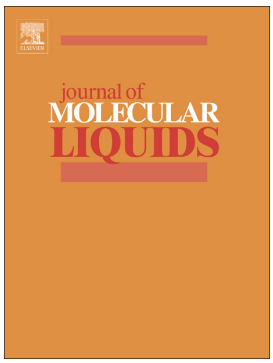

Microwave-assisted β-cyclodextrin/chrysin inclusion complexation: An economical and green strategy for enhanced hemocompatibility and chemosensitivity in vitro

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Microwave-Assisted β−Cyclodextrin/Chrysin Inclusion Complexation: An Economical and Green Strategy for Enhanced Hemocompatibility and Chemosensitivity *In Vitro*

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Abstract

Chrysin (5, 7-dihydroxyflavone) (CHR), a wonder flavonoid that nature has bestowed upon us, is endowed with a broad spectrum of pharmacological properties. Nonetheless, the practical uses of CHR are greatly impeded owing to its poor aqueous solubility and low oral bioavailability. Inclusion complexes (ICs) in β−cyclodextrin (β−CD) have been an effectual strategy for the same and herein, CHR/ β−CD solid ICs were accomplished by diverse approaches. The inclusion phenomenon was affirmed from different spectroscopic techniques. Molecular dynamic simulation studies ascertained the high stability of CHR/ β−CD IC. The microwave irradiation method, which is environmentally more benign, was found to be the optimum wherein the product (MW) exhibited better characteristics in terms of drug content and dissolution. *In vitro* hemolytic assay revealed no adverse effect on RBC morphology and the toxicity of CHR was significantly reduced by employing MW. Furthermore, the MW product demonstrated improved chemosensitivity potency in terms of intracellular uptake and cytotoxicity against MCF-7cells in comparison to pristine CHR. Our findings unambiguously validate the efficacy of MW as a superior drug formulation for pharmaceutical applications of CHR delivery.

Keywords: β−CD; Chrysin; Inclusion complex; Microwave irradiation; Chemosensitivity

1. Introduction

A major surge has been witnessed in the utilities of plant bioactive compounds in food, cosmetic and pharmaceutical industries owing to their biocompatibility, low toxicity and easy accessibility [1]. Such compounds mostly comprise polysaccharides, alkanoids and polyphenolics with well-documented pharmacological characteristics [2]. Flavonoids are a class of polyphenolics, which find special mention because of major functions such as anti-viral actions, inhibition of bacterial growth and protection of cells against damage caused by reactive oxygen species [3]. The high potency and low systemic toxicity enable these flavonoids as viable substitutes to conventional therapeutic drugs. And, Chrysin (5, 7-dihydroxyflavone) is one such marvel flavonoid that nature has bestowed upon us.

Chrysin (CHR), the principal component of Indian trumpet tree (*Oroxylum indicum*) and passion flower (*Passiflora incarnata*), is endowed with a broad spectrum of pharmacological properties like anti-inflammatory, anti-tumour, anti-hypertension, antibacterial, anti-haemolytic and an antioxidant [4]. The beneficial pharmacological activities of CHR have been articulated beautifully in a recently published review article [5]. Contemporary research has revealed its tremendous inhibitory effect on an array of carcinogenic cells [6-8]. In addition, CHR is one of the most potent Breast Cancer Resistant Protein (BCRP) inhibitors till date [9]. However, despite the huge applicability; the practical usage of CHR is impeded owing to its poor aqueous solubility and low oral bioavailability [5, 10]. Hence, effective solubilization of CHR is warranted in order to accomplish its clinical potential.

Cyclodextrins (CDs) and their derivatives have come a long way in improving the solubility, stability and bioavailability of the guest molecules encapsulated in their cavities [11-13]. CDs, as encapsulating agents, for various plant bioactive compounds are well-recognized wherein the biological, chemical and physical properties of the guest molecules

have improved significantly [2]. Inclusion studies on CHR and β−cyclodextrin (β−CD) in aqueous solutions have been investigated and an appreciable improvement in the solubility and anti-oxidant potential was observed [14]. The influence of β−CD, 2-hydroxypropyl-β−CD and heptakis (2,6-di-*O*-methyl) β−CD on the aqueous solubility of CHR have also been explored [15]. Water content of CHR and α−/β−CD nanoparticles synthesized by crystallization from water-ethanol solution has been studied [16]. Solid inclusion complex (IC) of β−CD and CHR prepared by co-crystallization process has been evaluated for its anti-microbial and anti-tumour potential [4]. The authors observed that the inclusion process not only increased the drug solubility but also its biological activities in terms of anti-oxidant potential, anti-bacterial and anti-tumor activities. ICs of sulfobutyl ether−β−CD (Captisol®) and CHR have been prepared by freeze-drying and assessed for aqueous solubility, dissolution, pH-stability and bio-efficacy [10]. CHR-loaded β−CD nanosponges were found to be potent towards anti-tumorous activity against HeLa cells as compared to pristine CHR [17]. Also, a quantum mechanical approach for CHR/ β−CD system has been carried out to shed light on the antioxidant mechanism of CHR to scavenge hydroxyl radical in solution [18].

Nevertheless, a comparative study on the solid ICs of CHR in β−CD formed by diverse methods is not available. Conventional methods of IC preparation mostly include physical mixing, kneading, and spray drying technologies. Though co-precipitation and freeze-drying methods generate true ICs, they are inevitably associated with certain shortcomings. The major drawbacks lie in the long processing time and the use of organic solvents [19]. In lieu of this, microwave-assisted chemistry has gained an upward thrust recently because this method accomplishes the reaction in a very short time span and is environmentally more benign by utilizing minimal amount of solvents, reducing the quantities of by-products and lowering the reaction temperature [20, 21]. Yet, complexation

studies of CHR with β−CD by the microwave irradiation method remains largely unexplored. Thus, the focus of the present work is to address the efficacy of different conventional methods of preparation of solid ICs of CHR with β−CD and compare them with that of the microwave irradiation method. The formation of true ICs was ascertained from different spectroscopic techniques. Molecular docking and simulation studies have been carried out to gain insight into the mode of inclusion of CHR with β−CD and to comprehend the stability of the complex. More importantly, the ICs have been assessed for their bio-potential in terms of hemocompatibility, intracellular uptake capacity and anticancer activity *in vitro*.

2. Materials and methods

Chrysin (CHR), β−cyclodextrin (β−CD), D_2O and MTT dye were purchased from Sigma Aldrich, India and used as received without any further treatment. Ethanol and DMSO were procured from SRL, India. DMEM, FBS, penicillin-streptomycin-neomycin solution, phosphate buffer and cell lysis buffer were obtained from Himedia, India. MCF-7 cells were from NCCS Pune, India. Deionized water from a Millipore Milli Q system was employed throughout. All other reagents were of analytical grade purity.

2.1. Studies on inclusion by UV-Vis spectroscopy

For UV-Vis studies, the concentration of CHR was kept constant at $1x10^{-5}$ M and the β−CD concentration was increased gradually up to 20 mM. The absorption spectrum was recorded in a Shimadzu UV-Vis spectrophotometer (UV-1800). The binding constant (K_b) for the drug and the β−CD complexation was calculated from the absorbance data using the modified Benesi-Hildebrand equation: [22]

$$\frac{1}{(A - A_o)} = \frac{1}{([\beta - CD\,]\alpha K_b)} + \frac{1}{\alpha} \qquad (1)$$

Here A_0 and A represent the absorbance of CHR in absence of β−CD and at various β−CD concentrations and α is a constant. From the double reciprocal plot of [1/ ($A-A_0$)] against 1/ [β−CD], the ratio of the intercept to the slope provided the value of K_b.

2.2. Phase solubility studies

Phase solubility studies were carried out in aqueous medium at room temperature according to the reported method by Higuchi and Connors [23]. An excess amount of CHR was added to 5 mL of aqueous solution containing various concentrations of β−CD (0−10 mM) in sealed glass containers. The proposed suspensions were shaken on a mechanical shaker for 7 days at room temperature to achieve equilibrium. After equilibrium was achieved, the samples were filtered and properly diluted. The concentration of CHR was determined spectrophotometrically from the absorbance at λ_{max}= 270 nm and comparing it with the calibration plot. The apparent stability constant K_s was calculated from the phase solubility diagram according to the following equation [24]:

$$K_s = \frac{slope}{S_0(1-slope)} \qquad (2)$$

where S_0 is the solubility of CHR in absence of β−CD.

2.3. Preparation of solid ICs

The solid inclusion complexes of CHR with β−CD were prepared in a 1:1 molar ratio (on the basis of the preliminary results obtained from UV-Vis and phase solubility studies) by adopting various methods, briefed below [19]:

(a) Physical mixture: A physical mixture (PM) of CHR and β−CD was prepared by homogenous blending of the components in a mortar for 15 minutes.

(b) Kneading: Minimal quantities of water-ethanol mixture (1:1 v/v) were added to a known amount of the CHR and β−CD physical mixture. The mixture was kneaded thoroughly to obtain a homogeneous paste. The product obtained (KN) was dried and ground to a fine powder.

(c) Co-precipitation: Briefly, calculated amount of β−CD was dissolved in distilled water. Desired amount of CHR, solubilised in ethanol, was slowly added to the β−CD

solution and continuously stirred for 24 h. The solution was then refrigerated overnight at 4°C. The precipitated complex (CP) was recovered by filtration and washed with ethanol to remove the uncomplexed drug. The residue (CP) was vacuum-dried for 48 h and utilized further.

(d) Freeze drying: The freeze-dried product (FD) was obtained by dissolving β−CD in water and adding stoichiometric amounts of CHR to the β−CD solution. The suspension was stirred for 48 h at room temperature. The resultant solution then was frozen at −20°C for 6 h and then lyophilized at −55°C for 48 h (Scanvac Coolsafe Freeze Dryer).

(e) Microwave irradiation: For the preparation of ICs by microwave irradiation, a physical mixture of CHR and β−CD was taken and dissolved in minimal amounts of water-ethanol mixture (1:1 v/v). The mixture was then subjected to microwave irradiation in a scientific microwave oven (Sineo UWave-1000) at a power of 300 W for 180 s at 60 °C. The product (MW) thus formed was washed with water-ethanol solvent mixture to remove the residual components and dried *in vacuo*. A control experiment was carried out to check the properties of CHR upon exposure to microwave irradiation under the same conditions as that of IC preparation. No change in CHR properties such as melting point, UV-Vis absorption characteristics and also XRD pattern was witnessed thereby suggesting no adverse effect of microwave radiation on CHR under the experimental conditions.

2.4. Characterization of ICs

FTIR. The samples were triturated with dry KBr, compressed to pellets and scanned in the range of 4000 to 400 cm^{-1} at room temperature in a Thermo Scientific Nicolet iS5 spectrophotometer.

X−RD. The powder X−RD profiles were collected on a Rigaku Ultima IV X−Ray diffractometer using Nickel-filtered Cu K_α radiation (1.54056 Å) and scanned from 10° to 30° at room temperature at a scan rate of 3°/min.

DSC. DSC was performed by Mettler Toledo DSC822 instrument on 5−10 mg of samples under N_2 atmosphere (purging rate: 40 mL/ min) from 50−300°C at a heating rate of 10°C/min.

FESEM. The morphology of the samples was observed on a Nova NanoSEM 450 field emission scanning electron microscope. The samples were sputtered coated with gold, mounted on metal stubs and visualized under the scanning electron microscope.

^{1}H NMR. Proton NMR experiments were carried out in a Bruker 400 MHz NMR at 298 K.

2.5. Assay of drug content

The total amount of CHR present in ICs prepared by CP, FD and MW was determined spectrophotometrically. 25 mg of each IC was dissolved in 10 mL of ethanol and subjected to ultrasonication for 15 min to allow complete dissolution of CHR. The solutions were centrifuged at 2500 rpm for 10 min to remove the β-CD. 3 mL of the supernatant was collected, filtered with a 0.22 μm filter (Himedia, India) and analysed subsequently. The percentage of drug content was calculated according to the equation:

$$Drug\ Content\ (\%) = \frac{Actual\ CHR\ content}{Expected\ CHR\ amount} \times 100 \qquad (3)$$

where actual CHR content is the amount of drug present in the solid IC and expected CHR amount refers to the calculated amount of CHR in 25 mg of IC considering 1:1 stoichiometry. Three independent measurements were carried out and the data presented are the average of the three values.

2.6. *In vitro* drug dissolution studies

The dissolution studies of free CHR and the prepared ICs were performed in 100 mL of phosphate buffer saline (PBS; pH 7.4). 25 mg of pristine CHR or its equivalent amount of the solid ICs were subjected to dissolution studies; with a constant stirring speed of 250 rpm maintained at 37°C [25]. At definite time intervals, 3 mL aliquots were withdrawn and assayed spectrophotometrically. The withdrawn samples were replenished with same volume of fresh medium. The sink conditions were maintained throughout the dissolution study. The experiments have been performed in triplicate and the mean data have been represented.

2.7. Molecular docking and molecular dynamics simulation studies

In order to understand the inclusion of CHR in β−CD cavity, we performed molecular docking. The 3D structure of β−CD was retrieved from CD glycosyltransferase − β−CD complex (PDB ID: 3CGT [26] and CHR from ChemSpider database (ID: 2068). The molecular docking of CHR and β−CD was then carried out using AutoDock Vina [27] following the procedure from our previous report [28]. Further, the docked β−CD-CHR complex with lowest binding energy (high binding affinity) was taken for dynamic study.

In order to comprehend the dynamic-stability of CHR in β−CD cavity, molecular dynamics (MD) simulation with explicit solvent was performed using GROMACS 4.5.5 [29] employing amber99sb-ildn force field [30] in periodic boundary condition. The topology parameter files for CHR was generated using Antechamber tool v 1.11 [31]. Keeping the CHR/ β−CD complex in the centre of cubic box, 1347 numbers of TIP3P water models and 0.15 M concentration of NaCl for solvation and electro-neutralization, were added respectively. Thereupon, steepest descent energy minimization was carried out until a tolerance of 1000 $kJ.mol^{-1}.nm^{-1}$ to ensure there are no steric clashes in the system. Next, the energy-optimized simulation system was equilibrated (position restrained) in two individual phases: NVT (0.1 ns) and NPT (1 ns). After equilibrium, the final MD simulation was carried

out for 20 ns time scale in duplicate for better sampling of results. The trajectory was saved in 10 ps interval. After obtaining the MD trajectories, the stability and compactness was observed by computing its root mean square deviation (RMSD) and Radius of gyration (Rg) of CHR/ β−CD complex. Further, to comprehend the nature of interaction, the intermolecular H−bonds between β−CD and CHR as a function of simulation time was computed. 2D graphs were plotted using Grace 5.1.21 program (http://plasma-gate.weizmann.ac.il/Grace/) and PyMOL (academic license) was used for studying the intermolecular interactions and structural visualization.

2.8. *In vitro* hemolysis using human RBCs

Hemolysis study was performed as per the reported method [32]. Briefly, erythrocytes were harvested in heparinized tubes (venous blood collected from a healthy human volunteer after written consent) by centrifugation. The supernatant plasma was discarded, and the sedimented erythrocyte cake was washed thrice with PBS (pH 7.4). After the final wash, erythrocytes were resuspended in saline (0.9% w/v). Thereafter, 2 mL of the erythrocyte suspension was incubated with 0.2 mL of test samples at 37°C for 2 h in a shaker and then centrifuged at 1000 rpm for 15 min. The supernatant was collected with utmost care and analyzed for hemoglobin content at 540 nm spectrophotometrically. PBS (pH 7.4) was considered as the negative control with 0% hemolysis and deionized water served as the positive control with 100% hemolysis. The hemolysis (%) was calculated from the following equation (4):

$$Hemolysis\ (\%) = \frac{(OD)_{sample} - (OD)_{-ve\ Control}}{(OD)_{+ve\ Control} - (OD)_{-ve\ Control}} \qquad (4)$$

The samples were then investigated in terms of their morphological changes under an optical microscope (Nikon H550S, Japan).

2.9. *In vitro* chemosensitivity evaluation

2.9.1. Intracellular uptake studies

The effect of CHR and the ICs on the intracellular uptake was conducted as per the reported method [33]. MCF-7 cells were inoculated in 24-well plates at a density of 1×10^5 cells/ well overnight. The cells were then treated with pristine CHR (40 μg/ mL) and the ICs at an equivalent drug concentration. They were incubated at 37 °C for 30 min for 2 h, 6 h and 12 h. After washing with PBS thrice, the cells were lysed by 1 mL of cell lysis buffer. Thereafter, the cell lysis solution was centrifuged at a speed of 1000 rpm for 15 min. The intracellular uptake was determined by a fluorescence microplate system.

2.9.2. Cytotoxicity assay

The cytotoxicity of pristine CHR, CP, FD and MW was evaluated in human breast adenocarcinoma cells (MCF-7) by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium reduction assay based on the conversion of yellow soluble tetrazolium salt to a purple formazan in living cells [34]. Briefly, cells were seeded at 1×10^5 cells/ well in a 96-well plate and incubated for 24 h. After incubation, the cells were subjected to treatment with CHR, CP, FD and MW for 24 h. At the end of the incubation period, 20 μL of thiazolyl blue tetrazolium bromide was added to each well and the cells were further incubated for another 4 h at 37 °C. After removing all the culture medium, 100 μL DMSO was added per well to dissolve the formazan crystals. The percentage of cell viability was measured on a microplate reader at $\lambda = 550$ nm. The cytotoxic effects of CHR and ICs on MCF-7 cells have been expressed as IC_{50} values (the drug concentration that reduced the absorbance of treated cells by 50% compared to untreated cells).

2.10. Statistical analyses

Data have been expressed as mean ± SD, statistical significance was determined by one-way analysis of variance (ANOVA) using MedCalc statistical software (ver. 17.8; MedCalc Software, Ostend, Belgium).

3. Results and discussion

3.1. Studies on drug inclusion by UV-Vis spectrophotometry

As illustrated in Fig. 1A, the absorbance of CHR was found to increase with the gradual addition of β−CD. The absorption spectrum of CHR displayed an intense band around λ_{max} = 270 nm (attributed to $\pi - \pi^*$ transition in the benzoyl ring) and a less intense band at around 340 nm (absorbance of the cinnamoyl group) [14]. A slight blue shift in the shorter wavelength peak was observed in the spectra upon increasing the amount of β−CD as compared to that in pure aqueous medium. This could be due to inclusion of the drug into the apolar cavity of β−CD indicating possible interactions between them. The increased absorbance of CHR in presence of β−CD suggests the complexation of the hydrophobic drug CHR with β−CD.

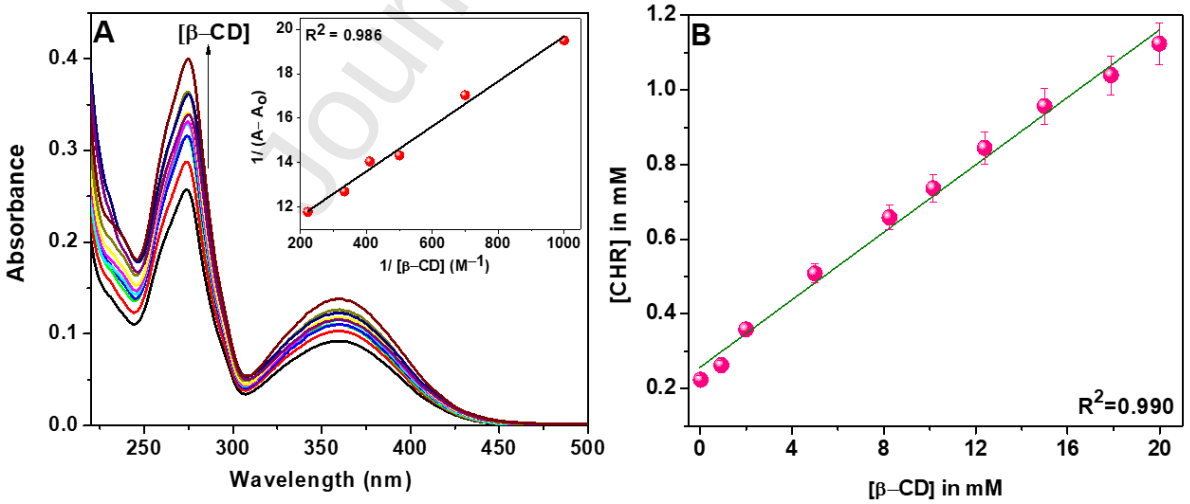

Fig. 1. (A) Absorption spectra of CHR at varying [β−CD]; [β−CD] = 0−20 $\times 10^{-3}$ M, [CHR] = 1×10^{-5} M; (Inset) Benesi–Hildebrand plot and (B) Phase solubility profile of β−CD−CHR in water.

From the absorption spectral study, the Benesi–Hildebrand plot was constructed (Inset of Fig. 1A) and its linearity indicated the formation of a 1:1 complex of CHR with β−CD. The binding constant (K_b) was found to be 945±25 M^{-1} from the plot, which agrees well with previous reported value [14].

3.2. Phase Solubility study

To analyse the effect of inclusion complexation of β−CD on the solubility of CHR; phase solubility studies were performed. The solubility of CHR increased proportionally with increased concentration of β−CD (Fig. 1B). This profile of phase solubility diagram has been classified as the A_L-type, and the stoichiometric molar ratio of β−CD to CHR was considered 1:1 [23]. From the slope and intercept of the approximate line, the stability constant between CHR and β−CD was determined to be 1050 M^{-1} which is in good agreement with other reported values for CHR/ β−CD complexes [10]. The thermodynamic parameter for the CHR/β−CD complexation was calculated from the Gibbs equation given by:

$$\Delta G = -2.303\ RT \log K_s \qquad (4)$$

The Gibbs free energy (ΔG) for the CHR/ β−CD complex formation was estimated to be −17,238 J/mol. The negative ΔG value suggested an energetically favourable complexation process.

3.3. Characterization of solid ICs

The formation of IC of CHR with β−CD prepared by different methods was affirmed from various spectroscopic techniques such as FTIR, XRD, DSC, SEM and ^{1}H NMR.

3.3.1. FTIR analyses

FTIR helps in determining the change in the bands of vibrations of the individual moiety in the process of complex formation. Figs. 2A and A’ illustrate the FTIR spectra of

pristine β−CD, pristine CHR and the ICs. The FTIR spectrum of β−CD showed key peaks at around 3378 cm^{-1} ($O-H_{str}$), 2919 cm^{-1} ($C-H_{str}$), 1026 cm^{-1} ($C-O-C_{bend}$) and 1156 cm^{-1}, 1082 cm^{-1} (primary CH_2-OH_{str}). The key peaks of CHR include the characteristics methylene absorption at 2712 cm^{-1} and the characteristic $C=O_{str}$ at 1652 cm^{-1}. The FTIR spectrum of PM was found to be a superposition of the parent components which indicated that there is no apparent interaction. In the FTIR spectrum of KN; the key peaks of CHR and β−CD were both present but with slight shifts from their original positions that suggested an incomplete and weak interaction. In contrast; the CP, FD and MW inclusion products (Fig. 2A') displayed significant shifts in the characteristic parental peak positions. The characteristic $C=O_{str}$ peak of CHR have shifted from 1652 cm^{-1} to 1644 cm^{-1} in CP; 1648 cm^{-1} in FD; and 1650 cm^{-1} in MW. On the other hand, prominent shifts were also witnessed in the key absorption positions of β−CD. The $O-H_{str}$ and $C-H_{str}$ peaks of pristine β−CD at 3378 cm^{-1} and 2919 cm^{-1} have shifted to 3385 cm^{-1}, 3389 cm^{-1}, 3386 cm^{-1} and 2925 cm^{-1}, 2926 cm^{-1}, 2926 cm^{-1} in CP, FD and MW respectively. In addition, the primary CH_2-OH_{str} absorption of β−CD at 1156 cm^{-1} has also shifted to 1164 cm^{-1}, 1160 cm^{-1} and 1168 cm^{-1} in CP, FD and MW correspondingly. As CHR gets encapsulated within β−CD cavity, there arose an interaction between them which altered the vibration modes and thus modified bands were observed in the spectra of the ICs. These spectral modifications altogether signified the inclusion of CHR in β−CD cavity.

3.3.2. X−RD analyses

Powder X-ray diffraction has been recognized as one of the useful tools to judge drug-CD complexation phenomenon wherein the diffraction profile of the IC is usually different from those of the individual components. The X−RD profiles of pristine β−CD, CHR and the ICs are displayed in Fig. 2B. The powdered X−RD profile of β−CD included signals at 12.5°, 23.0° and 25.8° indicating its highly crystalline nature. CHR showed key

peaks at 14.8°, 17.7° and 27.5° being consistent with previous reports that revealed the crystalline nature of its particles [4]. In the case of PM and KN, the signals corresponding to CHR were observed which pointed towards the presence of crystalline drug thus suggesting an incomplete complexation rendered by these methods. However, it was no longer possible to distinguish the characteristics signals of CHR in the diffractograms of CP, FD and MW. The key signal of CHR at 17.7° was slightly shifted to 17.5° in CP but completely different diffraction peaks were observed in FD and MW. Thus, the X−RD data strongly supported the formation of new entities of ICs by the co-precipitation, freeze-drying and microwave irradiation methods and also established that the crystallization of CHR was indeed disturbed.

3.3.3. DSC analyses

Characterization by DSC is paramount for the recognition of formation of ICs. The melting, boiling or sublimating points generally shift to different temperatures or disappear completely when guest molecules are encapsulated into β−CD cavities [35]. Fig. 2C displays the DSC thermograms of pristine β−CD, pristine CHR and the ICs. The DSC trace of β−CD showed a broad endothermic peak around 120 °C associated with its dehydration process. The thermogram of CHR was typical of a crystalline anhydrous substance with a sharp endotherm around 290 °C indicating its melting point [4]. The endotherms for PM and KN showed the presence of both melting peaks of β−CD and CHR, thereby suggesting incomplete complex formation. The DSC curves for CP, FD and MW products revealed the total disappearance of the CHR melting peak and only the dehydration peak from β−CD was observed with slight shifts to lower temperatures. The absence of CHR melting peak in the CP, FD and MW products suggests its inclusion in the β−CD in the molecular form. Thus, the DSC results also supported the formation of true ICs by the co-precipitation, freeze-drying and the microwave irradiation methods.

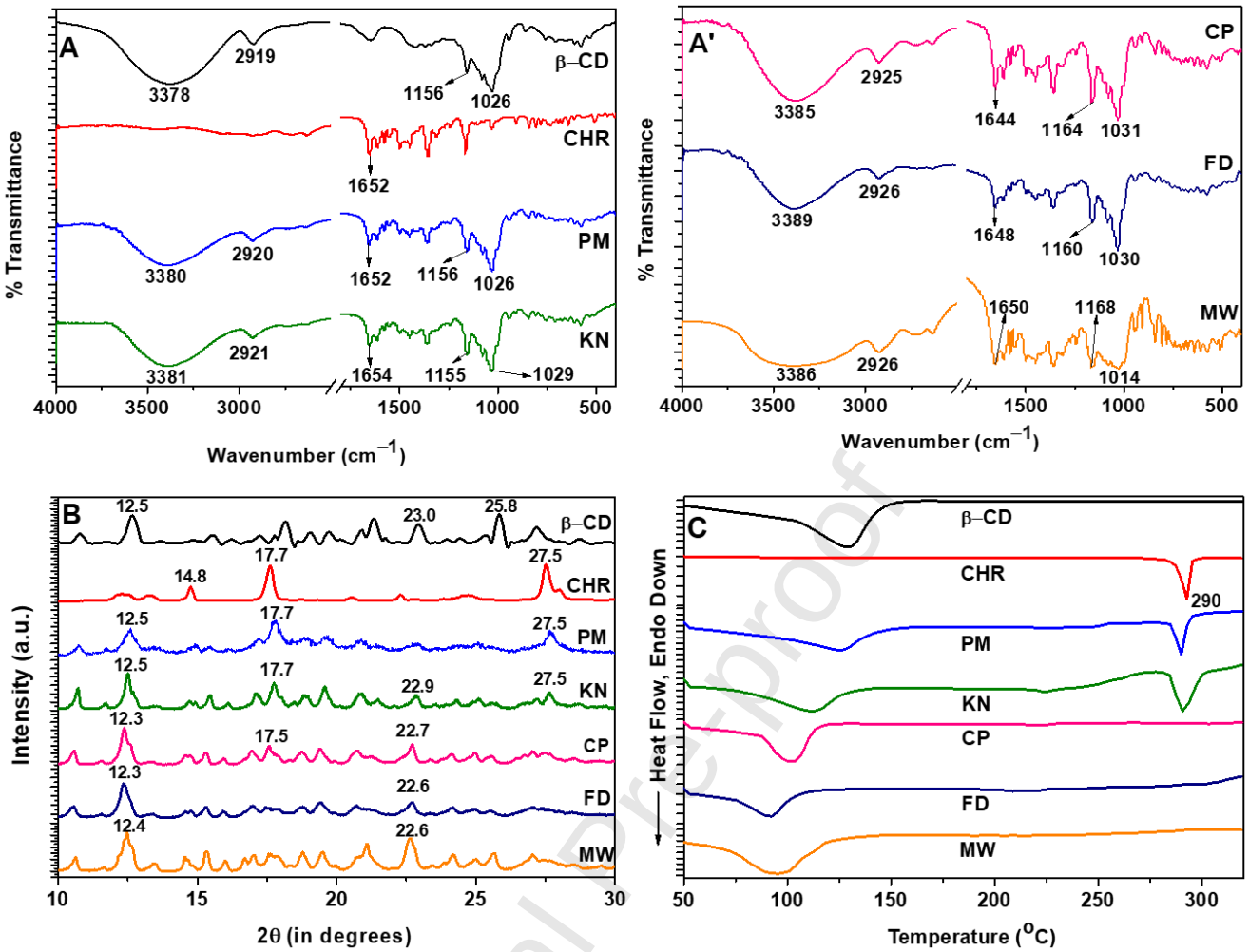

Fig. 2. (A), (A') FTIR spectra, (B) X−RD profiles and (C) DSC thermograms of β−Cyclodextrin (β−CD), chrysin (CHR), physical mixture (PM), kneaded (KN) and (B) co-precipitated (CP), freeze-dried (FD) and microwaved (MW) products.

3.3.4. Morphological visualization

FESEM is a qualitative method to study the morphological aspects of β−CD, drugs and inclusion products obtained by different methods. The images of β−CD, CHR, PM, KN, CP, FD and MW are shown in Fig. 3. The particles of β−CD and CHR were found to be crystalline with different dimensions and polyhedral in nature. The PM and KN consisted of both the particles of β−CD and CHR. The CP and FD comprised of agglomerated blocks; unlike the parent components. MW, however possessed particles with larger dimensions.

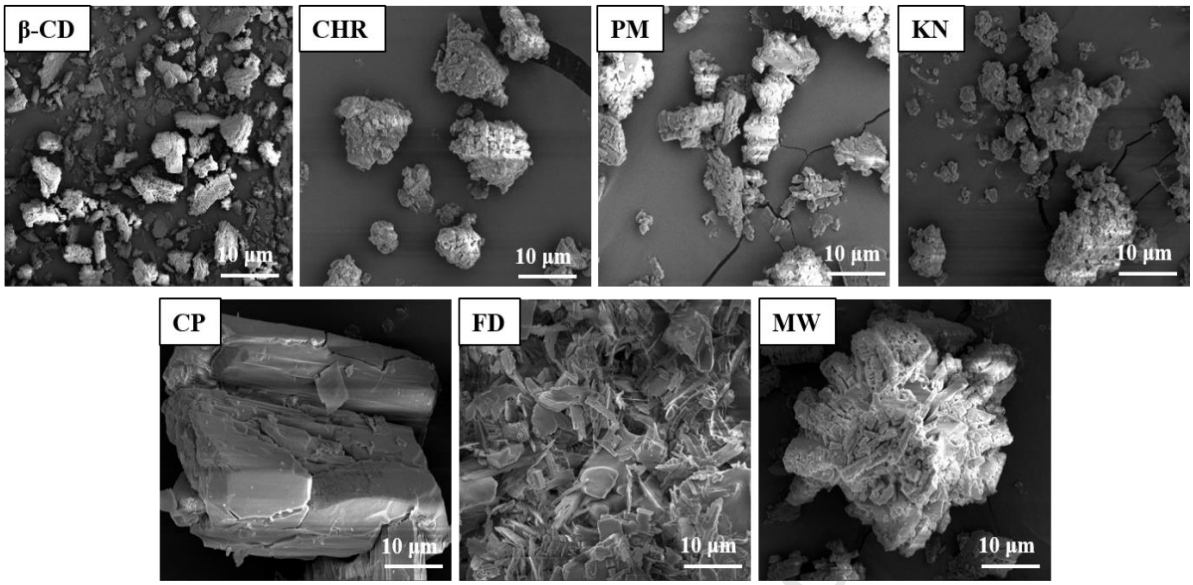

Fig. 3. FESEM images of β−Cyclodextrin (β−CD), chrysin (CHR), physical mixture (PM), kneaded (KN), co-precipitated (CP), freeze-dried (FD) and microwaved (MW) products

3.3.5. ^{1}H NMR analyses

Direct evidence for the formation of drug−β−CD IC can be obtained from ^{1}H NMR studies. β−CD possesses a hollow cone topology with H−3 and H−5 being the inner protons. The hydrophobic guests get included in the toroidal cavity of β−CD thereby affecting these inner protons. Thus, the chemical shift variations in the positions of H−3 and H−5 of the β−CD and IC are pivotal and reflect total and/ or partial inclusion. The ^{1}H NMR spectra of β−CD, CP, FD and MW in D_2O are presented in Fig. 4.

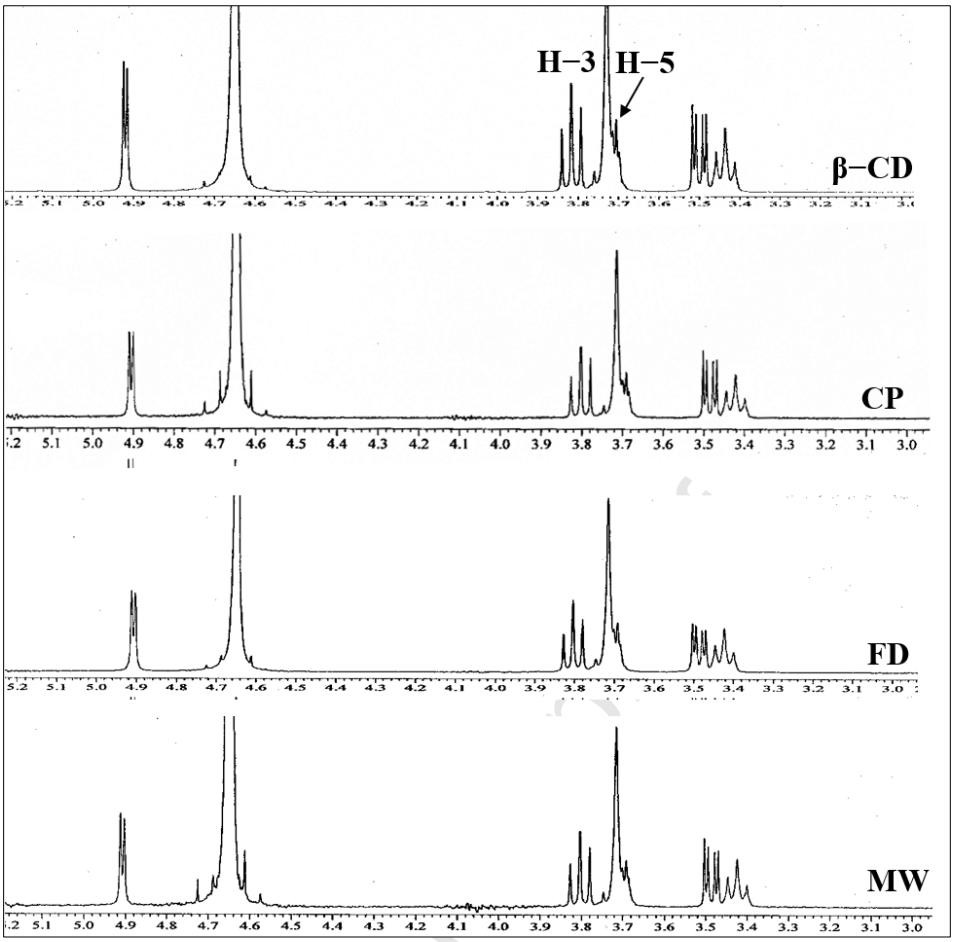

Fig. 4. Partial ^{1}H NMR spectra of β−Cyclodextrin (β−CD), co-precipitated (CP), freeze-dried (FD) and microwaved (MW) products in D_2O at 25 °C.

According to Greatbanks and Pickford, if [Δ(δH3)] ≤ [Δ(δH5)], then total inclusion of guest occurs inside β−CD cavity; and [Δ(δH3)] > [Δ(δH5)] indicates partial inclusion [36]. Table 1 presents the chemical shift in the positions of protons in native β−CD and the ICs.

Table 1. δ and Δδ of protons in β−CD, CP, FD and MW

Protons	$\delta_{\beta-CD}$	CP		FD		MW	
		δ	Δδ	δ	Δδ	δ	Δδ
H−3	3.817	3.802	0.015	3.804	0.013	3.803	0.014
H−5	3.705	3.684	0.021	3.688	0.017	3.686	0.019

From Table 1, it is noteworthy that the chemical shift values of H−3 and H−5 protons of β−CD showed prominent changes in the CP, FD and MW products. It was further observed that [Δ(δH3)] was always less than [Δ(δH5)]; which indicated total encapsulation of

CHR in β−CD cavity. Thus, the formation of true ICs by co-precipitation, freeze drying and microwave irradiation methods is confirmed from the ^{1}H NMR studies.

3.4. Molecular docking and MD simulation studies

In order to rationalize the experimental data and infer the mode of inclusion of CHR in β−CD cavity; we performed molecular docking by AutoDock Vina (Fig. 5A). As displayed in Fig. 5B, the lowest energy complex from docking simulation showed the complete inclusion of CHR in β−CD cavity.

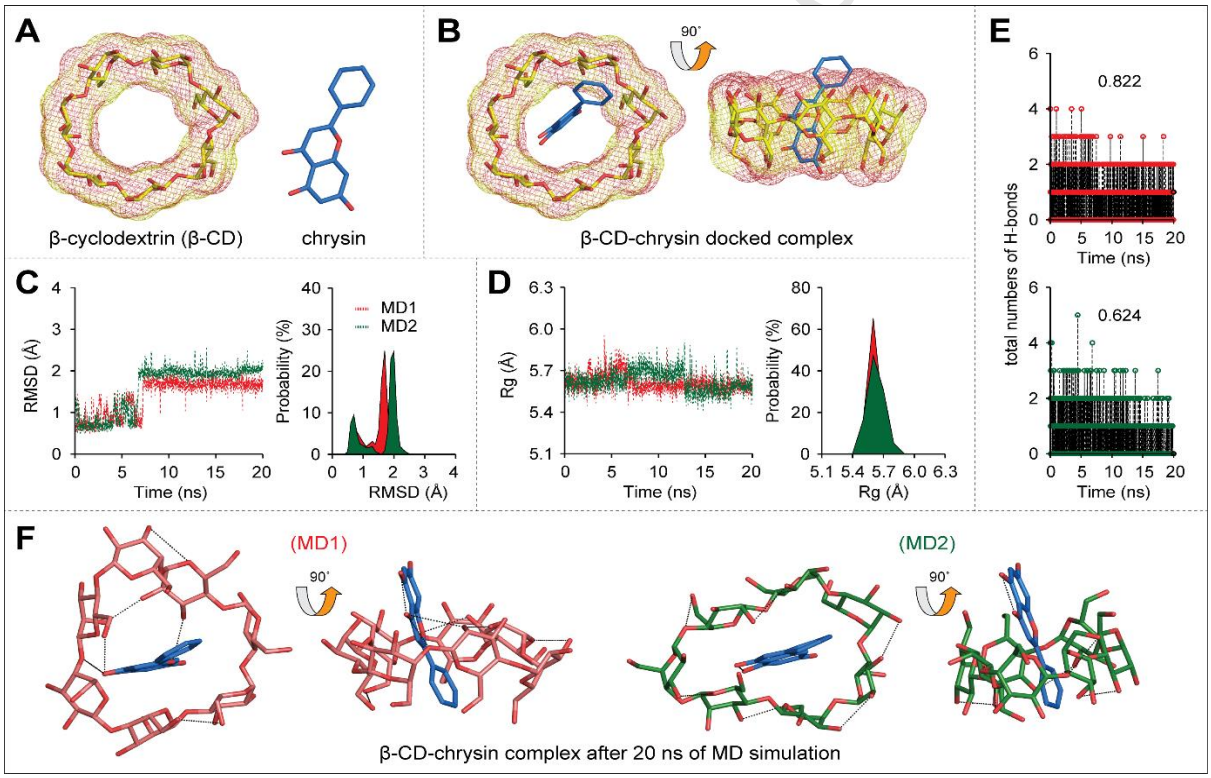

Fig. 5. Inclusion of CHR in β-CD cavity. (A) 3D structures of β-CD, and CHR; (B) β-CD-CHR docked complex. (C) Root mean square deviation (RMSD); (D) Radius of gyration of the CHR/ β−CD complex; (E) intermolecular H-bonds formed between β-CD and CHR as a function of simulation time and (F) structural overview of CHR/ β−CD after MD simulation.

Further, we performed the MD simulation of the CHR/ β−CD complex to understand the stability of the complex in dynamic condition. Post simulation, the stability and compactness of CHR/ β−CD complex was assessed by computing the root-mean square

deviation (RMSD) and Radius of gyration (Rg) with respect to simulation time. As illustrated in Figs. 5C and 5D, we observed a stable RMSD and Rg, which indicated the dynamic stability of CHR/ β−CD complex. H-bonds are one of the major intermolecular forces that define binding stability of bio-molecular entities. Hence, to understand the nature of H-bond interaction of CHR upon encapsulation in β−CD cavity; intermolecular H-bonds were calculated as a function of simulation time. Shown in Fig. 5E, we found stable and conserved pattern of H-bonds between β-CD and CHR in both the trajectories. Further, the post-MD snapshot of CHR/ β−CD complex (Fig. 5F) also revealed the stability of the complex, indicating the stable inclusion phenomenon CHR in β-CD cavity. Based on the above findings, the schematic of CHR/ β−CD IC could be represented in Fig. 6. This structure is in consistent with our proton NMR and molecular docking studies.

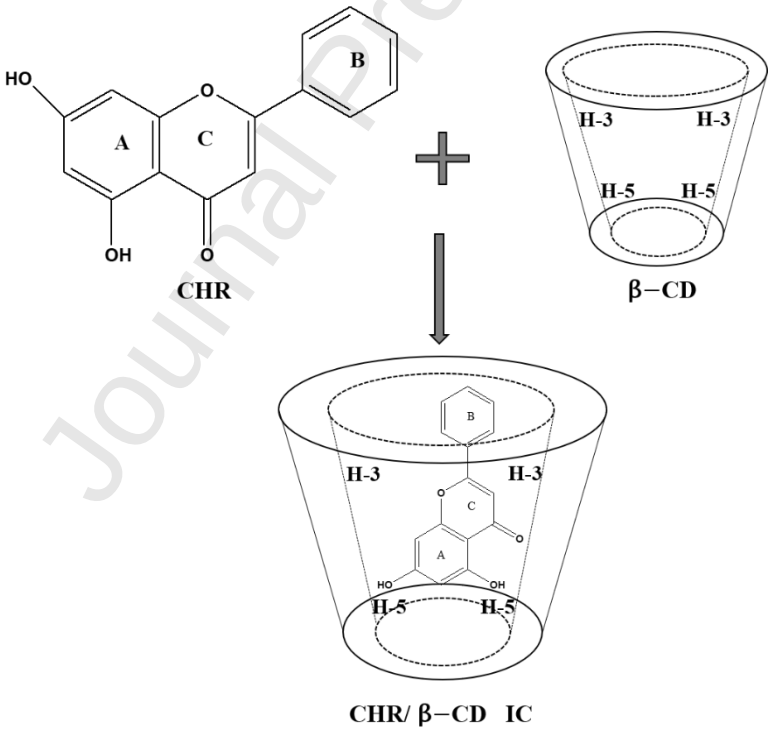

Fig. 6. Mode of inclusion for CHR/ β−CD complex.

3.5. Drug content assay

The results indicated that the drug content in the binary mixture was in the range of 94 –96%. The CP, FD and MW exhibited CHR content of 94.72 ± 0.30%, 95.53 ± 0.25% and

96.85 ± 0.15%. The findings suggested the relatively higher encapsulation efficiency of microwave methods over others.

3.6. *In vitro* drug dissolution studies

The dissolution curves of pure CHR and the ICs in phosphate buffer (pH 7.4) are depicted in Fig. 7.

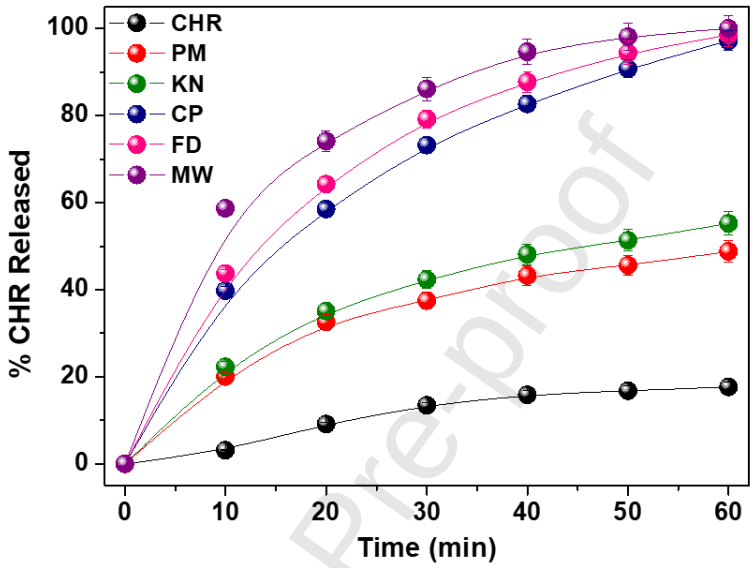

Fig. 7. Dissolution profiles of chrysin (CHR), physical mixture (PM), kneaded (KN), co-precipitated (CP), freeze-dried (FD) and microwaved (MW) products in phosphate buffer (pH 7.4).

As evident, the dissolution of pure CHR was very low even after 60 minutes (approx. 15%) which could be ascribed to its poor aqueous solubility. The dissolution was slightly enhanced when CHR was physically mixed with β−CD. This improvement in dissolution rate can be attributed to the improved drug wettability and *in situ* formation of readily soluble complexes [37, 38]. The dissolution rate of KN was more or less similar with that of PM. In stark contrast, the dissolution rates for the CP, FD and MW were much higher. However, the dissolution was found to be relatively high for MW as compared to all other products which indicated the efficacy of this method over others probably due to the relatively higher drug content in this product. This dissolution profile established the superiority of the microwave

irradiation method over the conventional methods for the generation of improved CHR/ β−CD ICs in terms of better dissolution characteristics.

3.7. *In vitro* hemolysis evaluation

The interaction between a biomaterial and an erythrocyte (RBC) membrane is paramount to access the biosafety of an administered foreign material and is usually reflected by a hemolysis test. Hemolysis refers to the release of hemoglobin from RBCs indicating the disruption of RBC membrane integrity by interacting with the biomaterials/ devices [39, 40]. Hemolysis test has been extensively used in biocompatibility evaluations of various biomedical materials. Fig. 8A shows the hemolysis induced by free CHR and the ICs on human erythrocytes. No hemolytic effect was observed for the ICs in the drug concentration ranging up to 40 μg /mL; permissible limit being < 5% for biomaterials (Figs. 8B). This confirmed that complexation significantly decreased the toxicity of pristine CHR and indicated the high biosafety of the formulations. In particular, MW demonstrated significant decrease in toxicity of CHR thereby validating the superiority of the product.

The samples subjected to hemolysis were further inspected for their morphological changes under an optical microscope and the images are depicted in Fig. 8C. As evident, direct contact between the RBCs and the samples did not evoke any adverse effect on the cell morphology. Quite visibly, no cell death was brought about by treatment with the ICs which further corroborates our hemolysis observation.

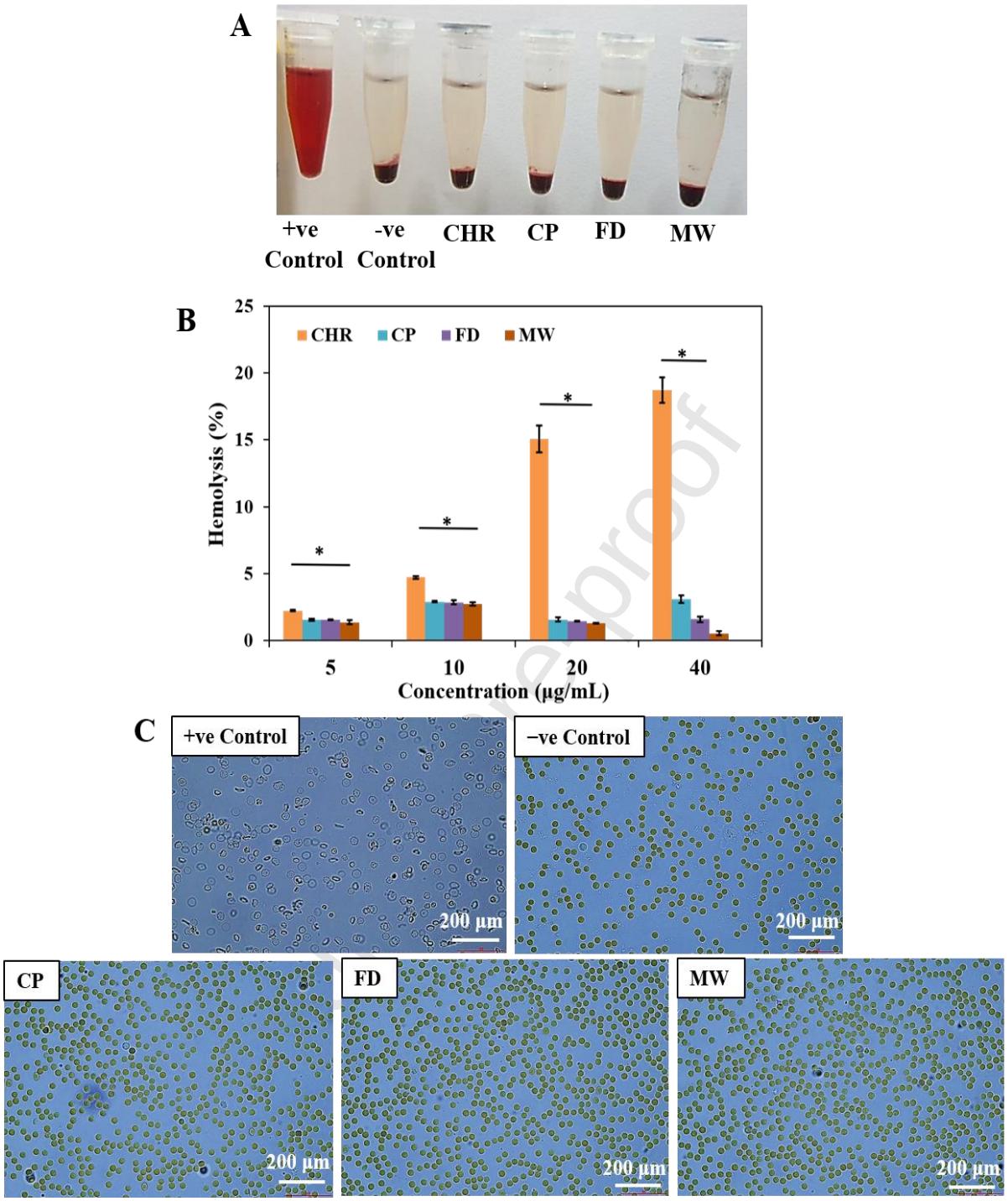

Fig. 8. (A) Digital photographs, (B) hemolysis rate and (C) morphology of red blood cells treated with deionized water (+ve control), PBS (−ve control), chrysin (CHR), co-precipitated (CP), freeze-dried (FD) and microwaved (MW) products. Data are presented as mean ± SD (n=3, *p < 0.001 with respect to CHR).

3.8. *In vitro* chemosensitivity estimation

3.8.1. Intracellular uptake propensity

CDs are safe excipients for various pharmaceutical applications. CDs based targeted and intelligent delivery systems have demonstrated terrific potential in cancer treatment [41]. Inclusion complexation of CDs with flavonoids have further resulted in better pharmaceutical products with a broad spectrum of applications [42]. It is well established that complexation with CDs offer numerous advantages in terms of solubility, stability and bioavailability of drugs [43, 44]. From our dissolution studies, we found an improvement in the solubility of CHR after complexation with β−CD. Thus, we hypothesized that ICs of CHR in β−CD could promote the cellular uptake of CHR because the frequency of partition into the plasma membrane would eventually increase upon increasing the solubility of CHR in aqueous medium. In this regard, the uptake efficacy of CHR by MCF-7 cells, the human breast carcinoma cells, after 12 h treatment was determined quantitatively in a fluorescence microplate system.

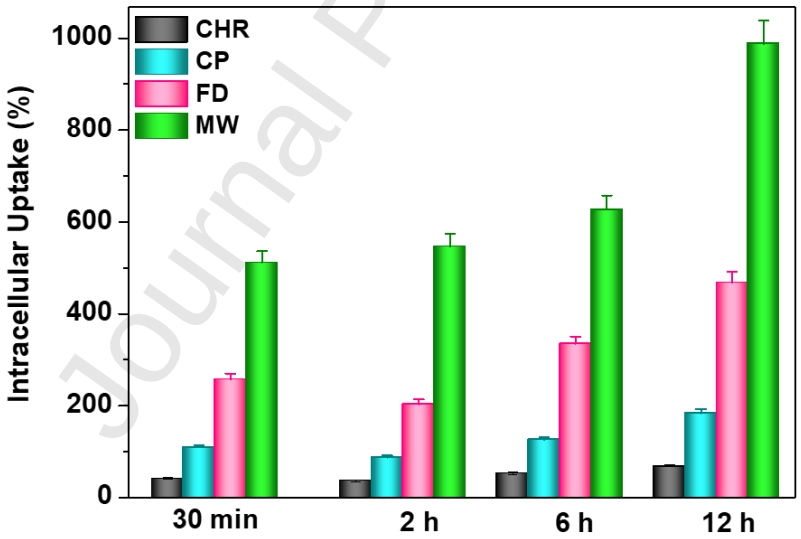

Fig. 9. Intracellular uptake capacity of chrysin (CHR) in MCF-7 cells treated with chrysin (CHR), co-precipitated (CP), freeze-dried (FD) and microwaved (MW) products for 12 h. Data are presented as mean ± SD (n=3).

As evident from Fig. 9, treatment of MCF-7 cells with pristine CHR did not induce any significant change in the intracellular uptake capacity of CHR even after 12 h indicating that the cellular uptake of CHR was minimal. However, complexation of CHR with β-CD

greatly improved the intracellular uptake capacity of CHR. In particular, a pronounced effect was witnessed for the MW inclusion product. This result could likely be explained by the enhanced solubilization of MW in comparison to CP and FD. Therefore, it was inferred that microwave-assisted inclusion complexation of CHR with β-CD might be beneficial to increase the cellular uptake efficacy of CHR in MCF-7 cells.

3.8.2. Cytotoxicity assay

Till date, CHR remains one of the potent Breast Cancer Resistant Protein (BCRP) inhibitors [9]. Thus, to further expand the applicability of ICs of CHR in cancer treatment, we have evaluated their cytotoxicity in human breast cancer cells (MCF-7 cells) by MTT assay. The cells were incubated with equivalent doses of 5 μg/ mL, 10 μg/ mL, 20 μg/ mL and 40 μg/mL CHR for a period of 24 h. Fig. 10 depicts the cytotoxicity profile of MCF-7 cells after treatment with CHR and the ICs. At all concentrations, a remarkable reduction in cellular viability of MCF-7 cells was observed in a dose-dependent manner by free as well as complexed CHR.

The cytotoxicity effect of CHR and the ICs on the MCF-7 cells have also been calculated in terms of inhibitory concentration at 50% (IC_{50}). Pristine CHR was observed to have an IC_{50} at 37.54 μg/ mL for 24 h treatment time. On the other hand, the IC_{50} values were evaluated to be 24.90 μg/ mL, 24.33 μg/ mL and 21.31 μg/ mL for CP, FD and MW products respectively for the same treatment time. This observation could be rationalized by the enhanced CHR content in MW thereby increasing its anticancer activity. Taken together, these results suggest that MW effectively inhibits the growth of MCF-7 cancer cells after short-term treatment.

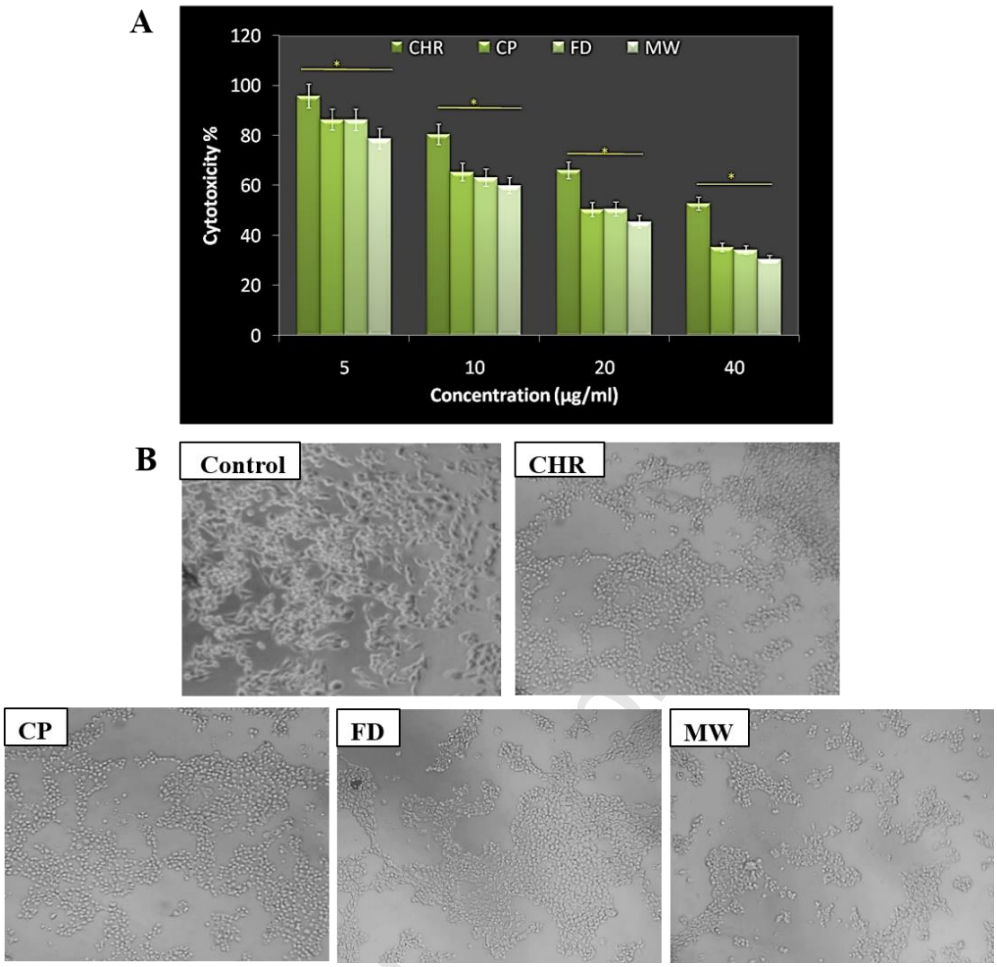

Fig. 10. (A) Cytotoxicity profile and (B) morphological changes of MCF-7 cells after treatment with chrysin (CHR), co-precipitated (CP), freeze-dried (FD) and microwaved (MW) products. Data are presented as mean ± SD (n=3, *p < 0.05).

Digital images (Fig. 10B) recorded form an inverted microscope at the highest drug concentration clearly demonstrated prominent growth inhibition and cell shrinkage of MW in comparison to pure CHR. The cytotoxic evaluations are in consistent with our previous findings from cellular uptake experiments. Thus, the chemosensitivity evaluation of CHR/ β−CD ICs were clearly indicative of MW as a superior drug formulation in cancer treatment.

4. Conclusion

In order to enhance the aqueous solubility and bioavailability of the potent flavonoid CHR; solid ICs of CHR in β−CD were accomplished by diverse approaches. The formation of true ICs by the co-precipitation, freeze drying, and microwave irradiation methods was

ascertained from various spectroscopic analyses. Inclusion phenomenon of CHR into β−CD cavity was also affirmed from molecular docking studies. Molecular dynamic simulation analyses revealed the high stability of the CHR/ β−CD complex. The bioavailability of CHR was greatly enhanced upon complexation. *In vitro* hemolytic assessment demonstrated the decrease in toxicity of CHR when ICs were employed. More importantly; the chemosensitivity of CHR was also increased post-complexation. The microwave irradiation method, which is environmentally more benign, was found to be the optimum wherein the MW inclusion product exhibited better characteristics in terms of drug content, dissolution, hemocompatibility, intracellular uptake and cytotoxicity. Thus, our findings unambiguously validate the efficacy of MW as a superior drug formulation for pharmaceutical applications of CHR.

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Notes

The authors declare no conflicts of interest.

Ethical Statement

All experiments have been conducted in accordance with the Guidelines of Indian Council of Medical Research (ICMR) for biomedical research on human participants. Experiments were approved by the human ethical committee at Ravenshaw University, Cuttack, Odisha, India. An informed consent was obtained from a human volunteer for this study.

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Microwave-Assisted β−Cyclodextrin/Chrysin Inclusion Complexation: An Economical and Green Strategy for Enhanced Hemocompatibility and Chemosensitivity *In Vitro*

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Conflicts of Interest

The authors declare no conflicts of interest.

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Soumya R. Jena: Conceptualization, Investigation

Jasmine Nayak: Conceptualization, Investigation

Usharani Subuddhi: Conceptualization, Resources, Writing-Review and editing, Supervision

Graphical Abstract

Microwave-Assisted β−Cyclodextrin/Chrysin Inclusion Complexation: An Economical and Green Strategy for Enhanced Hemocompatibility and Chemosensitivity *In Vitro*

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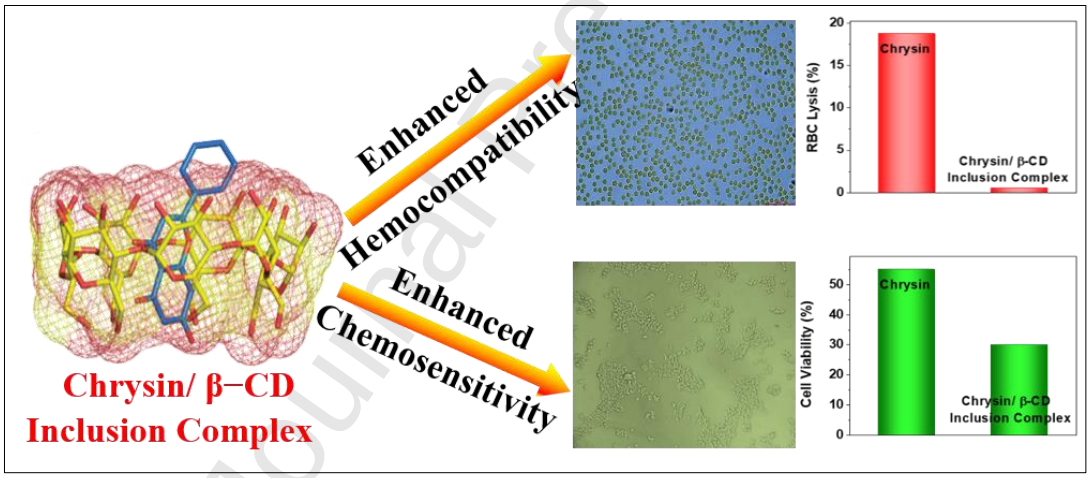

Highlights

- 1:1 solid inclusion complex (IC) of chrysin with β-cyclodextrin prepared
- Microwave irradiation method is time-saving and environmentally benign
- Microwaved IC has highest drug content, solubility and hemocompatibility
- Microwaved IC displayed superior chemosensitivity against MCF-7 cells