its anticancer effects. A potentially active water-soluble anti-cancer with biocompatible material from the LLA has been produced in the presence of BCD by the sonication method, and their formation was thoroughly validated using complementary analytical techniques. ¹H NMR and ROESY provided direct evidence of complexation, showing that LLA primarily interacts with the outer rim of the BCD cavity rather than achieving complete encapsulation. This observation was further supported by IR, Raman, and NMR analyses, which collectively confirmed the stability of the inclusion complexes (ICs). Surface characterization revealed notable changes in morphology and surface roughness following complex formation. In vitro

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assays demonstrated that LLA:BCD ICs effectively induced apoptotic cell death in MDA-MB231 triple-negative breast cancer cells, while exhibiting minimal cytotoxicity in normal lung fibroblast cells (MRC-5).

1. Introduction

Bringing a new drug to the market is both exciting and challenging, particularly in terms of formulation [1]. Low aqueous solubility is a major hurdle in developing new chemical entities (NCEs), affecting drug absorption, efficacy, and side effects [2]. Poor solubility leads to slow absorption, variable bioavailability, and reduced effectiveness. Since solubility determines a drug's systemic concentration and pharmacological response, it must be assessed early in drug discovery. Additionally, poor solubility can impact metabolism, permeability, drug interactions, and release profiles [3]. Administering poorly soluble drugs, especially BCS class II and IV compounds, is difficult [4], with many cytotoxic anticancer drugs falling into class IV due to low solubility and permeability [5]. While techniques like solubilization, cosolvency, and solid dispersion improve solubility and bioavailability, they often suffer from low drug loading and high dosage requirements [6,7]. Cyclodextrin (CD) complexation has emerged as a promising alternative, offering enhanced solubility and formulation efficiency [8].

CDs are versatile excipients widely used in pharmaceuticals [9]. These cyclic oligosaccharides, composed of glucopyranose units linked via 1,4-linkages, exist as α , β , and γ types with 6, 7, and 8 units, respectively. Their truncated-cone structure features a hydrophobic cavity that entraps hydrophobic molecules, enhancing solubility, stability, and bioavailability. CDs also protect drugs from degradation, mask taste, and reduce irritation. Inclusion complexation depends on CD size, guest molecule properties, and thermodynamic interactions. CDs have enabled the market entry of insoluble drugs, improving treatment for life-threatening diseases. Also, CD-based formulations include ziprasidone mesylate, voriconazole, and reformulated drugs like aripiprazole, diclofenac sodium, meloxicam, and insulin (nasal spray), benefiting both commerce and healthcare [10]. The inclusion complexation with non-toxic agents enhances the physicochemical properties of drugs. Many anticancer drugs are complexed with CDs to improve solubility, stability, bioavailability, and dissolution while reducing toxicity [11–17]. For example, doxorubicin complexed with γ -CD and hydroxypropyl substitution (HP- γ -CD) increased blood-brain barrier permeability by disrupting the membrane [18]. A BCD-PEG-folic acid conjugate enhanced the solubility of chlorambucil, while HP-BCD complexation with 9-nitrocamptothecin significantly improved antitumor activity with reduced toxicity [11].

Leelamine (LLA) is named as dehydroabietylamine, and it is a lysosomotropic, lipophilic diterpene amine extracted from pine bark, with a pK_a of 9.9 [19,20]. Previously noted for weak cannabinoid receptor agonism and limited PDK inhibition [19,21], it has gained attention for its potent lysosomotropic activity and strong antiproliferative effects [22,23]. LLA effectively inhibits tumorigenesis [24] in human breast cancer [25], melanoma [19,20,26], and prostate cancer cell lines [27,28]. Additionally, the LLA-based derivatives enhance their anti-tumor activity in the form of a Schiff base with a copper complex [29]. Given the pharmaceutical significance of LLA and its potential applications, this study has been strategically designed to investigate its interaction with BCD through the formation of ICs. Despite the well-established benefits of CD-based drug delivery systems, a comprehensive review of the literature reveals no prior reports on the ICs of LLA with BCD. This lack of research highlights a significant knowledge gap regarding the physicochemical properties, bioavailability, and anti-cancer potential of the LLA:BCD ICs. Additionally, LLA has been previously identified for its anti-cancer properties, but its clinical translation is hindered by poor solubility and bioavailability. By investigating the formation of ICs with BCD, this research aims to assess whether BCD can serve as a biocompatible carrier to enhance LLA's

therapeutic potential.

2. Materials and methods

2.1. Materials involved

The guest, LLA, and the host, BCD, were procured from Sigma-Aldrich Chemicals Company and used without further modification. As per the literature, the reported solvents for recrystallization were employed to purify all the compounds before use. Methanol (BDH) was utilized directly without additional purification to solubilize the guest, LLA. Throughout the entire experiment, triply distilled water was also used to ensure high purity and minimize potential contaminants.

2.2. Preparation of solutions for absorption and fluorescence spectral measurements

As LLA is water-insoluble, its stock solution is prepared in methanol. For spectral measurements, a solution of LLA at a concentration of 2.25×10^{-5} M is used. To prepare the inclusion complex, the LLA solution is gradually added to a pre-prepared BCD solution under continuous stirring. The mixture is stirred for 6 h using a magnetic stirrer to ensure proper interaction and complex formation. Following this incubation period, spectral measurements were recorded to analyze the interaction between LLA and BCD.

2.3. Preparation of inclusion complex (ICs)

Following a modified method [30,31], inclusion complexes (ICs) of LLA and BCD were synthesized using ultrasonic homogenization and freeze-drying. In order to sonicate the solutions of LLA (0.045 M) and an equimolar solution of BCD (1:1 molar ratio), Hielsher UP200ST ultrasonic devices are used for 90 min at 20 kHz. Then, the stirring process was started with the same solution and heating to 60 °C. The resulting clear solution indicated IC formation. Freeze-drying at -82 °C for 48 h produced white IC powders. This method was both time- and energy-efficient.

2.4. Computational method

The initial geometry of the guest molecule, LLA, was constructed using the HyperChem software, while the structure of the host molecule, BCD, was obtained from the Protein Data Bank. All geometry optimizations and subsequent calculations were performed using the Gaussian 16 program [32]. The LLA:BCD ICs were modeled by positioning the LLA molecule within the hydrophobic cavity of BCD, as supported by experimental NMR results (Scheme 1). Geometry optimizations for the isolated molecules (LLA and BCD) and the complex were carried out using the M06–2X functional with the 6–31 G(d) basis set, which includes polarization functions on all atoms. No geometrical constraints were applied during optimization, allowing the LLA molecule to freely reorient and interact within the BCD cavity throughout the calculation process. To confirm that all optimized structures corresponded to true minima on the potential energy surface, harmonic vibrational frequency analyses were performed, and the absence of imaginary frequencies was verified. The binding of LLA to the central cavity of BCD was assessed through structural relaxation and energetic stabilization. To quantify the interaction strength between host and guest, the complexation energy ($E_{\text{complexation}}$) was calculated using the following equation.

$$E_{\text{complexation}} = E_{\text{ICs}} - (E_{\text{BCD}} + E_{\text{LLA}}) + BSSE \quad (1)$$

where E_{complex} , E_{BCD} and E_{LLA} are the total energies of the complex, the host, and the guest, respectively. This approach allows for a direct comparison of the energetics of the free and bound states. To account for the artificial stabilization arising from the incompleteness of the basis set, the basis set superposition of error (BSSE) was corrected using the counterpoise method [33].

Geometry optimization and energy calculations were performed in both gas and aqueous solution, using the IEFPCM implicit-solvent model for water [34]. To further explore the thermodynamic stability of the inclusion complex, statistical thermodynamic computations were performed at 298 K. The enthalpy change (ΔH), entropy change (ΔS), and Gibbs free energy change (ΔG) for the complexation process were calculated using the following relations, 2–4:

$$\Delta H = H_{(\text{LLA:BCD ICs})} - (H_{\text{BCD}} - H_{\text{LLA}}) \quad (2)$$

$$\Delta S = S_{(\text{LLA:BCD ICs})} - (S_{\text{BCD}} - S_{\text{LLA}}) \quad (3)$$

$$\Delta G = G_{(\text{LLA:BCD ICs})} - (G_{\text{BCD}} - G_{\text{LLA}}) \quad (4)$$

These thermodynamic parameters provide quantitative insight into the driving forces of complex formation, with ΔG serving as the key indicator of spontaneity under ambient conditions. In addition, Non-Covalent Interaction (NCI) analysis was employed to investigate the weak intermolecular interactions between the host and guest, providing detailed insight into hydrogen bonding, van der Waals forces, and other non-covalent interactions that contribute to the stability of the ICs.

2.5. Details of statistical analysis

Experiments were performed at least three times ($n = 3$), with results presented as Mean $\pm$ Standard Deviation. Statistical analysis was conducted using one-way ANOVA, followed by Duncan's multiple range test (DMRT). A p -value ≤ 0.05 was considered statistically significant.

3. Results and discussion

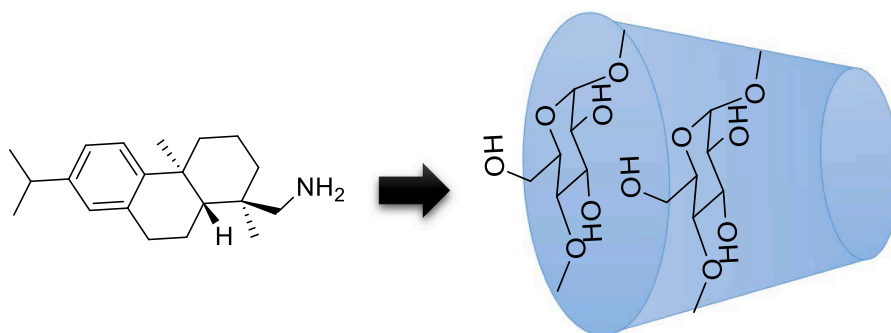
3.1. Interaction of LLA with BCD: absorption and fluorescence analysis

The inclusion complexation between LLA and BCD was systematically studied by evaluating changes in absorption and fluorescence spectra at pH 6.2. This pH was chosen under the assumption that LLA exists in a neutral form, which is essential for understanding its interaction with BCD. The absorption maxima of LLA undergo a notable red shift (10 nm) as BCD concentration increases, accompanied by a gradual rise in absorbance up to 12×10^{-3} M BCD (Fig. 1A, and Table S1). This red shift suggests a change in the microenvironment surrounding LLA, indicating successful encapsulation within the hydrophobic cavity of BCD. Beyond this concentration, the absorption maxima and absorbance remain constant, implying that the host-guest complexation reaches saturation, and additional BCD does not further influence LLA's absorption properties [35]. The red shift in absorption is a typical

phenomenon when hydrophobic interactions occur between a guest molecule, LLA, and the non-polar cavity of a BCD. In aqueous environments, LLA molecules tend to self-aggregate due to their hydrophobic nature. However, in the presence of BCD, these hydrophobic molecules are selectively entrapped inside the CD cavity, leading to enhanced solubility and stability. The observed increase in absorbance confirms the formation of stable inclusion complexes (ICs) between LLA and BCD, and named as LLA:BCD ICs. Such behavior is consistent with previous studies, which have shown that CDs enhance the solubility and optical properties of poorly water-soluble organic compounds and drugs [36]. The fluorescence spectra of LLA in the presence of BCD (Fig. 1B) provide further insight into the nature of the ICs. Interestingly, a slight blue shift (2.0 nm) in fluorescence maxima is also noted upon complexation. This minor shift suggests a conformational change in LLA, which alters its electronic environment. When LLA is encapsulated within the BCD cavity, it experiences a less polar environment compared to free LLA in water. This results in restricted molecular motion and reduced solvent interactions, thereby increasing fluorescence intensity. The enhancement in fluorescence intensity is characteristic of CD complexation, as the host-guest interaction stabilizes the excited-state energy of LLA, minimizing non-radiative decay processes [37,38].

As illustrated in the BH plots (Fig. 1C), the complexation between LLA and BCD is confirmed with a 1:1 stoichiometric ratio, indicating that one LLA molecule is encapsulated within a single BCD cavity. Such linear behavior is typically observed when host-guest inclusion proceeds through a well-defined binding equilibrium, and no substantial aggregation or higher-order complexation occurs within the studied concentration range. However, in the case of fluorescence measurement (Fig. 1D), a linear correlation is not obtained. At lower BCD concentrations, the primary 1:1 inclusion complex dominates, wherein LLA is partially or fully encapsulated in a single BCD cavity. However, as the concentration of BCD increases, additional host molecules may associate with the exposed hydrophobic regions of LLA, leading to the formation of higher-order aggregates or 1:2 (LLA:BCD) complexes [39]. Such secondary interactions, sometimes referred to as “external” or “sandwiched” complexes, have been reported in systems involving aromatic or amphiphilic guests with extended π -conjugation or flexible side chains [40]. These secondary LLA:BCD associations minimally affect the absorption spectrum (hence preserving the linear BH profile) but substantially disturb the fluorescence properties by altering the polarity, rigidity, or conformational dynamics of the encapsulated LLA [10].

To comprehensively evaluate the spontaneity of ICs, an in-depth analysis of thermodynamic parameters offers critical insights into the molecular interactions governing this process. Specifically, these parameters shed light on the displacement of LLA within the BCD cavity and the concomitant exchange of water molecules during the encapsulation event [41]. The Gibbs free energy change (ΔG) is a fundamental thermodynamic metric that delineates the feasibility of complexation and the temperature-dependent stability of the resulting ICs [42,43]. Negative values of ΔG indicate a thermodynamically favorable process, affirming spontaneous complexation under the given conditions.



Scheme 1. Schematic representation of the complexation process of LLA with BCD.

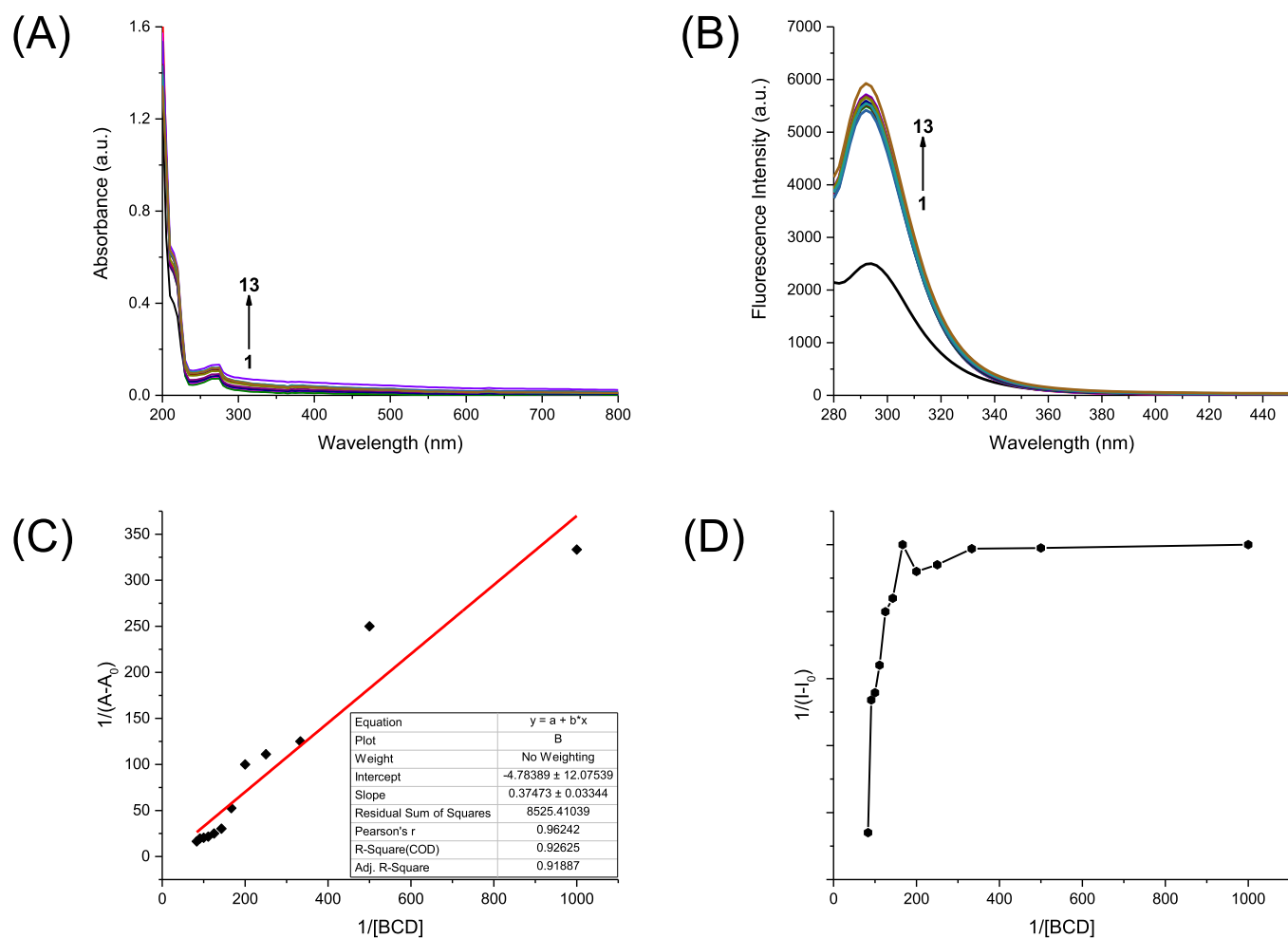


Fig. 1. Absorption (A) spectra of LLA with gradually increasing concentrations of BCD (1. Without BCD, 2. 0.001, 3. 0.002, 4. 0.003, 5. 0.004, 6. 0.005, 7. 0.006, 8. 0.007, 9. 0.008, 10. 0.009, 11. 0.010, 12. 0.011, and 13. 0.012 M). Fluorescence spectra (B) of LLA with gradually increasing concentrations of BCD (1. Without BCD, 2. 0.001, 3. 0.002, 4. 0.003, 5. 0.004, 6. 0.005, 7. 0.006, 8. 0.007, 9. 0.008, 10. 0.009, 11. 0.010, 12. 0.011, and 13. 0.012 M). BH plot of LLA with BCD by absorbance change (C), and fluorescence intensity change (D).

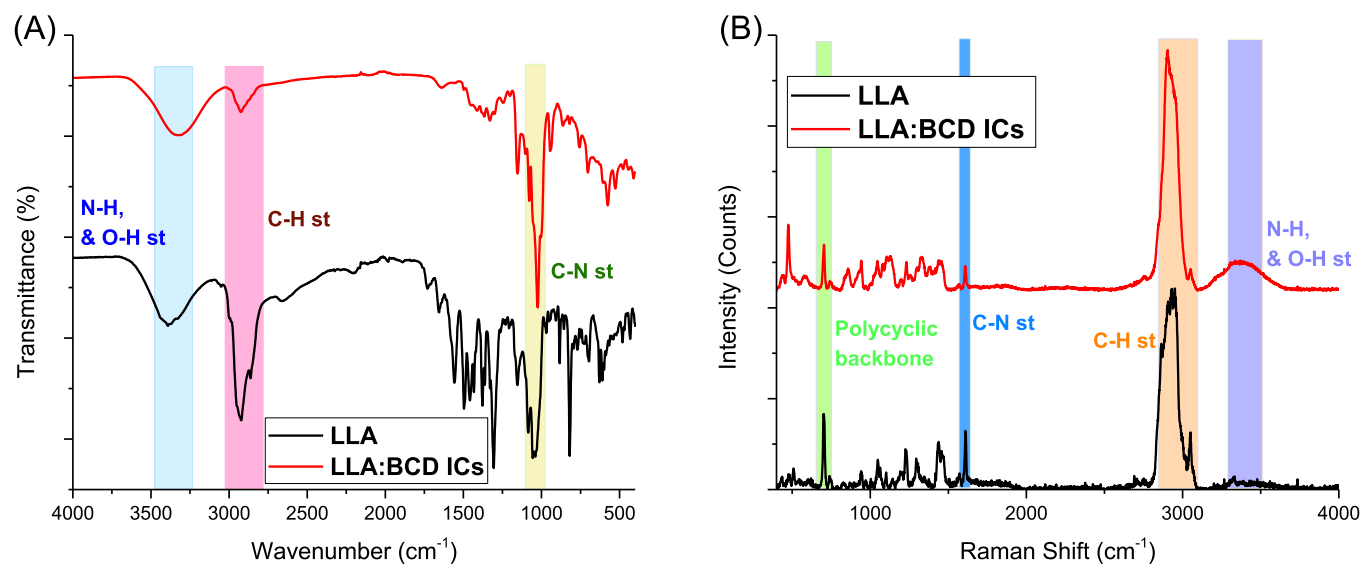


Fig. 2. FT-IR spectra (A), and Raman spectra (B) of LLA and LLA:BCD ICs.

Empirical data obtained under aqueous conditions at neutral pH and ambient room temperature reveal negative ΔG values (-9.447 kJ/mol based on absorbance variations derived from UV measurements) for the synthesized ICs (Table S2). These negative ΔG values provide robust confirmation of the spontaneous nature of the inclusion process, underscoring a substantial binding affinity between the BCD and LLA. Furthermore, the exergonic nature of the reaction underscores the thermodynamic favorability, indicating that the process is energetically straightforward at room temperature. This exothermic profile not only validates the intrinsic spontaneity of complexation but also provides essential insights into the molecular stability and structural integrity of the ICs.

3.2. FT-IR spectral analysis

FT-IR spectral analysis serves as a valuable tool for examining the interactions of guests with host molecules, such as CDs, making it instrumental in the study of functional groups and the identification of some important characteristic peaks of the guests. ICs can induce shifts or alterations in FT-IR spectra, indicative of interaction with other molecules [44,45]. In Fig. 2A, we present the FT-IR spectra of LLA in conjunction with the ICs involving BCD. The FT-IR spectrum of LLA exhibits characteristic absorption bands corresponding to the functional groups present in its structure. A broad absorption band appears at 3338 cm^{-1} , which is attributed to the stretching vibration of the primary amine ($-\text{NH}$) group. The presence of aliphatic C–H stretching vibrations is confirmed by strong bands at 2953 cm^{-1} (asymmetric) and 2855 cm^{-1} (symmetric). The N–H bending vibration of the amine group is observed at 1605 cm^{-1} , while the C–N stretching vibrations appeared at 1045 cm^{-1} , further supporting the amine functionality. For the comparison, FT-IR spectra of BCD have been provided in Figure S1. For BCD, a strong and broad fringe appeared at 3412 cm^{-1} , corresponding to the valence vibration of the O–H bond. The peak centering at 2921 cm^{-1} is attributed to the valence vibration of the C–H bond in the CH_2 - and CH_3 - groups of the host molecule. The asymmetric stretching vibration of the O–H bond in the C–O–H group and the C–H bond could be observed at 1632 cm^{-1} and 1155 cm^{-1} , respectively. The peak at 1030 cm^{-1} could be attributed to the valence vibration of the C–O bond in BCD [45,46]. The ICs, which shift in the positions of LLA stretching vibrations (e.g., carboxyl and carbonyl groups), indicate interactions such as hydrogen bonding between the carbonyl oxygen of LLA and the hydroxyl groups of the BCD cavity. A broader peak appears at 3389 cm^{-1} in the ICs as well, which is attributed to the presence of BCD and LLA (Table 1). This peak indicates the stretching vibration of O–H bonds within BCD, which becomes more prominent in the ICs. C–N stretching of LLA and C–O stretching of BCD are close to each other, and these are completely merged. These changes in the FT-IR spectra upon complexation provide strong evidence for the inclusion of functional groups from LLA into the cavity of BCD [47].

Table 1
Assignment of FT-IR frequencies of LLA and ICs.

Assignment	LLA (cm^{-1})	BCD (cm^{-1})	ICs (cm^{-1})	Changes or differences (cm^{-1})
N–H stretch	3338	NIL	3389 (broader)	+ 51
C–H stretch	2953/ 2855	2921	Slightly altered	NA
N–H bend	1605	NIL	1591	–14
C–N stretch	1045	NIL	Merged with BCD	NA
C–O–H stretch	NIL	1632	Weakened	NA
O–H stretch	NIL	3412	3389 (broader)	–23 (centered)
C–O stretch	NIL	1030	1039	+ 9

3.3. Raman spectral analysis

The Raman spectra analysis provides detailed insights into the interactions between LLA and BCD, as provided in Fig. 2B [48]. For the free LLA molecule, three characteristic Raman bands are observed: The C–H stretching vibration at 2945 cm^{-1} , the C–N stretching vibration at 1595 cm^{-1} , and a prominent band at 708 cm^{-1} corresponding to the polycyclic backbone vibrations. These peaks serve as molecular fingerprints of LLA. Upon ICs with BCD, these characteristic bands are still present but appear at slightly shifted positions: The C–H stretching mode shifts from 2945 to 2887 cm^{-1} , the C–N stretching shifts from 1595 to 1587 cm^{-1} , and the polycyclic backbone vibration moves from 708 to 719 cm^{-1} . Such systematic downshifts or upshifts in vibrational frequencies are indicative of host–guest interactions, confirming that LLA has been successfully encapsulated inside the hydrophobic cavity of BCD. In addition to these shifts, new Raman features emerge in the IC spectrum. A broad band at around 3345 cm^{-1} is observed, which corresponds to N–H and O–H stretching vibrations. This band originates from the hydroxyl groups of BCD and from hydrogen bonding interactions between the host and guest molecules. The slight displacement of this band relative to pure BCD suggests a perturbation in the hydrogen-bonding network due to inclusion. Hence, the observed spectral shifts and the appearance of additional O–H/N–H stretching modes strongly support the successful formation of the LLA:BCD ICs, consistent with previous reports on CD host–guest systems where vibrational shifts are diagnostic of encapsulation.

3.4. NMR and ROESY analysis

NMR spectroscopy provides detailed insights into drug and BCD interactions by revealing the drug's inclusion within the CD cavity, its orientation, binding affinity, and the specific parts of the drug that interact with the CD's hydrophobic cavity and polar surface. Advanced NMR techniques, such as ROESY experiments, are used to correlate these host–guest spatial relationships, while chemical shift changes and relaxation studies can characterize the strength and dynamics of the interactions and provide information on the complex's stability and structural changes [49].

3.5. Analysis of the proton NMR spectrum of LLA: BCD ICs

Numbering of the LLA (guest) molecule is shown in Fig. 3A, and a 3D representation of the compound is presented in Fig. 3B. The proton NMR spectrum of the LLA:BCD ICs is depicted in Fig. 3C. The spectrum clearly displays resonances corresponding to both the BCD and the LLA, indicating their presence in the ICs. Upon complexation, notable changes in the chemical shifts of specific protons were observed, suggesting interactions between the two components. For the BCD moiety, the hydroxy protons at the C-2 and C-3 positions and the CH_2OH group attached to the C-6 carbon exhibited slight downfield shifts in the range of 0.01 – 0.04 ppm. These deshielding effects imply hydrogen bonding or dipole–dipole interactions between the hydroxyl groups of BCD and the functional groups of LLA. Conversely, the remaining protons in the BCD framework experienced upfield shifts of approximately 0.03 – 0.10 ppm. This shielding effect is likely due to changes in the electronic environment surrounding the BCD cavity upon guest encapsulation. With respect to the guest molecule LLA, the angular methyl proton at the C-4a' position and the angular methine proton at H-10a' exhibited significant downfield shifts ranging from 0.06 to 0.11 ppm, suggesting their involvement in inclusion within the hydrophobic cavity of BCD. These protons likely experience deshielding as a result of being located near the electron-deficient interior of the host. In contrast, the remaining protons of LLA showed minor upfield shifts between 0.01 – 0.02 ppm, further supporting their altered magnetic environments due to complex formation. The overall pattern of chemical shift perturbations in both host and guest molecules strongly supports the formation of an inclusion

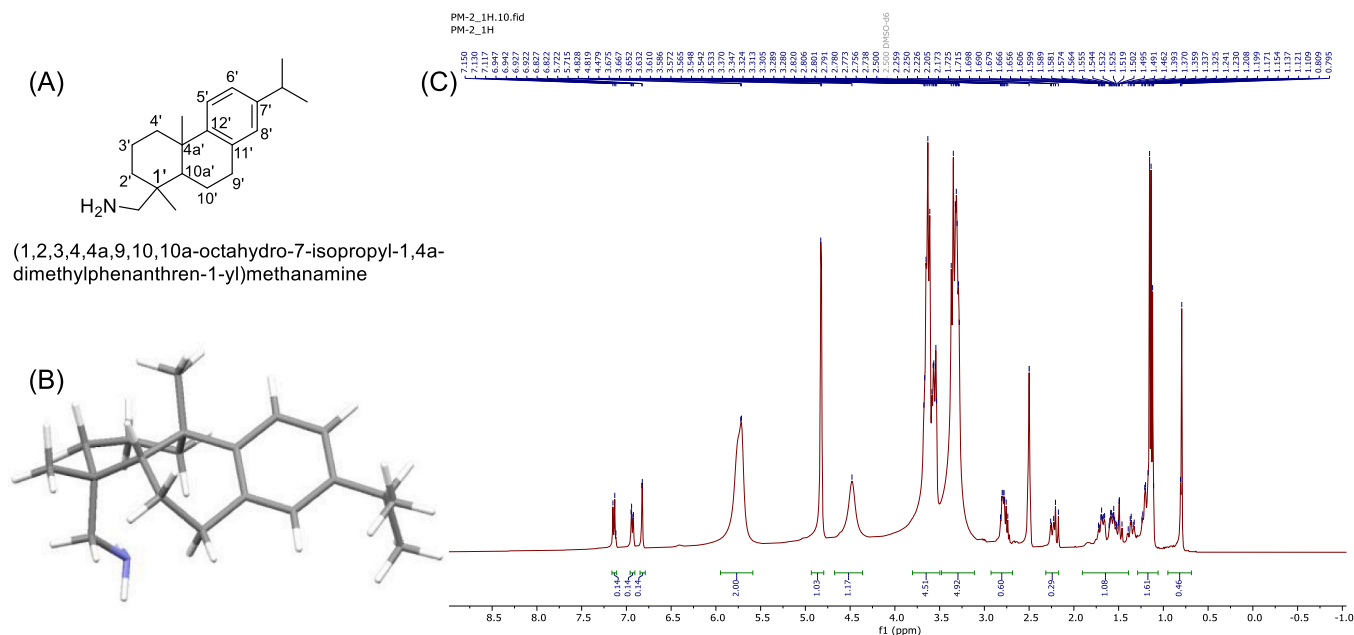


Fig. 3. Labelling of protons (A), 3D structure of LLA (B), and ¹H NMR spectra of LLA:BCD ICs (C).

complex. To further confirm this interaction, ROESY analysis is performed. The ROESY revealed spatial proximity between specific protons of LLA and those located within the cavity of BCD, providing definitive evidence for host–guest complexation. The chemical shift values of individual protons for pure BCD, free LLA, and the LLA:BCD ICs are summarized in Table S3, highlighting the shift variations upon complex formation.

3.6. Analysis of ROESY of β -CD:LLA ICs

The ROESY of the LLA:BCD ICs is presented in Fig. 4A. This spectrum provides critical insights into the spatial proximities between protons of the BCD and LLA molecules, confirming the inclusion phenomenon. Several key cross-peaks were observed, indicating dipolar interactions between spatially close protons (typically within 5 Å), even if they are not chemically bonded. These interactions are strong evidence for the

spatial orientation of the LLA molecule within the BCD cavity. Notably, cross-peak **a** at (7.14/2.22 ppm) and cross-peak **b** at (7.14/6.94 ppm) reveal strong ROESY correlations between aromatic protons H-5' and H-6' of the LLA moiety and the axial proton at C-4'. These correlations confirm that the aromatic ring of LLA is close to the central axial region of the molecule, supporting its partial insertion into the BCD cavity. The assignments of these aromatic protons, 7.14 ppm for H-5', 6.94 ppm for H-6', and 2.22 ppm for the axial proton at C-4', were validated through these cross-peaks. Another cross-peak of interest, labeled **c** at (7.14/2.85 ppm), shows a correlation between the aromatic proton H-8' and the methylene protons at C-9', indicating that the side chain of LLA is also oriented toward the inner cavity of BCD. Further supporting the spatial arrangement, cross-peaks **d**, **e**, and **f** were observed at (2.22/1.21 ppm), (2.22/2.73 ppm), and (2.22/7.14 ppm), respectively. These correlations confirm the proximity of the axial proton at C-4' with (i) the methylene group at C-9' (1.21 ppm), (ii) the angular methine proton at C-10a'

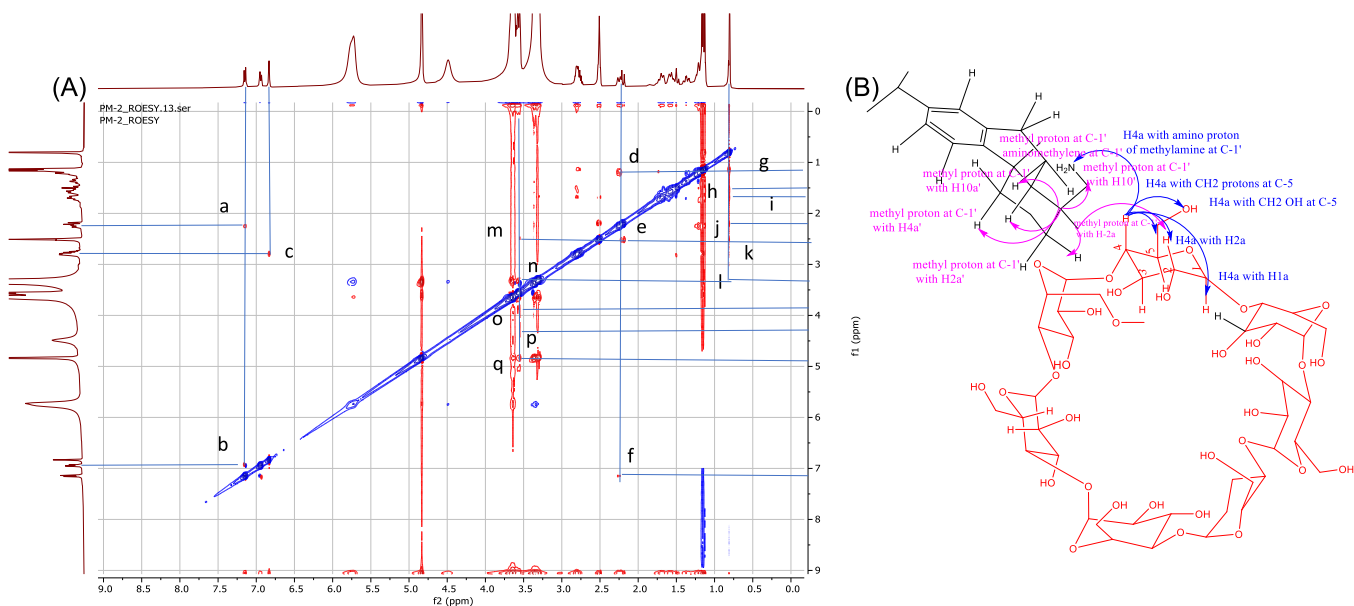


Fig. 4. ROESY of LLA:BCD ICs (A), and Important ROESY correlation to assign LLA protons (B).

(2.73 ppm), and (iii) the aromatic proton H-5' (7.14 ppm). Each of these assignments had been preliminarily established based on NMR data and is now strongly supported by the observed ROESY correlations. Hence, these ROESY cross-peaks (a–f) provide crucial spatial mapping of the guest molecule within the BCD cavity, confirming the successful formation of a well-defined ICs.

In addition to the previously discussed cross-peaks (a–f), a set of new cross-peaks **g–l** was observed, providing further confirmation of the ICs. Cross-peak **g** (0.80/1.21) reflects proximity between the methyl group at C-1' and the angular methine proton at H-10a'. Cross-peak **h** (0.80/1.51) is due to correlation with methylene protons at C-2'. Cross-peak **i** (0.80/1.70) represents interaction with methylene protons at C-10'. Cross-peak **j** (0.80/2.22) confirms spatial interaction with the axial proton at C-4'. Cross-peak **k** (0.80/2.70) suggests proximity to the methylene protons of the aminomethylene group at C-1'. The chemical shifts at 1.21 and 2.22 ppm had already been assigned in the previous discussion. The remaining proton resonances 1.51 ppm, 1.70 ppm, and 2.70 ppm were thereby confidently attributed to methylene protons at C-2', methylene protons at C-10', and aminomethylene protons at C-1', respectively. A particularly significant cross-peak, **l** (0.80/3.33 ppm), indicates a correlation between the methyl protons at C-1' of LLA and H-2 of the BCD ring. This intermolecular cross-peak provides direct evidence of the inclusion of the guest molecule into the hydrophobic cavity of BCD, specifically placing the C-1' methyl group near the narrow rim of the BCD. Additional important host–guest correlations were observed through cross-peaks **m–q**, occurring as follows. **m** (3.56/2.73 ppm), **n** (3.56/3.33 ppm), **o** (3.56/3.82 ppm), **p** (3.56/4.46 ppm), and **q** (3.56/4.82 ppm). Among these, cross-peak **m** indicates a correlation between the H-4 proton of the BCD ring and the amino proton of the aminomethylene group in LLA. This interaction enables the assignment of the 2.73 ppm signal to the amino proton at C-1' of the guest molecule. The remaining cross-peaks **n–q** confirm intra-host correlations between H-4a of β -CD (3.56 ppm) and various protons within the BCD structure itself, including: H-2 (3.33 ppm), H-1 (3.82 ppm), Methylene protons at C-5 (4.46 ppm), and Hydroxyl protons at C-5 (4.82 ppm). These interactions collectively support a stable and well-oriented inclusion of the guest molecule within the BCD cavity. The full network of ROESY interactions (cross-peaks **a–q**) is summarized in Fig. 4B, which visually demonstrates the spatial proximity and confirms the encapsulation of LLA within the CD cavity. Based on all observed chemical shifts and ROESY correlations, a structural model for the ICs is proposed. The data strongly suggest that the aminomethylene group bearing the cyclohexane ring of LLA is positioned near the narrow rim ("bucket mouth") of BCD, while

the remaining parts of the molecule extend outward from the cavity. The proposed spatial arrangement and orientation of the LLA:BCD ICs are illustrated in Figure S4.

3.7. Morphological images by FE-SEM analysis

FE-SEM is a widely utilized technique for examining the surface morphology and structural characteristics of pharmaceutical compounds, including LLA and its ICs [50]. In Fig. 5, FE-SEM images provide a comparative morphological analysis of pure LLA, BCD, and the corresponding ICs, offering insight into the structural modifications induced by complexation. The FE-SEM images reveal that pure LLA exhibits stick-like crystalline structures with a combination of smooth and rough surfaces, suggesting a well-defined crystalline nature. This morphology is indicative of strong intermolecular interactions within the LLA structure, which often contribute to its poor aqueous solubility. On the other hand, BCD displays a distinct rock-like morphology, consisting of both small and large irregularly shaped particles distributed throughout the sample [51,52]. This heterogeneous surface is characteristic of CD powders, which tend to form amorphous or semi-crystalline aggregates due to their hydrophilic exterior and hydrophobic cavity. In contrast to the individual LLA and BCD samples, the LLA:BCD ICs exhibit entirely different morphologies, as evident from FE-SEM images captured at different magnifications. The structural features of both free LLA and BCD disappear, indicating a significant modification in surface morphology upon the inclusion complexation. Instead of the crystalline stick-like LLA structures or the rock-like BCD particles, the ICs appear as irregularly shaped, amorphous aggregates with a smoother, less defined surface. This transformation suggests the successful encapsulation of LLA into the BCD cavity, leading to altered physical characteristics. The disruption of the original crystalline structure of LLA further implies that the LLA molecules are well-dispersed within the BCD matrix, forming a more homogeneous complex.

3.8. Surface analysis by powder XRD patterns

XRD analysis was performed to further confirm the formation of the LLA:BCD ICs. XRD serves as a powerful analytical tool for determining the crystallinity of pharmaceutical compounds and provides direct evidence of complexation by detecting structural changes in the guest molecule upon encapsulation [53]. The XRD pattern of pure LLA (Fig. 6) exhibits sharp, well-defined diffraction peaks at specific 2θ angles

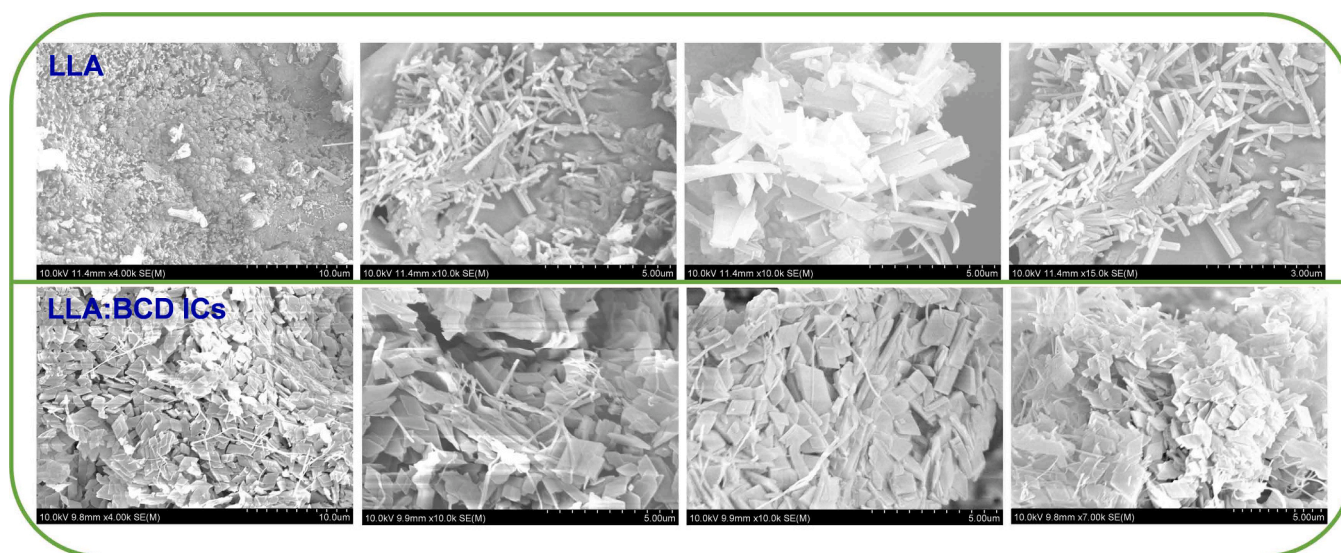


Fig. 5. SEM images of LLA, and LLA:BCD ICs.

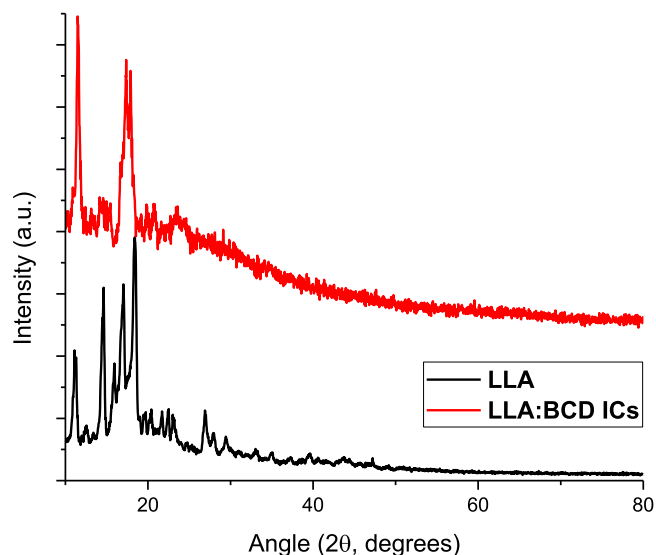


Fig. 6. Powder XRD patterns of LLA, and LLA:BCD ICs.

(11.11°, 14.67°, 15.98°, 17.03°, and 18.41°), confirming its crystalline nature. These intense peaks indicate a highly ordered molecular structure, a common characteristic of poorly water-soluble drugs. Similarly, BCD displays its typical diffraction peaks at 8.8°, 10.6°, and 12.5°, representing its "cage-type" crystalline structure. This pattern is characteristic of pure cyclodextrin, which tends to form a semi-crystalline lattice due to its unique molecular arrangement [54,55]. Upon

forming the LLA:BCD ICs, a notable reduction in peak intensity and, in some cases, a complete disappearance of LLA's characteristic diffraction peaks is observed. This significant change suggests a loss of crystallinity, indicating that LLA has undergone amorphization or transformed into a semi-amorphous state within the ICs. The reduction in crystallinity is a strong indication of successful encapsulation, as guest molecules typically experience disruption in their ordered lattice when forming ICs. Additionally, this structural change commonly increases the apparent solubility and the dissolution rate because an amorphous (or molecularly dispersed) drug has higher free energy and greater molecular mobility than the crystalline form, which often translates into faster drug release and improved oral (or local) bioavailability [56,57]. The stable interactions between LLA and BCD result in a homogeneous molecular dispersion within the CD cavity, preventing LLA from maintaining its crystalline structure [58,59].

3.9. Thermal stability of LLA and ICs by TG/DTA analysis

TG/DTA are critical analytical techniques employed in supramolecular assembly to evaluate thermal stability by monitoring weight changes and temperature variations under controlled heating conditions [60]. These techniques provide insights into the decomposition behavior, phase transitions, and interactions within ICs. Fig. 7 presents the TG curves for both pure LLA and its corresponding ICs. LLA, recognized for its stability as an organic drug molecule, exhibits no weight loss with a gradual increase in temperature up to 190°C, suggesting negligible volatile component loss. Beyond this temperature, LLA undergoes thermal decomposition, with a substantial weight reduction observed in the range of 200–284°C, which leads to complete

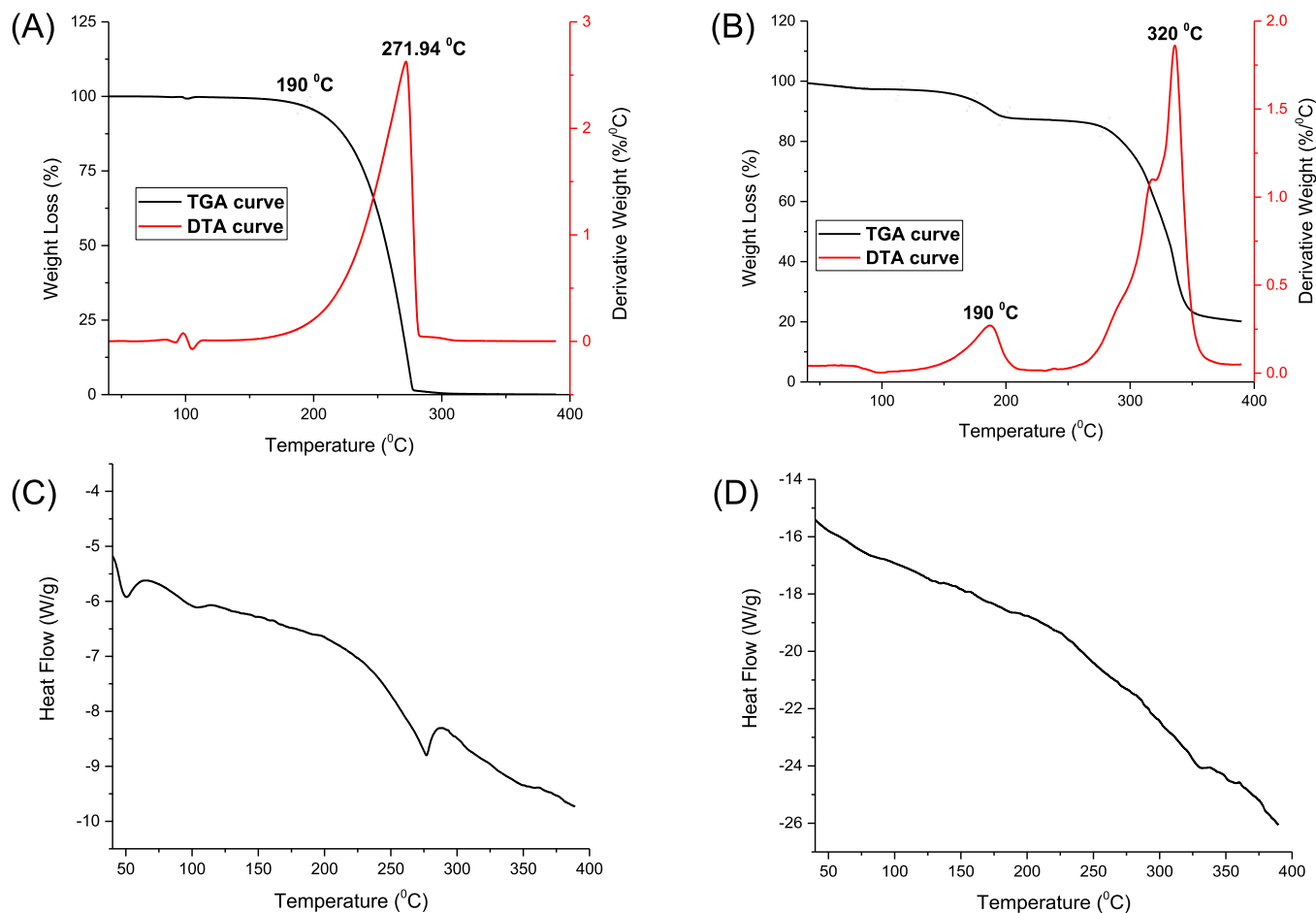


Fig. 7. TG/DTA curves of LLA (A), and LLA:BCD ICs (B). Heat Flow of LLA (C), and LLA:BCD ICs (D).

decomposition. The DTA analysis of LLA reveals a distinct endothermic peak at 271.94°C, signifying its thermal degradation, which commences after 215°C. In the case of ICs, a minor weight loss is detected below 100°C, attributed to moisture desorption, though this effect is relatively insignificant. The ICs remain stable up to 190°C, reflecting the thermal stability of LLA. A notable mass reduction of approximately 7 % (from 99 % to 92 %) occurs around this temperature range, corresponding to the partial decomposition of LLA. However, unlike pure LLA, the drug does not undergo complete decomposition at this stage. The presence of BCD in the ICs plays a crucial role in modulating the weight loss profile, as the encapsulation effect provides a protective barrier against rapid thermal degradation. This phenomenon suggests a delayed decomposition mechanism due to the stabilizing influence of BCD. Beyond 205°C, the ICs maintain thermal stability up to approximately 295°C, a notable improvement compared to pure LLA. Following this temperature, a sharp weight loss occurs, indicating the major decomposition phase and corresponding to the simultaneous breakdown of both BCD and LLA within the ICs. At this stage, around 60 % of the total mass is lost, marking the extensive degradation of the ICs. The corresponding DTA analysis further corroborates these findings, with distinctive thermal events observed at 190°C and 320°C, corresponding to the sequential decomposition of LLA and BCD, respectively. The thermogravimetric findings highlight the impact of molecular encapsulation on the thermal behavior of LLA [61,62]. The delayed degradation and altered decomposition pattern in ICs confirm the protective effect of BCD and enhance the thermal flexibility of LLA [63]. These results demonstrate the potential of supramolecular inclusion strategies in improving the stability and thermal characteristics of bioactive compounds. In addition, it is more useful for the LLA in the improvement of shelf-life and reducing degradation pathways that would otherwise lower potency.

3.10. Computational analysis of LLA:BCD ICs

The LLA molecule is firmly encapsulated within the wider cavity of BCD, contributing significantly to the overall stabilization of the ICs. The complexation energy was calculated to be $-14.9 \text{ kcal.mol}^{-1}$ in the gas phase and $-15.2 \text{ kcal.mol}^{-1}$ in aqueous solution at the M06-2X/6-31 G (d) level of theory. These negative values indicate that the formation of the complex is thermodynamically favorable and spontaneous in both phases, reflecting a strong and energetically preferred interaction between the host and guest molecules. Fig. 8 shows more stable optimized geometries of LLA:BCD in the water phase, revealing the inclusion of LLA with the wider cavity of BCD, thus contributing to the stabilization of the ICs.

The highest occupied molecular orbital (HOMO) and Lowest unoccupied molecular orbital (LUMO) are fundamental descriptors in

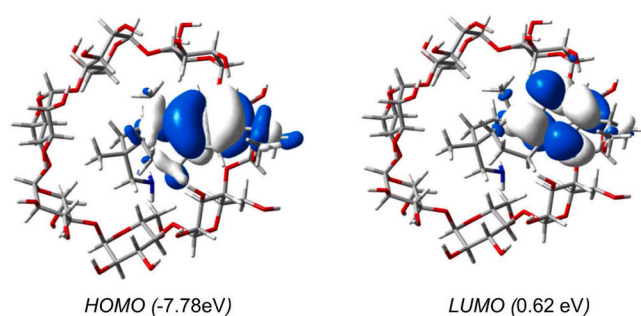


Fig. 9. The HOMO and LUMO energy diagram of ICs.

quantum chemistry, as they govern frontier orbital interaction and overall chemical reactivity of molecules. The representation of the HOMO and LUMO orbitals is shown in Fig. 9. From these orbital energies, several global parameters can be derived, including the frontier orbital energy gap ($\Delta E_{\text{gap}} = E_{\text{LUMO}} - E_{\text{HOMO}}$), chemical potential (μ), global hardness (η), and electrophilicity index (ω). These are calculated using the following equations, 6–8:

$$\mu = \frac{1}{2}(E_{\text{HOMO}} + E_{\text{LUMO}}) \quad (6)$$

$$\eta = \frac{1}{2}(E_{\text{HOMO}} - E_{\text{LUMO}}) \quad (7)$$

$$\omega = \frac{\mu^2}{2\eta} \quad (8)$$

The calculated FMO parameters for BCD, LLA, and their ICs (LLA:BCD) in both gas and aqueous phases are summarized in Table 2. In the gas phase, the free LLA molecule exhibits the smallest energy gap

Table 2

FMO, HOMO-LUMO gaps, and global descriptors for BCD, LLA and LLA:BCD system calculated at M06-2X level theory in gas and aqueous phase (IEFPCM water).

Phase	Compound	E_{HOMO} (eV)	E_{LUMO} (eV)	ΔE (eV)	μ (eV)	η (eV)	ω (eV)
Gas	LLA:BCD	-7.78	0.62	8.40	-3.58	4.20	1.52
	BCD	-8.81	1.78	10.59	-3.51	5.29	1.17
	LLA	-5.81	0.39	6.20	-2.71	3.10	1.18
Water	LLA:BCD	7.71	0.71	8.42	-3.50	4.21	1.45
	BCD	-8.81	2.36	11.17	-3.23	5.58	0.94
	LLA	-7.46	0.98	8.44	-3.24	4.22	1.24

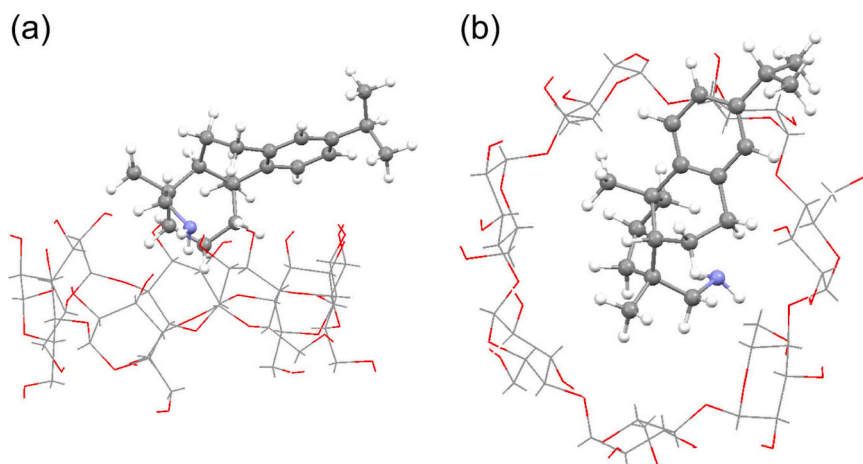


Fig. 8. Top (a), and side (b) views of optimized structures of ICs.

(6.20 eV), signifying the highest chemical softness and inherent reactivity, whereas BCD shows the largest gap (10.59 eV), indicating greater stability and hardness. Upon the formation of ICs, the energy gap of LLA:BCD increases to 8.40 eV, falling between the values of its individual components, which implies that the encapsulation of LLA within the cavity of BCD stabilizes its frontier orbitals.

In the aqueous phase, a similar trend is observed, with the ICs showing an intermediate energy gap (8.42 eV) between LLA (8.44 eV) and BCD (11.17 eV), confirming that solvation slightly modulates the electronic distribution while maintaining the stabilization effect. The negative chemical potential values for all systems reflect their electron-rich nature. Furthermore, the ω reveals that the ICs (1.45 eV) behave as a stronger electrophile compared to the individual LLA (1.24 eV) and BCD (0.94 eV). This enhanced electrophilicity suggests that host–guest interactions not only stabilize the electronic configuration but also increase the electron-accepting ability of the ICs, which could be relevant to its reactivity and possible biological performance [64,65].

Beyond the FMO analysis, the thermodynamic parameters provide a deeper insight into the driving forces leading the inclusion process. The calculated enthalpy, entropy, and free energy values for BCD, LLA, and the LLA:BCD (ICs) are summarized in Table 3. In the gas phase, the enthalpy change ($\Delta H = -11.57 \text{ kcal}\cdot\text{mol}^{-1}$) is negative, indicating that complex formation is exothermic and energetically favorable. This stabilization originates from non-covalent interactions, such as hydrogen bonding and van der Waals contacts, between LLA and the hydrophobic cavity of BCD. However, the entropy change ($\Delta S = -26.46 \text{ cal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$) is strongly negative, reflecting the loss of configurational freedom of the guest molecule (LLA) and the ordering of the host cavity upon inclusion. Consequently, the overall free energy change ($\Delta G = -3.68 \text{ kcal}\cdot\text{mol}^{-1}$) confirms that the inclusion process remains spontaneous at room temperature. In the aqueous phase, the inclusion is even more favorable, with a larger exothermic enthalpy change ($\Delta H = -55.52 \text{ kcal}\cdot\text{mol}^{-1}$) and a moderate entropy decrease ($\Delta S = -19.37 \text{ cal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$). The resulting free energy change ($\Delta G = -49.88 \text{ kcal}\cdot\text{mol}^{-1}$) demonstrates a strong thermodynamic driving force for the formation of ICs in solution. These findings indicate that the enthalpic stabilization, mainly due to host–guest hydrogen bonding and van der Waals interactions, dominates over the unfavorable entropy contribution.

To complement the thermodynamic results, NCI (non-covalent interaction) and RDG (reduced density gradient) analyses were conducted to gain deeper insights into the nature of the host–guest interactions stabilizing the ICs. The NCI analysis quantitatively supports the role of weak interactions in stabilizing the ICs. Visual inspection of the NCI isosurfaces (Fig. 10A) indicates multiple green patches within the cyclodextrin cavity, corresponding to dispersive van der Waals contacts between the hydrophobic inner wall of BCD and the aliphatic chain of LLA. These dispersive regions coincide with interatomic distances in the range of 3.2–3.6 Å, typical for van der Waals stabilization. In addition, small blue isosurfaces, though less intense, appear near hydroxyl groups of BCD interacting with polar groups of LLA, consistent

with weak hydrogen bonds. Measurement of selected contacts confirms hydrogen bond distances of 2.0–2.3 Å for O–H...N interactions, values that fall within the range of moderately strong non-covalent hydrogen bonding.

The RDG scatter plot (Fig. 10B) provides complementary evidence. The broad point around sign $(\lambda_2)\rho \approx 0$ corresponds to van der Waals interactions, while the tail extending toward negative values reflects the presence of hydrogen bonding contacts. The steric repulsion indicated by the positive sign $(\lambda_2)\rho$ values corresponds to the confinement of LLA within the cavity of BCD, which enforces close packing. Taken together, these results reveal that the inclusion process is stabilized by a synergistic combination of van der Waals dispersion forces and directional hydrogen bonding, with steric contacts shaping the geometry of the encapsulated guest. The predominance of dispersive interactions explains the moderate binding free energy observed in the thermodynamic analysis, whereas the specific hydrogen bonds provide additional stability and help orient LLA within the host cavity.

3.11. Biocompatibility assays of LLA and ICs on MRC-5 cell lines

Biocompatibility assays were conducted on human lung fibroblast cells (MRC-5) to evaluate the toxicological profiles of free LLA and its ICs across a range of concentrations. These assays aimed to compare the cytotoxic effects of each treatment with the untreated control group, thereby establishing a relative measure of cellular tolerance. Cell viability was quantitatively measured using the Alamar Blue assay [66], a reliable and sensitive indicator of metabolic activity, which indirectly reflects cell health and proliferation. The results, presented as mean $\pm$ standard deviation ($n = 3$), are detailed in Fig. 11 and Table S10. As illustrated in Fig. 11, MRC-5 cells exhibited differential survival rates depending on the concentration and formulation of the compounds. Treatment with free LLA demonstrated a concentration-dependent cytotoxic response, with notably reduced cell viability observed at higher concentrations. This suggests that LLA alone may exert significant toxic effects when administered at elevated doses. Conversely, at lower concentrations, the cytotoxicity of LLA was markedly diminished, and cell survival improved correspondingly. In contrast, the ICs displayed a more favorable cytotoxicity profile. At concentrations up to 0.4 mg/mL, IC-treated cells maintained high viability levels comparable to the control group, indicating minimal or negligible toxic impact. Mild cytotoxicity was observed only at the highest tested concentration (2 mg/mL) after 72 h of incubation, suggesting that the encapsulation of LLA within the ICs helps modulate and mitigate its toxic effects. These findings emphasize the significance of evaluating cytotoxic responses in normal human cell lines to ensure the biocompatibility of candidate therapeutic agents. The data clearly indicate that ICs offer a safer alternative to free LLA by reducing toxicity without compromising efficacy, thereby enhancing their potential for further development in therapeutic applications. The improved biocompatibility of ICs in MRC-5 cells supports their continued investigation as a promising delivery system for bioactive compounds like LLA.

Table 3
Thermodynamic data for BCD, LLA and LLA:BCD.

Phase	Parameters	BCD	LLA	LLA:BCD	Δ (ICs)
Gas	H (Hartree)	4272.331400	834.670085	5107.019917	-0.018432
	ΔH (kcal/mol)	-	-	-	-11.57
	S (cal·mol ⁻¹ ·K ⁻¹)	568.94	228.76	755.54	-26.46
	G (Hartree)	4272.501029	834.738291	5107.245181	-0.005861
	ΔG (kcal·mol ⁻¹)	-	-	-	-3.68
Water	H (Hartree)	4272.331400	834.676632	5107.096504	-0.0884720
	ΔH (kcal/mol)	-	-	-	-55.52
	S (cal·mol ⁻¹ ·K ⁻¹)	356.94	142.00	479.57	-19.37
	G (Hartree)	4272.501029	834.744149	5107.324672	-0.0794940
	ΔG (kcal·mol ⁻¹)	-	-	-	-49.88

* H - Thermal Enthalpy, S - Entropy, and G - Free energy

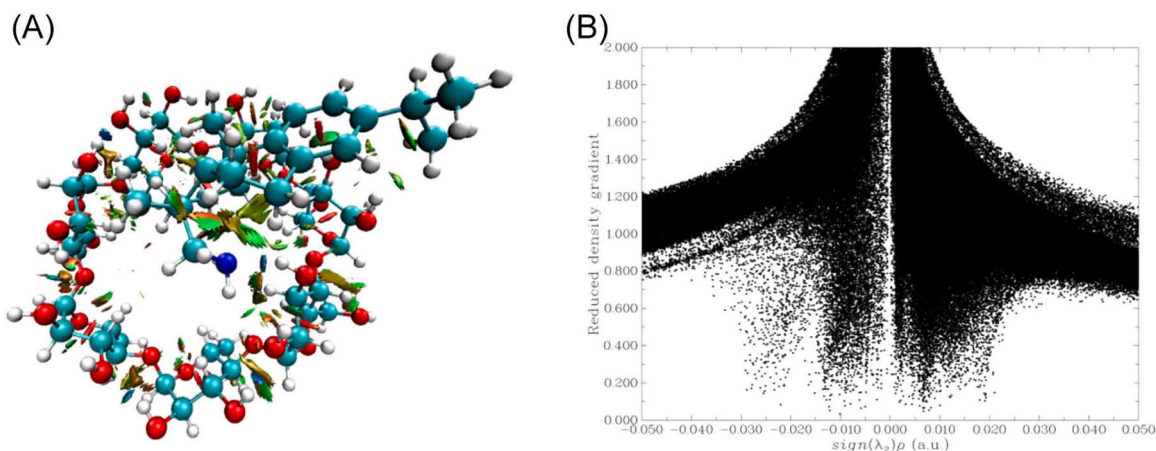


Fig. 10. NCI isosurface plot (A), and 2D RDG scatter plot of LLA:BCD ICs (B).

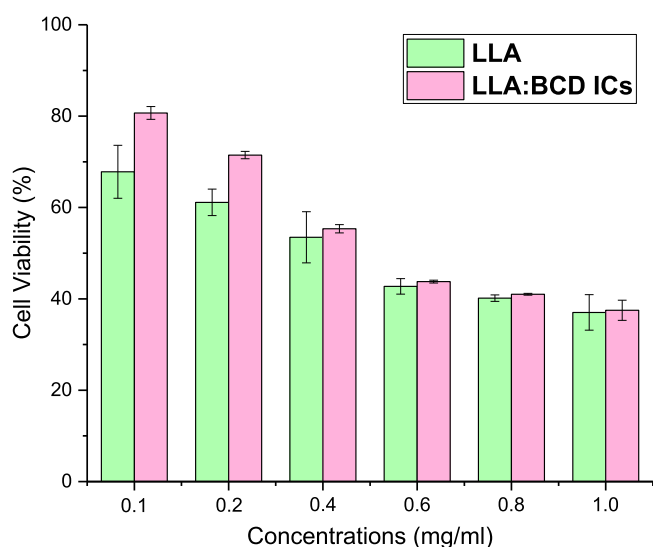


Fig. 11. Alamar blue biocompatibility assay of LLA, and LLA:BCD ICs against MRC-5 cell lines.

Following treatment, a morphological assessment of MRC-5 cells was conducted using a field microscope to evaluate cellular integrity and detect potential signs of cytotoxicity visually. As shown in Fig. 12, cells exposed to free LLA (1.0 mg/mL) exhibited pronounced morphological alterations, including cell shrinkage, membrane blebbing, and loss of adherence, typical indicators of cellular stress and apoptosis. In contrast, cells treated with the ICs of LLA and BCD retained a more normal, fibroblast-like morphology, displaying fewer structural changes relative to the LLA-only group. This suggests that encapsulation of LLA within the BCD cavity confers a protective effect, likely due to reduced free LLA availability at the cellular membrane. To further quantify cell death, apoptosis rates were measured using dual staining with Annexin V and propidium iodide (PI), which discriminate between early apoptotic, late apoptotic, and necrotic cells. Flow cytometric analysis revealed that treatment with 1.0 mg/mL of free LLA induced 8.04 % apoptosis in MRC-5 cells, whereas the IC-treated group showed significantly reduced apoptosis at 4.11 % (Fig. 12). Notably, this lower apoptotic rate in the ICs group was closer to that of the control, indicating that BCD encapsulation not only moderates cytotoxic effects but may also enhance the biocompatibility of the active compound, LLA.

3.12. Cytotoxic assay on MDA MB 231 cancer cell lines

A comprehensive evaluation of cytotoxicity and apoptosis was conducted to investigate the effects of varying LLA concentrations and their ICs on the human breast cancer cell line (MDA-MB-231). The primary objective was to compare the cytotoxic potential of free LLA and its ICs formulation relative to an untreated control group. In vitro cell viability was quantitatively assessed using the Alamar Blue assay, a reliable method for measuring cell metabolic activity. The results for the MDA-MB-231 cell line, expressed as mean $\pm$ standard deviation ($n = 3$), are illustrated in Fig. 13A. At concentrations below 0.4 mg/mL, both LLA and ICs exhibited minimal cytotoxicity, with cell viability remaining relatively high and comparable to the control. However, following a 72-hour incubation period, a dose-dependent cytotoxic effect was observed at higher concentrations, particularly at 200 μ g/mL, where a marked reduction in cell viability was detected. Interestingly, some higher concentrations produced a stronger toxic response, while others had a more moderate impact, indicating potential variability in compound-cell interactions or uptake kinetics. At lower concentrations, such as 25 μ g/mL, MDA-MB-231 cells exhibited only slight reductions in viability, suggesting a threshold-dependent response (Fig. 13B). A comparative analysis with non-cancerous human lung fibroblast cells (MRC-5) revealed that these normal cells displayed a significantly higher tolerance to LLA and its ICs than the MDA-MB-231 cancer cells (Table S11). Notably, while high concentrations of ICs exhibited potent inhibitory effects on MDA-MB-231 cancer cells, they caused minimal cytotoxicity in MRC-5 cells. This selective toxicity underscores the potential therapeutic value of ICs in targeting cancer cells while sparing healthy tissue.

These findings demonstrate that LLA and ICs, possess effective cytotoxic activity against MDA-MB-231 cells at higher concentrations while maintaining biocompatibility with non-cancerous cells. However, to fully understand the intracellular mechanisms underlying this selective cytotoxicity, further investigation is required to confirm the inactivation or modulation of downstream signaling pathways and apoptotic markers at the molecular level.

Confocal microscopy was employed to visualize and compare the morphological changes in MRC-5 and MDA-MB-231 cells treated with different formulations, offering critical insights into cellular responses to LLA and ICs. As illustrated in Fig. 14, cells in the control group exhibited intact morphology with no observable structural alterations, serving as a baseline for comparison. In contrast, cells treated with free LLA showed moderate morphological disruptions, indicative of early stages of cellular stress or apoptosis. Importantly, ICs-treated MDA-MB-231 cancer cells demonstrated more pronounced morphological alterations compared to both the control and LLA groups, suggesting enhanced cellular uptake and therapeutic efficacy of the ICs formulation in

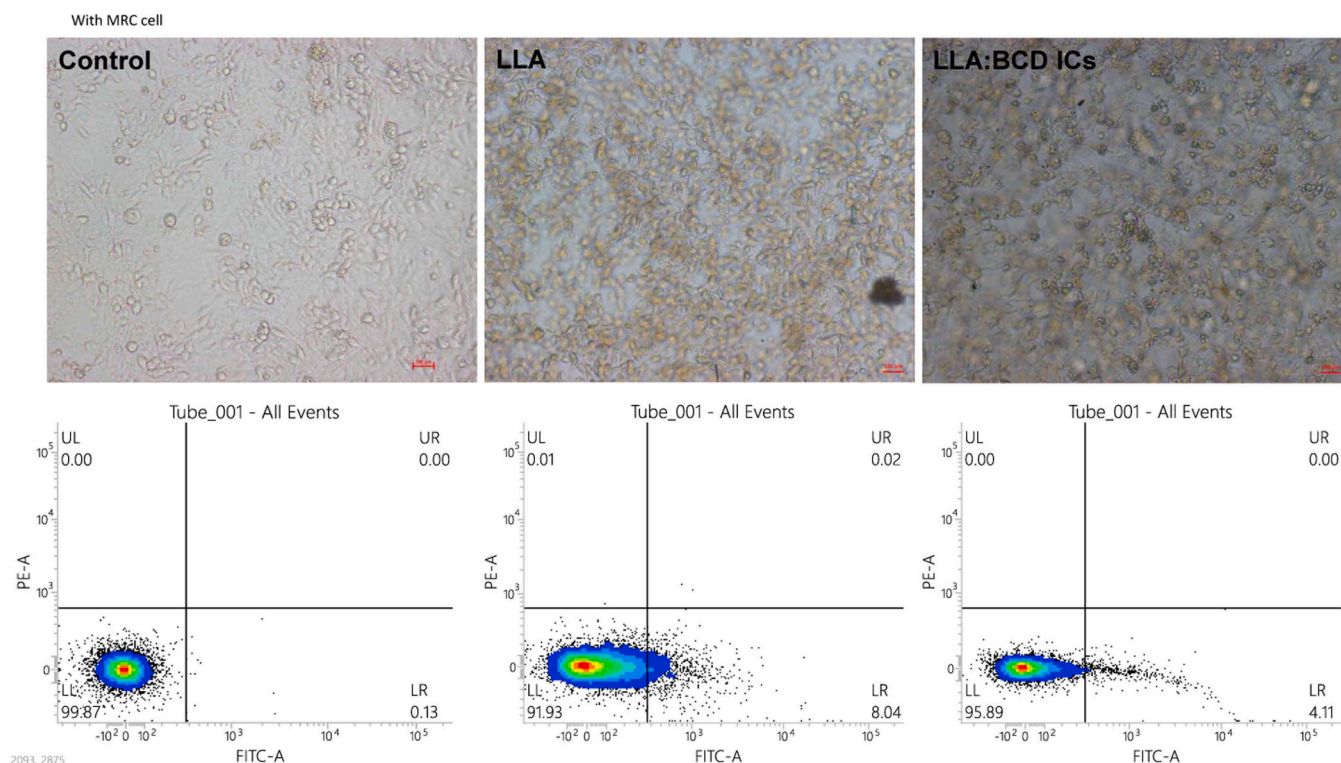


Fig. 12. Bright field cell images of control, LLA, and LLA:BCD ICs. FACS data in control, LLA, and LLA:BCD ICs.

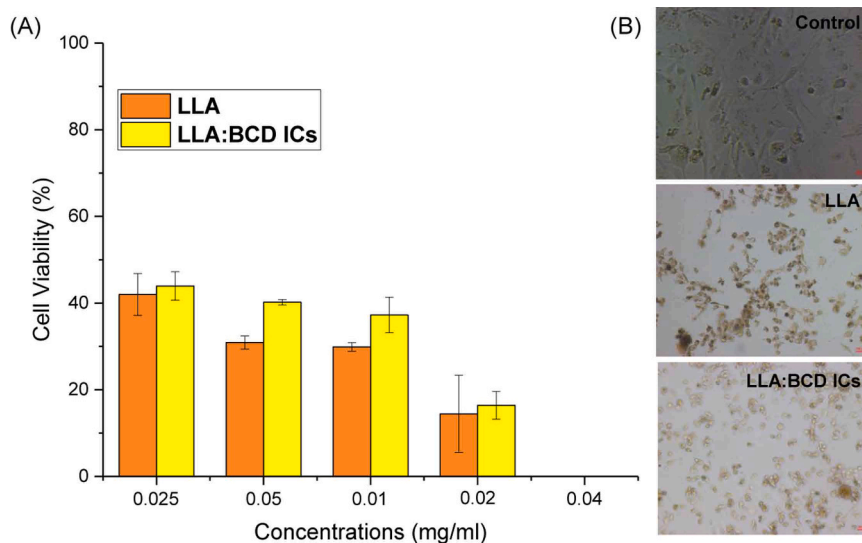


Fig. 13. Cytotoxicity assay of LLA, and LLA:BCD ICs against MDA MB 231 cell lines (A), and bright-field cell images of control, LLA, and LLA:BCD ICs (B).

inducing cancer cell death. When comparing responses across cell types, MRC-5 cells exhibited minimal structural changes upon treatment with either LLA or ICs, indicating good biocompatibility and low cytotoxicity toward normal fibroblast cells. Conversely, MDA-MB-231 cells showed significant morphological changes, particularly following ICs treatment, highlighting the selective cytotoxicity of ICs toward cancer cells over normal cells. To further confirm the in vitro toxicity and cell death pathways activated by LLA and ICs in MDA-MB-231 cells, apoptosis analysis was performed using flow cytometry, as shown in Fig. 14. Cells were treated with 1.0 mg/mL concentrations of LLA and ICs, and dual staining with Annexin V and Calcein AM was employed to differentiate between viable, early apoptotic, and late apoptotic/necrotic cells. The control group exhibited high viability with minimal apoptosis, reflected

by 99.88 % of cells residing in the live cell quadrant. Upon treatment with LLA, 30 % of cells were located in the lower-left (LL) quadrant (viable), 2.68 % in the upper-right (UR) quadrant (late apoptotic/necrotic), and 66.11 % in the lower-right (LR) quadrant (early apoptotic), indicating significant induction of apoptosis. In the ICs-treated group, the LL quadrant showed 53.50 % viable cells. In comparison, the UR and LR quadrants displayed 0.73 % and 45 %, respectively, pointing to a balanced apoptotic response with reduced late-stage cell death compared to LLA alone.

These findings suggest that ICs, while inducing notable early apoptosis in cancer cells, limit excessive necrosis or late apoptosis, potentially reducing inflammation and tissue damage associated with cell lysis. Moreover, the relatively preserved morphology and low

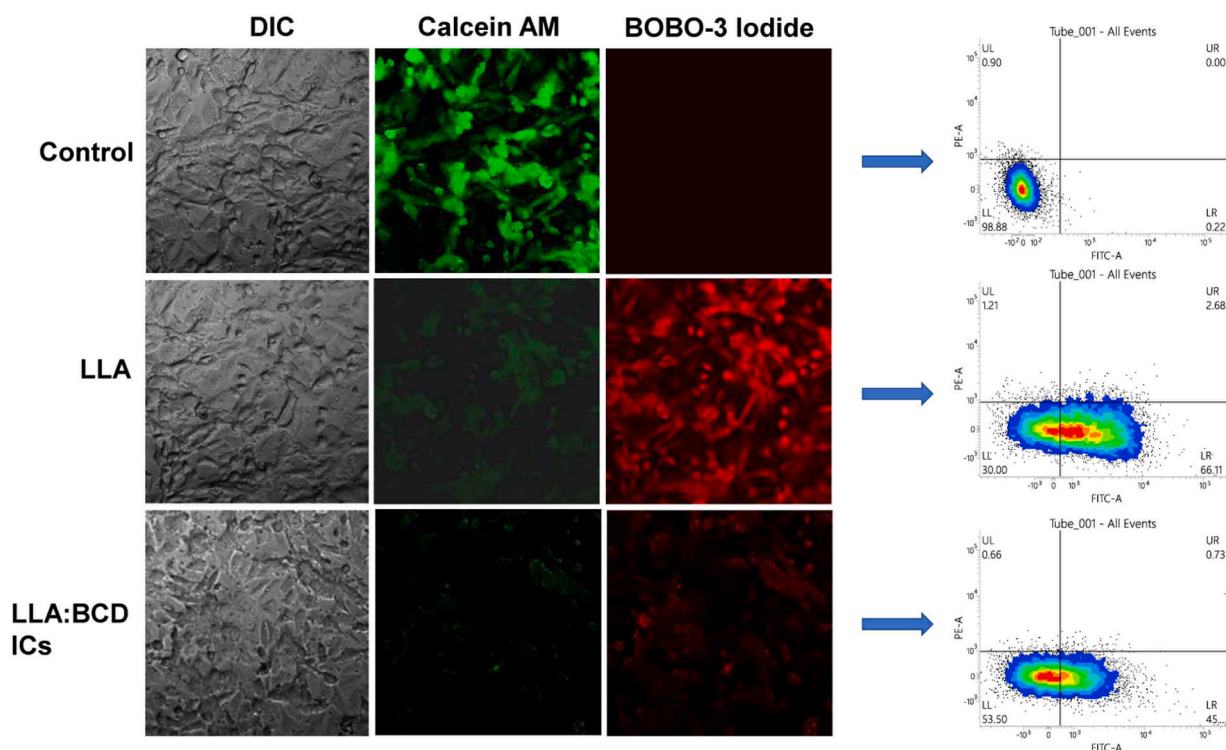


Fig. 14. Live/dead cell staining of lung fibroblastic cells in the control, green represents live cells and red in dead cells; the scale bar indicates 40 μm . FACS data in control, LLA, and LLA:BCD ICs.

apoptosis rates in MRC-5 cells reinforce the selectivity and safety of the ICs formulation. Collectively, these results underscore the therapeutic potential of ICs in targeting cancer cells while minimizing adverse effects on healthy tissues.

Importantly, complexation with BCD noticeably enhances the solubility of LLA, thereby increasing the apparent concentration of bioavailable drug in the extracellular environment. This raised local concentration can promote greater cellular uptake through passive diffusion or endocytic pathways. However, encapsulation within the BCD cavity can transiently reduce direct membrane partitioning, as the hydrophobic moieties responsible for passive diffusion are sequestered inside the BCD structure [67]. Consequently, the extent of cellular uptake is governed by the dynamic equilibrium between free LLA and the LLA:BCD ICs. Rapid dissociation of the ICs at the cell surface facilitates membrane permeation and internalization, whereas excessively stable complexes may hinder drug release and delay cellular uptake.

3.13. Limitations and future directions of the current study

This study emphasizes the complexation of LLA within the cavities of BCD, while its biological evaluation, particularly biocompatibility and cytotoxicity, remains at a preliminary stage. Initial findings are promising, but comprehensive investigations are needed to establish the pharmacological significance of LLA:BCD ICs. The current work lacks detailed mechanistic insights into how complexation influences cellular uptake, metabolic pathways, and interaction with biomolecules. In addition, no comprehensive in-vivo pharmacological studies have been conducted to assess therapeutic efficacy, bioavailability, biodistribution, or long-term safety. The stability of the ICs under physiological conditions and their potential immunogenicity also remain unexplored. Addressing these limitations through systematic biological, pharmacokinetic, and toxicological studies is essential before translating the LLA:BCD ICs into a clinically relevant therapeutic candidate.

4. Conclusions

A sonication-assisted method was successfully developed for the preparation of LLA:BCD ICs, and their formation was comprehensively confirmed through multiple analytical techniques. Furthermore, ^1H NMR and ROESY provided clear evidence of inclusion, demonstrating that the LLA molecule interacts primarily with the outer rim of the BCD cavity, without achieving full encapsulation. This interpretation is in excellent agreement with the complementary results from IR, and Raman studies, which collectively confirm the formation of ICs. Surface characterization further revealed that the inclusion process significantly altered the roughness and morphology of the ICs prepared with CDs. Importantly, in vitro assays established that LLA:BCD ICs effectively induced apoptotic cell death in MDA-MB231 triple-negative breast cancer cells, while exhibiting minimal cytotoxicity toward normal human lung fibroblast cells (MRC-5). Taken together, these findings demonstrate that the sonication-derived LLA:BCD ICs are not only structurally well-characterized and stable but also biocompatible and selectively cytotoxic against cancer cells. This highlights their potential as promising bio-composite derivatives for the improved treatment of triple-negative breast cancer.

Supporting Information

The [supporting information](#) contains detailed information on the experimental procedure for assessing cytotoxic assay, instrumental details, proton NMR spectra of BCD, and LLA. In addition, the optimized geometrical parameters of LLA, BCD, and LLA:BCD ICs in gas and aqueous phase.

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CRediT authorship contribution statement

Rajaram Rajamohan: Conceptualization, Methodology, Resources, Formal analysis, Writing—original draft. **Eswaran Kamaraj:** Software, Formal analysis, Writing—original draft. **Perumal Muthuraja:** Formal analysis. **Sekar Ashokkumar:** Methodology, and Formal analysis. **Kuppusamy Murugavel:** Formal analysis. **Seho Sun:** Writing—review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.colsurfa.2025.139128](https://doi.org/10.1016/j.colsurfa.2025.139128).

Data availability

Data will be made available on request.

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