



A biocompatible anti-inflammatory material from loxoprofen with native and methyl-substituted β -cyclodextrin

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ABSTRACT

The increasing demand for readily soluble and biocompatible pharmaceuticals has led to the development of active pharmaceutical ingredients that utilize cyclodextrins (CDs) in the form of inclusion complexes. These products enhance the solubility and bioavailability of drugs. In this study, we developed inclusion complexes (ICs) of the anti-inflammatory drug loxoprofen (LXP) using native β -cyclodextrin (BCD) and its methyl derivative (MCD). The formation of these complexes was confirmed through absorbance, fluorescence measurements, proton NMR, and ROESY analyses, indicating a 1:1 stoichiometric ratio between LXP and CDs. Proton NMR and cross-peaks of ROESY measurements revealed that LXP is partially encapsulated in both CDs. Computational data from DFT calculations further supported these findings, confirming the stability of the complexes. The inclusion of products significantly enhanced the solubility, bioavailability, and anti-inflammatory efficacy of LXP, suggesting improved therapeutic potential for inflammatory conditions. These results highlight the potential of CDs in strengthening the delivery and efficiency of LXP.

1. Introduction

The continuous search for innovative medicines to manage diseases effectively remains a challenging task, as it requires striking a delicate balance between therapeutic efficacy and patient safety. Although remarkable progress has been made in drug discovery, with numerous new pharmaceutical compounds entering the market, a significant number of medical conditions still lack highly effective treatment options. Many diseases, including chronic and life-threatening conditions, require novel therapeutic approaches that can address current limitations in drug performance and patient outcomes. Presently, many active pharmaceutical drugs demonstrate efficacy in treating specific diseases and conditions. However, they often come with inherent limitations that hinder their full potential. While some of these challenges remain unresolved [1], researchers are actively exploring various strategies to mitigate these drawbacks [2]. One of the most critical concerns associated with conventional drugs is their lack of biocompatibility [2–5], which can lead to unintended adverse effects on healthy tissues and

organs. Non-biocompatible drugs may trigger immune responses, toxicity, or other detrimental physiological reactions, ultimately reducing their safety profile [6]. Another major drawback of conventional drugs is poor solubility [7,8], which significantly affects their bioavailability. Drugs with low aqueous solubility face challenges in dissolving efficiently within the body, often necessitating higher dosages to achieve therapeutic levels in the bloodstream [9]. This increased dosage requirement may lead to potential side effects, increased toxicity, and a greater burden on the patient. Therefore, drug release behavior is a crucial factor in determining the overall impact of a medication on the body, influencing both its therapeutic effectiveness and patient compliance.

To address the aforementioned major concerns, several techniques have been employed, including the use of solubilizers, hydrotropes, cosolvents, prodrugs, pH modulation, salt modification, micronization, and solid dispersions [8]. However, these methods have certain limitations. Long-term use of surfactants can be potentially toxic, cosolvents may become diluted, increasing the risk of vascular occlusion, and salt

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modification is only applicable to drugs with ionizable groups [10]. In response to these limitations, novel strategies have been developed, such as the formation of cyclodextrin (CD)-based inclusion complexes, which significantly improve drug solubility, biocompatibility, and controlled release [11]. CDs are cyclic oligosaccharides composed of multiple D-(+)-glucopyranose units linked by α -1,4 glycosidic bonds [12]. They possess a hydrophilic external surface and hydrophobic internal cavities [13]. Drugs can be incorporated into these internal cavities through non-covalent interactions, forming stable inclusion complexes [14]. Among the various types of CDs, β -cyclodextrin (BCD) is the most commonly used natural variant [15], as it offers several advantages: (i) it converts drugs into microcrystalline or amorphous powders, enhancing their stability and dissolution; (ii) it eliminates unpleasant odors or tastes, improving patient compliance; and (iii) it prevents drug-drug and drug-additive interactions, thereby enhancing drug formulation and efficacy.

CD-based complexes with carboxylic acid-containing anti-inflammatory drugs have been developed to enhance solubility [16,17]. Loxoprofen (LXP), a non-steroidal anti-inflammatory drug (NSAID) with a carboxylic acid group, is chemically known as α -methyl-4-[(2-oxocyclophenyl-methyl) benzene acetic acid. Poorly soluble in water, LXP has been in use since 1981, with its preparation, metabolism, and applications well-documented [18,19]. Recent advancements, including nano-emulsification, have helped mitigate LXP's side effects [20]. Solubility measurements in the presence of supercritical carbon dioxide (CO_2) range from 1.35×10^{-5} to 1.28×10^{-3} under varying temperatures and pressures [21]. LXP has shown potential for improved solubility through inclusion complexation with native CD [22]. Additionally, an in-depth investigation has been conducted on the complexation of LXP with hydroxypropyl-substituted cyclodextrin (HCD). This study demonstrates a significant enhancement in the anti-inflammatory properties of LXP upon complexation. Furthermore, the inclusion complex exhibits improved safety and biocompatibility, making it a promising candidate for therapeutic applications [23]. To date, no comprehensive study has examined the formation of inclusion products of LXP with BCD and (methylated cyclodextrin) MCD in both solution and solid states to enhance its anti-inflammatory (AI) efficacy while minimizing toxicity. This study addresses this gap by preparing LXP:BCD and LXP:MCD using the ultrasonication process and freeze-drying. The resulting solid products are characterized with analytical techniques. Additionally, a preliminary in vitro evaluation of their AI and anti-arthritis properties is conducted using BSA denaturation analysis.

2. Materials and methods

2.1. Materials

The chemicals used for this experiment, LXP (Molecular Formula, Molecular weight, and purity are $\text{C}_{15}\text{H}_{18}\text{O}_3$, 246.31 g/mol, and 99 %, respectively, BCD (Degree of substitution (DS): 4.9 [24], CAS No.: 7585-39-9. Molecular Formula, Molecular Weight, nature and purity are $\text{C}_{42}\text{H}_{70}\text{O}_{35}$, 1134.98 g/mol, white powder, and 97 %, respectively), and MCD (Degree of substitution (DS): 2.6 [24], CAS No.: 128446-36-6. Molecular Formula, Molecular Weight, nature and purity are $\text{C}_{56}\text{H}_{98}\text{O}_{42}$, 1310 g/mol, white powder, and 99 %, respectively) are bought from Sigma-Aldrich Chemical Co. (Seoul, South Korea). For the making of solutions, distilled water was used as the solvent.

2.2. Preparation of LXP:CDs

In one setup, an accurate weight of LXP (0.2173 g), and the host molecule, BCD (1.0066 g), are dissolved in 10 mL of ethanol and 20 mL of distilled water, respectively. In a separate setup, another accurately weighed sample of LXP (0.1913 g) and MCD (1.0056 g) was dissolved in 10 mL of ethanol and 20 mL of distilled water, respectively. Each

resulting solution was clear and homogeneous. To ensure thorough mixing, the CD solutions are sonicated in an ultrasonic bath (operating at 26 kHz and 200 W, Hielscher UP200St, South Korea) for 5 min. The inclusion products are prepared by slowly adding the LXP solution dropwise into the CD solutions separately, maintaining a 1:1 M ratio. The ultrasonication process was continued for up to 2 h at 60.0 °C. The final mixtures remained clear and colorless, reflecting that the inclusion products are water-soluble. A freeze-drying process was employed for product isolation. The solutions are first filtered using a 0.45 μm syringe filter and then frozen at -83.0 °C for 48 h. After this, the lyophilization was carried out at -80.50 °C under a pressure of 1.09 Pa for four days using a freeze dryer. The process yielded a fine white powder, which was designated as the inclusion products (LXP:BCD and LXP:MCD).

3. Results and discussion

3.1. Absorption and fluorescence spectral analysis of LXP in the presence of CDs

Fig. S1 presents the absorption spectra of LXP in an aqueous solution at approximately pH 7, both in the absence and presence of CDs (BCD, and MCD). LXP exhibits two prominent absorption maxima at 220 nm and 265 nm, which correspond to its characteristic electronic transitions [23,25]. Upon the incremental addition of BCD and MCD, a gradual increase in absorbance is observed at both maxima (Tables S1 and S2). This increase persists with increasing CD concentrations, ultimately reaching the highest concentrations tested. Two critical aspects of these observations warrant further discussion to enhance clarity and understanding: (i) Despite the addition of CDs, no significant shift in the spectral maxima at 220 nm and 265 nm is detected. This suggests that the fundamental electronic structure of the LXP molecule remains unaltered, implying that its core chromophoric system is unaffected by the presence of CDs. The absence of a shift in the absorption maxima confirms that no significant changes in the conjugation system or molecular geometry of LXP occur upon interaction with CDs. (ii) A notable increase in absorbance is observed as the CD concentration increases, even though the concentration of LXP remains unchanged. This phenomenon strongly suggests that LXP interacts with CDs, potentially forming inclusion products [26].

The fluorescence spectra of pure LXP exhibit two distinct emission maxima at 239 nm and 360 nm. These maxima are characterized by relatively low fluorescence intensity, indicating that LXP is inherently weakly fluorescent. The observed emission peaks are primarily attributed to charge transfer transitions occurring within the LXP molecule in an aqueous medium. The addition of CDs to the LXP solution leads to notable changes in fluorescence behavior, revealing key interactions between LXP and CDs. Two significant findings emerged from the fluorescence analysis: (i) As the concentration of CDs increases, the fluorescence peak at 360 nm gradually diminishes and eventually disappears. This observation suggests that the charge transfer process responsible for this emission is inhibited in the presence of CDs. The suppression of the charge transfer process likely results from the encapsulation of LXP within the CD cavity, which restricts solvent interactions and alters the electronic environment of the molecule. (ii) Similar to the absorption spectra, no shift in fluorescence maxima is observed upon CD addition. This further supports the conclusion that LXP undergoes no significant structural modifications when interacting with CDs [27]. The absence of spectral shifts reinforces the idea that the primary mode of interaction involves non-covalent encapsulation rather than chemical transformation or molecular rearrangement.

The binding constants for the interactions between LXP and CDs, as determined from both absorption and fluorescence spectral data (Fig. 1), have been calculated and are presented in Table S2. Under aqueous conditions at neutral pH and room temperature, the inclusion process exhibits negative ΔG values (Table S3), indicating its exergonic nature. This implies that the complex formation occurs spontaneously without

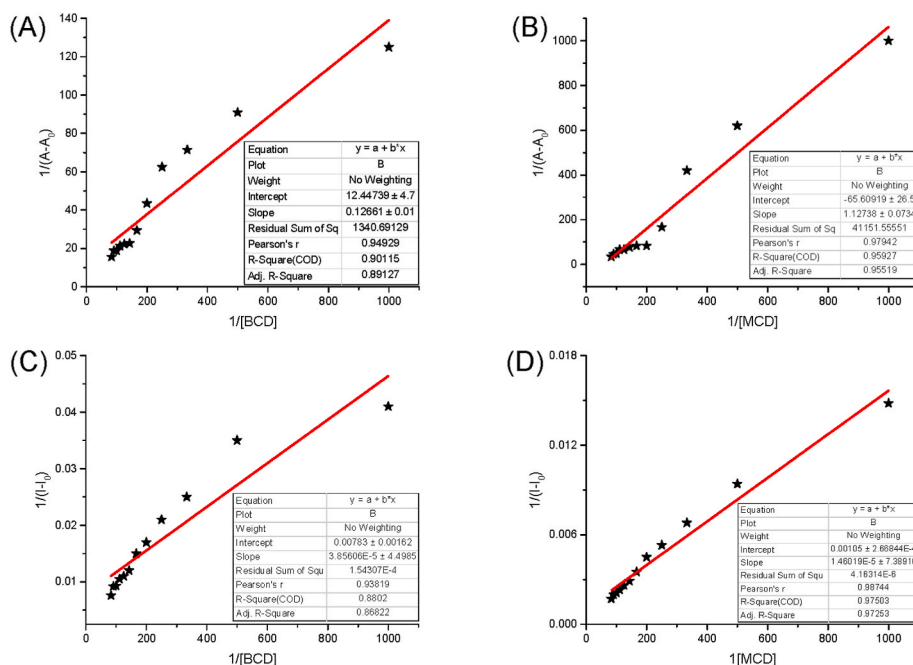


Fig. 1. BH absorbance plot of LXP:BCD (A) and LXP:MCD (B). BH fluorescence plot of LXP:BCD (C) and LXP:MCD (D).

the need for external energy input. The thermodynamic favorability of the process highlights the stability of the inclusion products, offering crucial insights into the molecular interactions governing their formation.

3.2. ^1H proton NMR spectral assignment of LXP:BCD

The ^1H NMR spectrum of LXP:BCD is shown in Fig. S3. In the spectrum, proton signals corresponding to both LXP and BCD are observed. For the guest molecule LXP, almost all protons exhibited a shielding effect, with chemical shifts ranging from 0.01 to 0.03 ppm, particularly in the cyclopentanone region, which experienced greater shielding. The acidic proton of propionic acid remained unaffected, as did the other protons of the guest molecule. Similarly, in BCD, the H2, H3, and protons of the methyl and methylene groups attached to C-5 are unaffected. However, the hydroxyl proton attached to the methylene at C-5 is deshielded by 0.07 ppm. The methine protons of H3 and H4 exhibited

shielding effects of approximately 0.09 and 0.2 ppm, respectively. These observations confirm the formation of inclusion products between LXP and BCD, which the ROESY further validated. The proton chemical shifts of BCD, LXP, and LXP:BCD are collectively presented in Table S4.

3.3. ROESY correlations of LXP:BCD

The ROESY of LXP:BCD is shown in Fig. 2A. In the ROESY, cross-peaks a (1.33/2.40), b (1.33/2.92), c (1.33/3.36), and d (1.33/3.63) are observed and used to assign the H-2' protons of LXP unambiguously. The proton signal at 1.33 ppm has already been assigned to the methyl proton of the C-3' carbon of LXP. This signal exhibits a strong correlation with the proton at 3.63 ppm, which has been assigned to H-2'. Based on this correlation, the assignment is made without ambiguity. Additionally, other cross-peaks indicate correlations between the methyl proton of LXP and the methylene protons at C-8 of LXP, and the methine proton (H4) of BCD. Furthermore, the hydroxyl proton of the methylene group

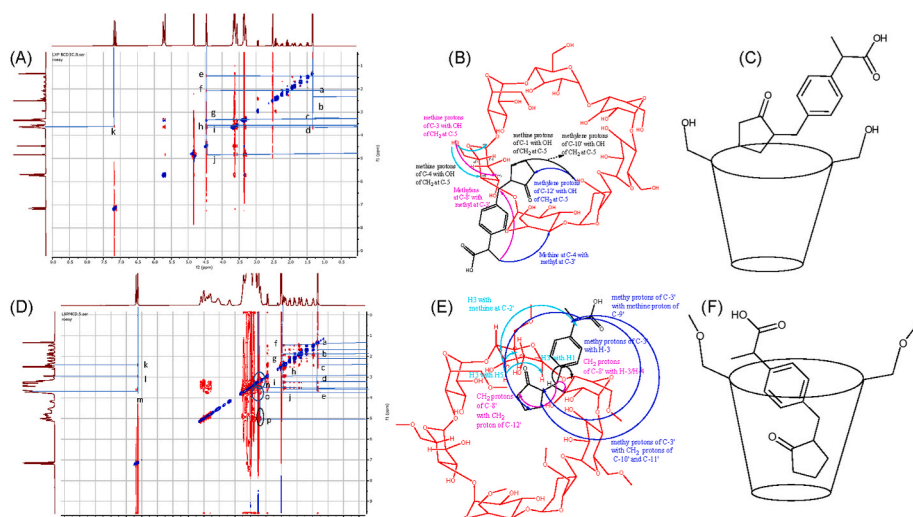


Fig. 2. ROESY of LXP:BCD (A), Correlation peaks (B), and proposed structure (C), ROESY of LXP:MCD (D), Correlation peaks (E), and proposed structure (F).

attached to C-5 of BCD exhibits cross-peaks **e-j** at 4.45/1.43, 4.45/2.05, 4.45/3.36, 4.45/3.67, 4.45/3.54, and 4.45/4.82 ppm, respectively. These cross-peaks reveal interactions between the hydroxyl proton of the methylene group at C-5 of the BCD ring and the methylene protons at C-12' and C-10' of LXP at 1.43 and 2.05 ppm, respectively. The remaining cross-peaks indicate correlations between the hydroxyl proton of the methylene group at C-5 of BCD and H4, the methylene protons at C-5, and the H2 and H3 protons of the BCD ring. Additionally, a cross-peak is observed at **k** (7.18/3.63), which reveals a correlation between the aromatic proton of LXP and the methine proton of LXP. These protons have already been assigned without ambiguity. The observed correlations strongly suggest that the LXP moiety is positioned outside the BCD cavity, interacting with the hydroxyl proton of the methylene group attached to C-5, as well as with H4 and the methylene protons at C-5. This confirms the formation of an inclusion product between BCD and LXP. Based on the NMR results and the correlations observed in the ROESY, it is strongly suggested that the guest molecule (LXP) remains outside the BCD cavity while forming strong interactions. The proposed structure of the inclusion product is depicted in Fig. 2C, and important ROESY correlations are shown in Fig. 2B.

3.4. ^1H NMR spectral assignment of LXP:MCD

The ^1H NMR spectrum of LXP:MCD is shown in Fig. S4. In the spectrum, proton signals corresponding to both LXP and MCD are observed. For the guest molecule LXP, almost all protons exhibited a shielding effect, with chemical shifts ranging from 0.01 to 0.03 ppm, particularly in the cyclopentanone region, which experienced greater shielding. The acidic proton of propionic acid and the aromatic protons remained unaffected. Similarly, in MCD, the H1, H2, H3, H4, OMe, and equatorial hydroxyl proton at C-2 showed shielding effects ranging from 0.2 to 0.8 ppm. In contrast, the methylene protons attached to C-5 and the H5 protons are deshielded by 0.02–0.08 ppm. Based on the above observations, the formation of an inclusion product of LXP with MCD is confirmed. This is further validated by ROESY analysis. The proton chemical shifts of MCD, LXP, and LXP:MCD are collectively presented in Table S5.

3.5. ROESY correlations of LXP:MCD

The ROESY of LXP:MCD is shown in Fig. 2D. In the ROESY, cross-peaks **a** (1.32/1.63), **b** (1.32/2.22), **c** (1.32/2.20), **d** (1.32/3.28), and **e** (1.33/3.59–3.67) are observed. The proton signal at 1.32 ppm has already been assigned to the methyl proton of the C-3' carbon of LXP. This signal exhibits a strong correlation with 3.63 ppm, which has been assigned to H-2' and the methylene proton of MCD at C-5. Based on this correlation, H-2' is assigned unambiguously. Additional cross-peaks indicate correlations between the methyl proton of LXP and the methylene protons at C-11' and C-10', as well as between H-9' of LXP and H-4/H-3 of the MCD ring. Furthermore, the methylene proton connecting the aromatic ring and cyclopentanone (C-8') of the guest molecule exhibits cross-peaks **f-j** at 2.42/1.43, 2.42/1.92, 2.42/2.92, 2.42/3.28, and 2.42/3.65 ppm, respectively. These peaks indicate interactions between one of the methylene protons of C-8' and the methylene protons at C-12' and C-8' of LXP at 1.43, 1.92, and 2.92 ppm, respectively. The remaining cross-peaks reveal correlations between the methylene proton at C-8' and H-4/H-3, the methylene protons at C-5 of the MCD ring, and H-2' of LXP. Additionally, cross-peaks **k** (7.12/3.63), **l** (7.12/2.92), and **m** (7.12/2.43) are observed, indicating correlations between the aromatic proton of LXP and the methine proton H-2' and the methylene proton at C-8' of LXP. These protons have already been assigned without ambiguity. Furthermore, cross-peaks **n** (3.24/3.64), **o** (3.24/3.71), and **p** (3.24/4.78–5.07) are detected. Among these, cross-peak **n** reveals a correlation between H-3 of the host and H-2' of the guest. The other two cross-peaks indicate interactions between H-3 and H-1/H-5, as well as equatorial hydroxyl protons at C-2 and C-3. These correlations strongly

suggest that part of the LXP molecule, particularly the cyclopentanone group, is slightly inserted into the MCD cavity, interacting with the H-3/H-4 protons of the MCD ring. This confirms the formation of the LXP:MCD inclusion product. Based on the NMR results and the ROESY correlations, it is strongly suggested that the LXP is partially positioned inside the cavity while forming strong interactions. The proposed structure of the inclusion product is depicted in Fig. 2F, and important ROESY correlations are shown in Fig. 2E.

3.6. FT-IR and Raman spectral analysis

The FT-IR and Raman spectra of LXP and inclusion products with BCD and MCD provide crucial structural insights, as illustrated in Fig. S5. The characteristic FT-IR absorption bands (Fig. S5A) of pure LXP exhibit distinct peaks at 1706 cm^{-1} and 1739 cm^{-1} , which correspond to the stretching vibrations of carboxyl ($-\text{COOH}$) and carbonyl ($-\text{C}=\text{O}$) functional groups. Additionally, a prominent band at 2952 cm^{-1} is attributed to the asymmetric C-H stretching of the aromatic ring, while the peaks at 1154 cm^{-1} and 1511 cm^{-1} can be ascribed to C-H bending and CH_2 scissoring vibrations, respectively [23]. The FT-IR spectrum of BCD, and MCD, displays a broad O-H stretching band centered at approximately 3291 and 3352 cm^{-1} , characteristic of intermolecular hydrogen bonding within the CD structure [28]. Notably, in the inclusion products, the O-H stretching band undergoes broadening and shifts to around 3350 cm^{-1} , indicating the presence of strong hydrogen-bonding interactions between LXP and the host molecules, BCD and MCD. Among these, the broadening effect is more pronounced in the LXP:MCD than in the LXP:BCD, suggesting a stronger interaction between LXP and MCD. These spectral modifications further support the successful encapsulation of LXP within the CD cavities and the involvement of hydrogen bonding in the stabilization of the inclusion products [29,30]. The observed trends in FT-IR data align well with NMR and ROESY results, reaffirming the proposed interaction and binding mode between LXP and the CDs.

The Raman spectra of LXP exhibit three prominent peaks at 3052 cm^{-1} , 2953 cm^{-1} , 1452 cm^{-1} , and 1207 cm^{-1} , corresponding to CH_2 asymmetric stretching, CH_2 symmetric stretching, CH_2 scissoring, and CH_2 twisting vibrations, respectively. Additionally, overtone peaks are observed at 2852 cm^{-1} and 1647 cm^{-1} , further supporting the vibrational characteristics of LXP. The Raman spectra of BCD and MCD display a broad peak centered at 3405 cm^{-1} , which is attributed to the presence of hydroxyl ($-\text{OH}$) groups within the cyclodextrin cavity [23, 31]. Furthermore, distinct peaks appear at 2955 cm^{-1} and 2967 cm^{-1} , corresponding to the C-H stretching vibrations of aliphatic bonds in BCD and MCD, respectively. Upon complexation with LXP, the Raman spectra of the inclusion products reveal a noticeable reduction in the intensity of the broad $-\text{OH}$ stretching peak at around 3400 cm^{-1} compared to pure CDs (Fig. S5B). This attenuation suggests that the hydroxyl groups of the host molecules are engaged in hydrogen bonding interactions with LXP [32]. Notably, the extent of this reduction differs between the LXP:MCD and LXP:BCD. The broad peak at 3400 cm^{-1} is significantly diminished in intensity for LXP:MCD compared to LXP:BCD, indicating a stronger interaction between LXP and MCD. These spectral variations corroborate the findings from NMR and FT-IR analyses, reinforcing the evidence for successful formation of inclusion products between LXP and CDs.

3.7. Surface analysis by FE-SEM measurement

FE-SEM analysis is conducted to investigate the morphological characteristics of pure LXP and its complexes BCD and MCD [33,34], as illustrated in Fig. 3. The analysis of pure LXP revealed the presence of both small and large stone-like particles exhibiting a colloid-like nature. The particles are irregular in shape and varied in size, which is typical of untreated crystalline substances [35]. Such morphological features indicate the crystalline and natural state of LXP. In stark contrast, the

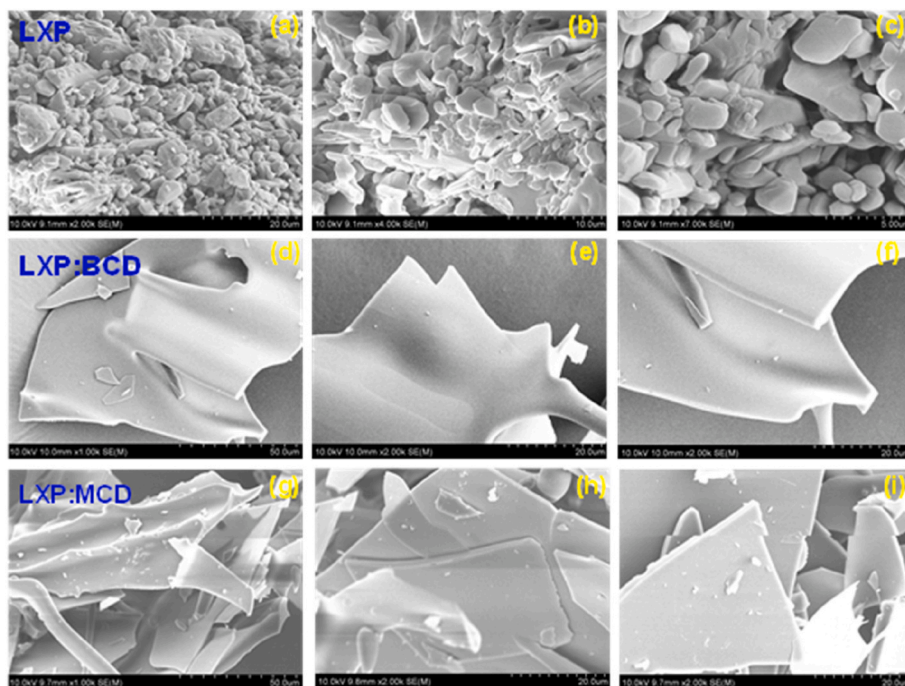


Fig. 3. FE-SEM images of LXP (a–c) [23], LXP:BCD (d–f), and LXP:MCD (g–i).

complexes with BCD and MCD displayed markedly different morphology compared to the raw LXP. The inclusion products exhibited a unique glass-like structure with uneven crystalline deposits on their surfaces. This altered morphology suggests significant structural reorganization, consistent with the changes observed in FT-IR analysis, confirming the successful complexation of LXP with the CDs. Notably, the SEM images revealed that the complexes comprised smaller and finer particles compared to pure LXP, indicating a more homogeneous and compact structure. Both complexes also demonstrated a layered configuration, suggesting a distinct physical arrangement as a result of complexation with BCD and MCD [36–38]. This layered morphology could be attributed to the encapsulation of LXP within the CD cavities, leading to a modified physical state. The observed changes in particle size, shape, and surface texture reflect the influence of CD complexation on the overall morphology and physical properties of LXP.

3.8. Analysis of thermal behavior and surface nature

Thermogravimetry (TG) and Differential Thermal Analysis (DTA) are fundamental techniques in supramolecular-based chemistry, particularly in assessing the thermal stability of inclusion products. These methods allow for the monitoring of weight changes and temperature variations under controlled conditions, providing valuable insight into the degradation behavior of complex molecules [39,40]. Fig. 4 presents the TG curves for LXP and its inclusion products, highlighting their thermal stability profiles. LXP, a well-known organic drug molecule, is characterized by its high thermal stability. In the TG analysis, LXP shows minimal mass loss with a gradual temperature increase up to 195 °C. This suggests that LXP remains stable in the lower temperature range, with only slight evaporation of volatile components, such as moisture. However, as the temperature exceeds 195 °C, LXP begins to decompose. The significant weight loss between 200 °C and 297 °C corresponds to the thermal degradation of LXP, which results in complete decomposition by the end of this temperature range. DTA analysis of LXP confirms

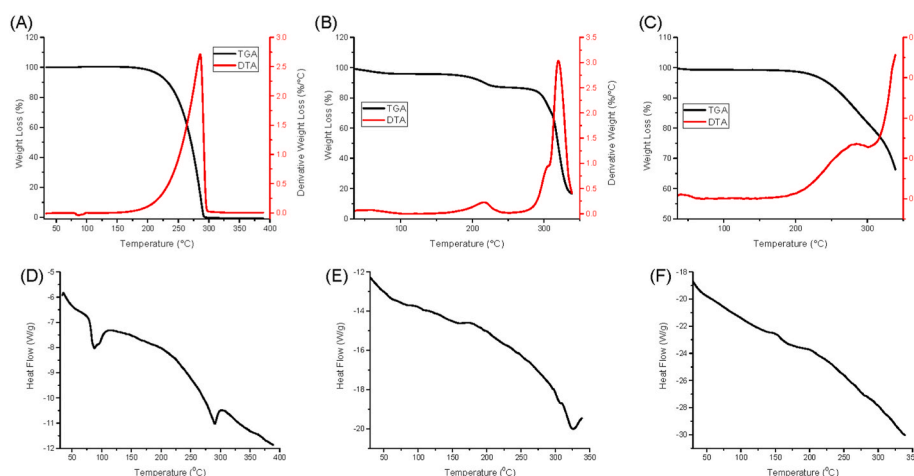


Fig. 4. TG/DTA of LXP (A) [23], LXP:BCD (B), and LXP:MCD (C). Heat flow diagram of LXP (D) [23], LXP:BCD (E), and LXP:MCD (F).

this, with a prominent peak at 292 °C, marking the onset of thermal degradation after 254 °C. This indicates that LXP's degradation is initiated at a relatively high temperature, further demonstrating its stability as an individual molecule.

In contrast, the inclusion product of LXP:BCD exhibits different thermal behavior. Below 100 °C, only a slight and insignificant weight loss is observed, attributed to the removal of residual moisture from the inclusion products. This is typical for inclusion products, where the encapsulated molecules often retain some amount of water within the structure. Between 100 °C and 185 °C, no significant weight loss occurs, suggesting that the complex remains stable in this temperature range. However, a marked weight loss is observed between 185 °C and 230 °C, with approximately 10 % of the mass lost, which is indicative of the degradation of LXP within the complex. Interestingly, the BCD cavity appears to offer some protection to the LXP molecule, as the degradation is not as pronounced as in the free LXP. This protection may result from the encapsulation of LXP within the BCD structure, which could hinder its exposure to thermal stress. At around 290 °C, there is a sharp drop in weight, signaling the onset of BCD degradation. This temperature marks the degradation of BCD, after which the entire inclusion product starts to break down, confirming that the inclusion product's stability is influenced by both components. For the LXP:MCD, the thermal behavior is slightly different. Between 185 °C and 230 °C, no significant weight loss is observed, indicating that MCD stabilizes LXP in this temperature range. However, at 210 °C, significant weight loss begins, reflecting the thermal decomposition of LXP observed in the free form. Unlike the sharp degradation observed for LXP alone, the weight loss for LXP:MCD continues gradually after 210 °C. This slow and steady loss is primarily attributed to the impact of MCD, suggesting that MCD may be degrading at a slower rate than BCD. The DTA analysis also reflects these differences: LXP:BCD shows thermal peaks at 215 °C and 315 °C, while LXP:MCD exhibits peaks at 270 °C and 315 °C. These distinct peaks further highlight the differences in thermal behavior between the two inclusion products. These findings underscore the enhanced stability of LXP when complexation with CDs. The inclusion of LXP into the cavities of BCD and MCD significantly improves its resistance to thermal degradation, demonstrating the role of these CDs in stabilizing the drug. This enhancement in thermal stability may also confer a better capacity to endure temperature fluctuations, making these inclusion complexes more suitable for various applications in pharmaceutical formulations [41,42].

When the drug guest molecules enter CDs, significant structural modifications typically occur. Firstly, the guest molecule, which is initially in a crystalline state, often undergoes a transition to an amorphous or partially amorphous form due to the disturbance of its ordered lattice structure. Secondly, this inclusion process induces changes in the diffraction patterns, leading to distinct alterations rather than a simple superimposition of the individual diffraction profiles of the host and guest molecules [43]. Fig. S6 illustrates the XRD patterns of the LXP, along with its inclusion with BCD and MCD. The pure LXP exhibits sharp and intense diffraction peaks at the angles 14.48°, 16.21°, 17.35°, 19.02°, 20.85°, and 25.51°, which are characteristic of its well-defined crystalline nature, with no indications of amorphous phases [23]. The diffraction pattern of pure BCD shows characteristic sharp peaks at 6.8°, 9.6°, 11.0°, 12.9°, 18.3°, and 19.1°, signifying its semi-crystalline structure [44]. In contrast, MCD exhibits a broad peak around 18.8°, which is indicative of its predominantly amorphous nature [45]. Upon the formation of the Inclusion, significant changes in the XRD patterns are observed. The diffraction pattern of the LXP:BCD exhibits characteristics of a semi-amorphous structure, while the LXP-MCD complex presents a predominantly amorphous profile. Notably, the characteristic diffraction peaks of pure LXP are either significantly reduced in intensity or completely absent in the inclusion product, suggesting a disruption in its crystalline structure. The interaction with BCD and MCD does not lead to significant modifications in the surface properties of LXP [46, 47]. Instead, the diffraction profiles of the inclusion products are largely

dominated by the host molecules, particularly in the case of MCD, where the amorphous nature is more pronounced. Additionally, minor shifts in the diffraction peak positions of both the guest and host molecules are observed, indicating possible structural rearrangements upon complexation. This absence or shift in diffraction peaks confirms that LXP has been successfully incorporated into the CD cavities, leading to the loss of its crystalline nature [48]. The stable intermolecular interactions between LXP and CDs contribute to the observed partial or complete amorphization of the resulting inclusion products [49]. This transformation is a crucial factor in enhancing the solubility and bioavailability of the guest molecule, further demonstrating the effectiveness of CD-based complexation in modifying the physicochemical properties of LXP.

3.9. Computational evidence for the LXP with BCD and MCD

The interaction of LXP with BCD and MCD in the form of complexes involves weak interactions, such as hydrogen bonding and energy [50–54]. To gain a comprehensive understanding of the complexation process, the energy calculations are performed to investigate the relative orientation of LXP within the cavity of CDs in the virtual state. These calculations are designed to explore how LXP aligns itself with the CD cavities, which is crucial for understanding the stability and nature of the formed complexes. Four distinct orientations, labeled orientations 1, 2, 3, and 4, are considered for each complexation scenario to identify the most stable configuration. The analysis revealed that LXP does not fully penetrate the cavities of BCD or MCD in any of the examined orientations. This observation aligns with prior findings (proton NMR and ROESY with cross-peak k), which suggested that LXP interacts primarily with the outer rim of the CD cavity rather than being fully encapsulated. This indicates a superficial yet stable interaction pattern facilitated by hydrogen bonding rather than a complete inclusion within the hydrophobic cavity of the CDs. Among the evaluated orientations, orientations 2 and 1 are identified as the most stable and optimized configurations for LXP:BCD and LXP:MCD, respectively, based on energy considerations (Fig. 5). The stability of these orientations can be attributed to the favorable hydrogen bonding interactions and optimal spatial arrangement between LXP and the outer rim of the CD cavities [55,56]. In contrast, the other orientations displayed significantly higher energy values, suggesting less favorable or unstable complexation states. Table 1 summarizes the complexation energies, where negative values indicate thermodynamically favorable interactions in a vacuum environment. Notably, orientation 1 for LXP:BCD and orientation 4 for LXP:MCD exhibited the most favorable complexation energies of –56.710 kJ/mol and –52.247 kJ/mol, respectively. These negative energy values confirm the spontaneous and energetically favorable nature of the complexation process [57,58].

The substantial energy differences between the optimized and non-optimized orientations further emphasize the specificity of the hydrogen bonding interactions that stabilize the inclusion products (Table 2). Additionally, Fig. 6 illustrates the hydrogen bonding interactions occurring in the different orientations of both complexes. This visual representation provides valuable insights into the specific bonding patterns, including the atoms involved and the spatial configuration that contributes to the overall stability of the inclusion products. The analysis clearly demonstrates that hydrogen bonding is the predominant force driving the complexation process, supporting the conclusion that LXP interacts with the external rim of the CD cavities rather than being fully encapsulated.

3.10. Correlation of the experimental and theoretical results of LXP:BCD, and LXP:MCD

The inclusion products of LXP with BCD and MCD have been thoroughly investigated through a combination of experimental and theoretical techniques, offering valuable insights into the molecular

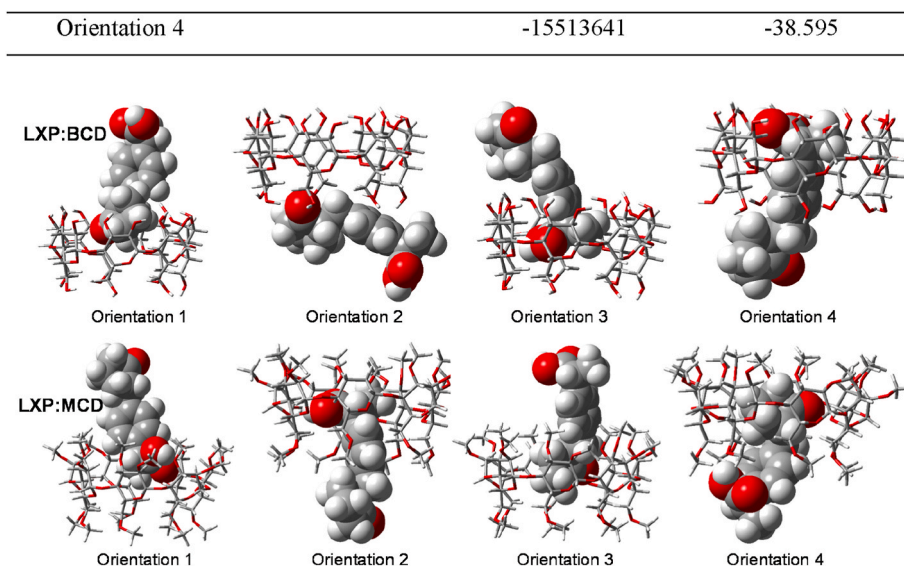


Fig. 5. Possible orientations of LXP:BCD, and LXP:MCD.

Table 1

Complexation energy of LXP with BCD and MCD.

Molecules	Method	E _{Opt} (kJ/mol)	ΔE (kJ/mol)
BCD	B3LYP/6-31G(d,p) in water	-11225100	
LXP		-2121863	
Orientation 1		-13346984	-21.266
Orientation 2		-13346996	-32.818
Orientation 3		-13346988	-24.417
Orientation 4		-13346992	-28.355
MCD		-13391739	
LXP		-2121863	
Orientation 1		-15513645	-43.058
Orientation 2		-15513635	-32.293
Orientation 3		-15513625	-22.579
Orientation 4		-15513641	-38.595

Table 2

Hydrogen bond distance in Host:Guest molecules in water.

Bonding parameter	Bond Distance (Å)	Bonding parameter	Bond Distance (Å)
LXP:BCD			
Orientation 1		Orientation 2	
H171–O100	3.01	H154–O144	2.72
O173–H4	2.59	H172–O19	2.98
H159–O17	2.59	H170–O40	2.55
H154–O119	2.92	O173–H62	1.94
		H157–O103	2.83
Orientation 3		Orientation 4	
O177–H70	2.69	H164–O61	2.97
H179–O34	2.20	O177–H45	2.48
H167–O35	2.98	H167–O82	2.61
H155–O17	2.96	H179–O79	2.06
H181–O119	2.97	H168–O103	3.00
LXP:MCD			
Orientation 1		Orientation 2	
O240–H79	2.64	O240–H61	2.33
H242–O69	1.78	H242–O67	2.03
H238–O51	2.60	H218–O103	2.83
H218–O33	2.78	H238–O104	2.68
H245–O104	2.70		
Orientation 3		Orientation 4	
H217–O33	3.00	O236–H83	2.58
H227–O104	3.01	H220–O71	2.88
O236–H79	2.70	H230–O31	3.02
H223–O51	2.88	H234–O33	3.05
		H233–O104	2.28

interactions and structural characteristics of these products. NMR spectroscopy, particularly ROESY, provided crucial information on the spatial arrangement of the LXP molecule within the CD cavities. The experimental data revealed that in the inclusion product with BCD, the LXP molecule does not fully encapsulate within the BCD cavity. Instead, it interacts predominantly with the outer rim of the BCD cavity. This finding suggests that LXP remains partially exposed to the surrounding environment while interacting with the hydrophilic outer surface of the BCD, which may influence its solubility and stability. In contrast, the inclusion product formed between LXP and MCD shows a different molecular interaction. The cyclopentanone part of LXP is found to be encapsulated within the MCD cavity, a result that is likely attributed to the additional methyl groups on MCD. These methyl groups create a more hydrophobic environment within the cavity, providing a more favorable setting for the inclusion of the lipophilic cyclopentanone moiety of LXP. This kind of encapsulation suggests that MCD offers a stronger, more stable complexation, which could be beneficial for improving the drug's stability and its sustained release profile. The difference in the inclusion modes between BCD and MCD is significant, as it indicates that the nature of the CDs (native vs. methylated) can play a critical role in determining the mode of interaction with the drug molecule.

To validate and further refine the structural orientation of both inclusion products, theoretical calculations are performed, considering four different input configurations for each complex. The computational studies provided insights into the most stable inclusion modes and the preferred orientations of the LXP molecule within the cavities of both BCD and MCD. These theoretical results are consistent with the experimental data, further supporting the proposed inclusion modes. The calculated energies of the inclusion complexes provided additional confirmation that the experimentally observed interactions are indeed the most stable configurations, which corroborates the experimental findings from NMR and IR spectra. The consistency between the experimental data and theoretical calculations is significant, as it strengthens confidence in the proposed inclusion modes. The identification of stable inclusion structures and the agreement between experimental and computational results underscore the reliability of the methodologies used to investigate these complexes.

3.11. Biocompatibility study

The biocompatibility of LXP and its inclusion products of LXP:BCD and LXP:MCD are systematically evaluated using MRC-5 fibroblast cells.

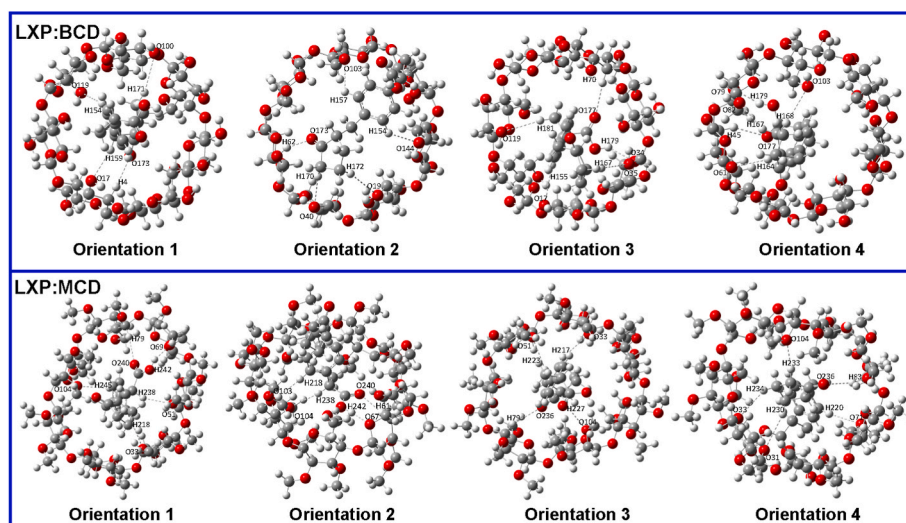


Fig. 6. Hydrogen bonding interaction of LXP:BCD, and LXP:MCD.

This assessment is carried out to compare the cytotoxic effects of LXP in its free form and when complexed with BCD and MCD, providing insight into their potential biomedical applications. A range of concentrations is tested to determine the safe dosage limits and to analyze how the inclusion products influence the cytotoxicity profile of LXP. Cell viability is quantified using the Alamar Blue assay, a widely recognized method for assessing metabolic activity and cellular health. The results, expressed as mean $\pm$ standard deviation ($n = 2$), are presented in Fig. 7A and Table S7. Fig. 7D illustrates the survival rates of MRC-5 cells upon exposure to varying concentrations of LXP and its inclusion products over three days. The findings reveal that at concentrations up to 0.4 mg/mL, all samples, including free LXP, LXP:BCD, and LXP:MCD exhibited no significant toxic effects on MRC-5 cells, indicating their biocompatibility at lower doses. However, as the concentration increased to 2 mg/mL, a slight cytotoxic response is observed after three days of exposure, suggesting dose-dependent toxicity. This effect is more pronounced in free LXP compared to its inclusion products, highlighting the role of complexation in mitigating cytotoxicity. A comparative analysis of the inclusion products revealed a significant enhancement in cell viability. Notably, LXP:BCD exhibited a cell viability reduction of approximately 5 % at the highest tested concentration, whereas LXP:MCD demonstrated a comparatively lower reduction of about 13 % when compared with free LXP. This suggests that LXP:MCD provides superior biocompatibility, which can be attributed to the enhanced solubility and

controlled release of LXP from the MCD cavity.

The impact of LXP and its inclusion products on MRC-5 cell morphology is examined using bright-field microscopy (Fig. 7D). Significant morphological alterations are observed in cells treated with free LXP compared to the untreated control group, indicating potential cytotoxic effects. These changes included cell shrinkage, irregular shapes, and a decrease in cell density, which are indicative of stress-induced cellular responses. In contrast, cells treated with LXP:CD inclusion products exhibited fewer morphological alterations, suggesting that complexation with CDs may mitigate the direct cytotoxic effects of LXP by improving its solubility and bioavailability. To further evaluate the cytotoxic effects, apoptosis rates are assessed using flow cytometry at a concentration of 1.0 mg/mL for both LXP and its inclusion products. This analysis utilized Annexin V and propidium iodide (PI) staining to distinguish between early and late apoptotic cells. The flow cytometry results (Fig. 8B) revealed a lower percentage of apoptotic cells in the LXP:CD-treated groups compared to those treated with free LXP, indicating that inclusion complexation reduced apoptosis induction. Notably, all treated samples demonstrated minimal apoptotic activity relative to the control group, suggesting that neither LXP nor its inclusion products triggered significant apoptosis under the tested conditions. This finding implies that the cytotoxicity observed at higher LXP concentrations may not be due to programmed cell death but rather to other stress-related cellular responses. Further validation of these

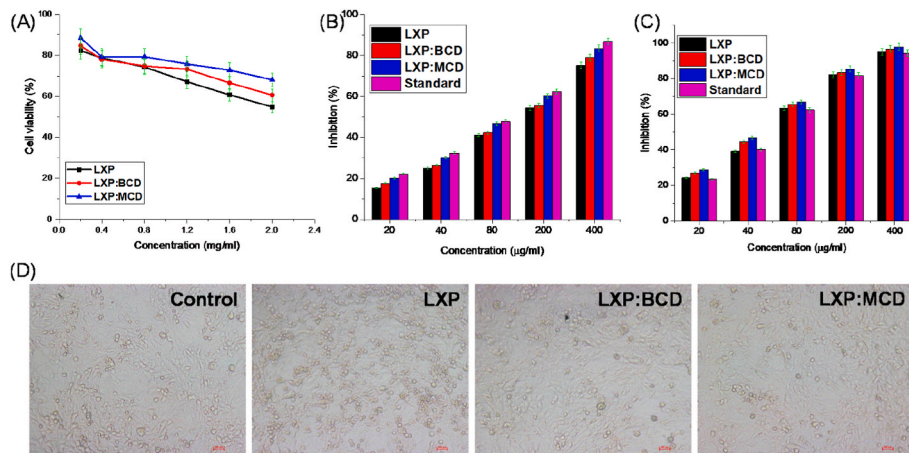


Fig. 7. Alamar blue biocompatibility study (A), anti-inflammatory activity of LXP, and LXP:CDs (B), anti-arthritis activity of LXP, and LXP:CDs (C), and bright field cell images of LXP [23], and LXP:CDs (D).

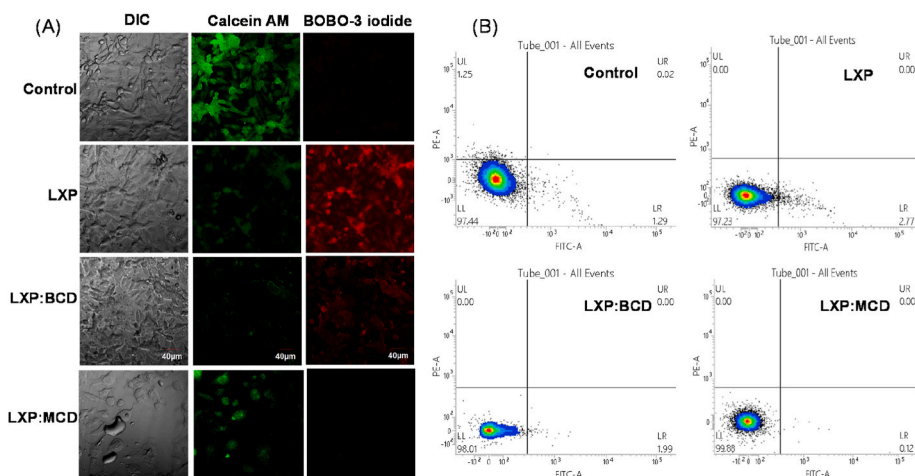


Fig. 8. LXP [23] and LXP:CDs induced apoptosis against MRC-5 cells [Note: Live and dead cell staining of lungs fibroblastic cells on control, green image denotes live cells and red in dead cells; scale bar is about 40 μm] (A), and FACS output for LXP [23], and LXP:CDs (B).

observations is provided through confocal microscopy analysis of live/dead cell staining (Fig. 8A). The control group displayed no dead cells, confirming the baseline viability of untreated MRC-5 cells. While cells exposed to free LXP exhibited signs of cellular damage, including increased membrane permeability and compromised structural integrity, those treated with LXP:CDs maintained a more intact morphology. This suggests that the inclusion of LXP within the CD cavities reduces its direct toxicity, likely due to improved solubility, controlled release, and reduced interaction with the cell membrane.

3.12. Anti-inflammation (AI) and anti-arthritis (AA) potentials of LXP:BCD, and LXP:MCD

A primary evaluation of the AI activity of LXP, LXP:BCD, and LXP:MCD is carried out using the albumin denaturation assay. This assay is widely employed to assess the AI potential of drugs based on their ability to inhibit the protein denaturation process, a key process associated with inflammatory responses and diseases such as rheumatoid arthritis [59, 60]. Protein denaturation in this assay is induced using BSA under physiological conditions (pH 7.4), and the AI potential of the tested compounds is quantified by measuring the extent of inhibition of this denaturation process. A higher percentage of inhibition corresponds to stronger AI properties [61]. To systematically analyze the AI efficacy, the inhibition of protein denaturation is evaluated at five different concentrations. The results demonstrated that LXP exhibited a concentration-dependent increase in inhibition percentage across all tested concentrations, closely paralleling the performance of the standard anti-inflammatory drug, diclofenac sodium (Fig. 7B, and Table S8). This dose-dependent inhibitory effect confirms the significant AI potential of LXP as a free drug. Given the promising AI activity observed with free LXP, further investigations are conducted to evaluate the AI potential of LXP:BCD and LXP:MCD, at identical concentration ranges. Interestingly, the results indicated that the release of LXP from these samples is more pronounced compared to the free drug, resulting in a more effective inhibition of BSA denaturation. Both LXP:BCD and LXP:MCD exhibited robust protective effects, with inhibition rates exceeding 78 % and 83 %, respectively. Notably, LXP alone also provided substantial protection, reaching an inhibition rate of approximately 75 %. The interaction pattern of LXP with BCD and MCD plays a crucial role in modulating its biological activity. Structural analysis and binding studies suggest that in the LXP:BCD, LXP primarily interacts with the outer rim of the BCD cavity rather than being fully encapsulated within it. This interaction likely occurs through hydrogen bonding at the external surface, leading to a partial inclusion that may influence the stability and release behavior of LXP [62]. In contrast, within the LXP:

MCD, LXP is more effectively accommodated inside the hydrophobic cavity of MCD, resulting in a stronger host-guest interaction. The deeper encapsulation of LXP within MCD enhances its solubility and alters its release kinetics, leading to improved bioavailability. This difference in the mode of interaction between the two CDs directly affects the biological activity of LXP.

The BSA protein denaturation assay systematically evaluated the *in-vitro* AA activity of LXP, LXP:BCD, and LXP:MCD. This well-established method assesses the AA potential of compounds based on their ability to inhibit protein denaturation, a key pathological process associated with inflammatory arthritis. Since protein denaturation plays a crucial role in the progression of arthritic conditions, compounds that effectively inhibit this process are considered to have significant therapeutic potential. LXP exhibited a dose-dependent increase in the inhibition of protein denaturation across all tested concentrations, closely reflecting the activity of diclofenac sodium, the standard reference drug (Fig. 7C, and Table S9). The progressive increase in inhibition suggests that LXP has a potent AA effect, capable of stabilizing protein structures and preventing inflammation-associated damage. Interestingly, the LXP:BCD and LXP:MCD demonstrated a notable enhancement in protein denaturation inhibition, with their activity increasing consistently as the concentrations are elevated [63]. This enhanced effect is likely due to the improved solubility and bioavailability of LXP when incorporated into CDs. Notably, both LXP and its inclusion products exhibited strong inhibition activity even at the lowest tested concentration (20 μg/mL), surpassing the performance of the standard diclofenac sodium. This trend continued across all tested concentrations, including the highest dose (400 μg/mL), indicating that the complex forms of LXP provide sustained and highly effective AA activity. The comparable inhibition percentages observed for LXP, LXP:BCD, and LXP:MCD suggest that LXP is efficiently released from the CD complexes upon interaction with the biological environment, ensuring optimal therapeutic activity. Furthermore, in addition to inhibiting protein denaturation, the inclusion products demonstrated a remarkable ability to protect erythrocyte membranes from lysis induced by heat and hypotonic stress. This additional protective effect reinforces the enhanced therapeutic potential of the LXP:CD, as erythrocyte membrane stabilization is a critical factor in mitigating inflammatory damage in arthritis [64,65].

3.13. Limitations and future directions of the current study

While this study primarily focuses on the complexation of LXP within the cavities of CDs, the biological evaluation, specifically its anti-inflammatory and anti-arthritis effects, has only been conducted at a preliminary level. Early results indicate promising biological activity,

yet a more thorough investigation is essential to fully explore the pharmacological significance of the LXP:CDs. To gain deeper insights into the molecular-level mechanistic pathways driving these effects, advanced cellular assays, including Western blot analysis, should be conducted to identify key signaling pathways and protein expressions influenced by the LXP:CDs. Additionally, further *in vitro* and *in vivo* studies are crucial to comprehensively assess the therapeutic potential, bioavailability, and metabolic stability of this innovative drug complex. A deeper understanding of its biological interactions will unlock its potential for clinical application in treating inflammatory and arthritic conditions.

Future research on LXP:CDs systems should explore the influence of various CD derivatives on complex stability, orientation, and release behavior. Quantum mechanical calculations, umbrella sampling, and multiscale simulation approaches have been successfully applied to curcumin, formononetin, luteolin, and other bioactive molecules [66–70]. Advanced computational methods, as discussed in the above articles, can further clarify host–guest interactions and their impact on solubility and stability. Evaluating these complexes in biological models may reveal improvements in bioavailability and reduced gastric side effects.

4. Conclusions

We successfully synthesized and characterized the inclusion products of LXP with CDs (BCD, and MCD), confirming a 1:1 stoichiometric ratio through BH plots. FT-IR, Raman, NMR, and ROESY analyses revealed that LXP interacts with the outer rim of BCD and is partially encapsulated in MCD. Structural and computational analysis showed favorable complexation energy, supporting experimental results. Inclusion of LXP in the cavities of BCD and MCD enhances its thermal stability by TGA analysis. The LXP:CDs complexes exhibited excellent biocompatibility on MRC-5 cells, demonstrating their activity for biological applications. *In-vitro* studies revealed enhanced anti-inflammatory and anti-arthritic activities, highlighting improved solubility and bioavailability of LXP. This approach provides a promising strategy for enhancing the efficacy of other poorly water-soluble nonsteroidal anti-inflammatory drugs, offering a pathway for further therapeutic exploration.

CRedit authorship contribution statement

Rajaram Rajamohan: Conceptualization, Methodology, Resources, Formal analysis, Writing – original draft. **Perumal Muthuraja:** Formal analysis. **Sekar Ashokkumar:** Methodology, and Formal analysis. **Mani Muralikrishnan:** Software, Formal analysis. **Kuppasamy 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jics.2025.102346>.

Data availability

The datasets used or analyzed during the current study are available

from the corresponding author upon reasonable request.

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