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NMR (^{1}H, ROESY) Spectroscopic and Molecular Modeling Investigations of Supramolecular Complex of β-Cyclodextrin and Curcumin

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Abstract:
In this paper we have investigated the solubility enhancement of curcumin through inclusion complexation by β-cyclodextrin as well as the topology and geometry of interaction between curcumin and carrier. For this purpose, phase solubility of curcumin was assessed using Higuchi and Connors method and the inclusion complex was characterized by 1D ^{1}H and 2D ROESY NMR analysis and finally were confirmed by molecular modeling. The phase solubility diagram demonstrated the A_L-type which confirms an increase in curcumin solubility by increasing the concentration of β-Cyclodextrin. ^{1}H NMR and ROESY spectra results showed a cross-peak between H-3 proton of β-Cyclodextrin and the aromatic rings group of curcumin. This revealed the hydrophobic interactions between aromatic rings of curcumin and the cavity of β-Cyclodextrin. Finally, the enthalpy of formation was obtained from molecular modeling results which in turn indicated that the process is exothermic and low-energy interactions are involved in the inclusion complex formation.

Keywords: β-Cyclodextrin, Curcumin, NMR, ROESY, Molecular Modeling

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1. Introduction

Curcumin (CUR) or bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5dione (Fig. 1a), an orange polyphenol found in turmeric, is a very valuable medicinal plant with health benefits including anti-inflammation (Julie & Jurenka, 2009), anti-microbial (De, Kundu, Swarnakar, Ramamurthy, Chowdhury, Nair, et al., 2009), anti-oxidant (Menon & Sudheer, 2007) and anti-cancer (Yallapu, Jaggi, & Chauhan, 2010) activity. It has been revealed that curcumin, an Indian traditional dietary ingredient, is responsible for the reduction of colon, lung and prostate cancer in India (Dahmke, Boettcher, Groh, & Mahlknecht, 2014). Clinical data have shown that curcumin inhibits the accumulation of triglycerides in rat liver (Shoji, Nakagawa, Watanabe, Tsuduki, Yamada, Kuwahara, et al., 2014).

Unfortunately, apart from the benefits there are some drawbacks such as low solubility in water and intolerance to acidic or basic conditions attributed to the CUR (Paramera, Konteles, & Karathanos, 2011). These shortcomings prevent commercial applications of CUR. To overcome these drawbacks, encapsulation, a technique that has recently been used to reduce or eliminate the restrictions on a drug in the pharmaceutical industry, has been proposed (López-Tobar, Blanch, Ruiz del Castillo, & Sánchez-Cortés, 2012). Several studies have been conducetd on CUR solubility enhancement through encapsulation of drug in different carriers (Bisht, Feldmann, Soni, Ravi, Karikar, Maitra, et al., 2007; Y.-M. Tsai, Chien, Lin, & Tsai, 2011; Wang, Leung, Kee, & English, 2009). These carriers include nanoparticles, nanocapsules, liposomes and various derivatives of cyclodextrins (CD). Among all mentioned carriers, CDs are the ones which show about 100 fold increase in the solubility of CUR and eliminate the limitations of therapeutic properties (Dandawate, Vyas, Ahmad, Banerjee, Deshpande, Swamy, et al., 2012). Previous studies have shown that drug complexation with cyclodextrin not only improves the drug solubility and stability but also could increase its pharmaceutical efficacy (Yallapu, Jaggi, & Chauhan, 2010).

CDs are doughnut-shaped oligosaccharides (Rafati, Hashemianzadeh, Nojini, & Safarpour, 2007) consisting of 6(α-), 7(β-) or 8(γ-CD) D-(+)-glucopyranose units linked by α-(1,4) bounds with a hydrophobic cavity that is capable of forming inclusion complex with hydrophobic guests of suitable size and shape and an outer hydrophilic surface (Fig. 1b) (Alexandrino, Calderini, Morgon, & Pessine, 2013; Michel, Chitanda, Balogh, Yang, Singh, Das, et al., 2012; Veiga, Fernandes, Carvalho, & Geraldes, 2001; Yadav, Prasad, Kannappan, Ravindran, Chaturvedi, Vaahtera, et al., 2010; Zarrabi, Adeli, Vossoughi, & Shokrgozar, 2011). β-cyclodextrin (β-

CD) has the ability to form inclusion complexes with CUR resulting in significant improvement in the solubility and stability of guest molecule (Y. Tsai, Tsai, Wu, & Tsai, 2010; Yallapu, Jaggi, & Chauhan, 2010).

The research's main focus is on the confirmation of inclusion complex formation by using different characterization techniques. NMR is one of the most powerful techniques suitable for studying inclusion complex formation in solution phase (Pessine, Calderini, & Alexandrino; Upadhyay & Ali, 2009). β-CD molecule consists of H-1, H-2, H-3, H-4 and H-5 protons that as shown in Fig. 1b, H-3 and H-5 are located inside the cavity. If the guest molecule is entered inside the cavity close to the mentioned protons, the relevant signals would be slightly displaced in ^{1}H NMR (Jiang, Zhang, Shi, & Jia, 2011; Leyva, Moctezuma, Strouse, & García-Garibay, 2001). Besides ^{1}H NMR, 2D ROESY NMR could also give useful information on the parts of the guest molecule that has interaction with the CD cavity (Upadhyay & Ali, 2009) since the NOE cross peaks are observed between the protons that are closer than 0.4 nm in spaces in the ROESY spectrum (Veiga, Fernandes, Carvalho, & Geraldes, 2001).

In this work, CUR solubility enhancement and topology of the interaction between CUR and β-CD is investigated through NMR (^{1}H and ROESY) analysis confirmed by molecular modeling. Although solubility studies could confirm the inclusion complex formation, the exact geometry of the complex system could not be deduced. To the best of our knowledge, 2D NMR and molecular modeling of such supramolecular complex is reported for the first time in this paper. The possible structure of CUR and β-CD inclusion complex in aqueous solution was investigated along with thermodynamic parameter such as Gibbs free energy (ΔG) and enthalpy of formation (ΔH).

2. Materials and methods

2.1. Materials

β-Cyclodextrin (β-CD, 98%, Sigma Aldrich) and Curcumin (CUR, >99%, Sigma Aldrich) were used as received. All solvents (Acetone and DMSO-d_6) were of high purity and were obtained from Merck Company (Germany).

2.2. Preparation of β-CD:CUR inclusion complex

40 mg β-CD and 12 mg CUR were dissolved in 8mL deionized water and 0.5mL acetone in two 20mL glass vials (Fisher Scientific, Pittsburgh, PA, USA), respectively. These solutions were then mixed and put on the

stirrer for 24h without a cap to evaporate the acetone. Then, the solution was centrifuged (1000 rpm , 5min) and the supernatant containing β-CD-CUR inclusion complex was freeze dried (Yallapu, Jaggi, & Chauhan, 2010) (VaCo5, Zirbus, Germany; -50°C, 130×10^{-3} mBar). The β-CD-CUR inclusion complex was stored at 4 °C until further use.

2.3. Determination of CUR loading

In order to determine the loading efficiency, β-CD-CUR inclusion complex (1 mg) was dissolved in DMSO (50 ml). The sample was then placed on a stirrer for 24 hours at room temperature in the dark to extract the CUR in DMSO. The sample was then centrifuged (14000 rpm) to make a clear supernatant of CUR in DMSO and remove clumps of β-CD. The amount of loaded CUR in the inclusion complex was then measured using the standard plot of CUR in DMSO and UV-Vis spectrophotometry (Biochrom Biowave).

2.4. Phase solubility studies

The phase solubility studies were performed using Higuchi and Connors method (Higuchi & Connors, 1965). Briefly, different concentrations of β-CD (0, 2, 6, 8, 10 and 20 mM) were prepared in deionized water in glass capped vials. To each vial 1 mg CUR was added. The vials were then sealed and placed in an oscillating water bath with a temperature of 30 ±2 °C until the equilibrium was reached. Samples were centrifuged after 72 hours (1500 rpm, 7min) to remove the unsolved CUR and then filtered through 0.22 µm membrane filters (Millipore). Then, the absorption of dissolved CUR by UV-Vis spectrophotometry was recorded at 425 nm. The phase solubility diagram was plotted according to the concentration of dissolved CUR vs. concentration of β-CD. The stability constant was also calculated from Eq. (1):

$$K_S = \frac{slope}{S_0(1-slope)} \quad (1)$$

Where, S_0 is the concentration of CUR in the absence of β-CD. The slope in the formula is the slope of the phase solubility diagram.

2.5. NMR investigations

All H-NMR, and H-H 2D ROESY NMR analysis were recorded for three samples β-CD, CUR and the inclusion complex (1:1 molar β-CD:CUR) dissolved in DMSO-d_6 at 298 K using Bruker Ultrasheet-400 MHz Spectrometer. The DMSO resonance at 2.48 ppm was used as internal reference to report chemical shift values. Chemical shift changes (Δδ) were calculated according to Eq. (2):

$$\Delta\delta = \delta_{(\text{complex})} - \delta_{(\text{free})} \quad (2)$$

A rotational overhauser enhancement experiment (ROESY) for detection of intermolecular nuclear overhauser effects (NOEs) between β-CD and CUR was conducted for the inclusion complex (1:1 molar β-CD:CUR) at 298 K. The 2D ROESY spectrum was collected with mixing time of 500 ms under the spin lock condition.

2.6. Molecular modeling studies

Molecular modeling is the sum of theoretical methods and computational techniques that is used to predict molecular behaviours specifically interactions between molecules. In this method the preferred orientation for molecules to bond to each other is calculated, which is used to form a stable complex. This preferred orientation is found by comparing strength of associations or binding affinities. Molecular modeling of the inclusion complex of β-CD and CUR was conducted to find probable combinations of drug and host molecule. At first 3D model of two β-CDs and one CUR were designed by Hyperchem software. To run the docking calculation Hex Server was used. CUR was considered as ligand and β-CDs as receptors. Their geometry was optimized based on thermodynamically stable conformations. The complex enthalpy of formation was calculated with Hyperchem software and based on Eq. (3).

$$\Delta H = \Delta H_{f\text{complex}} - \left(\Delta H_{f\text{CUR}} + 2\Delta H_{f\beta-\text{CD}}\right) \quad (3)$$

3. Results and discussion

3.1. Phase solubility studies

The phase solubility diagram based on Higuchi and Connors method is shown in Fig. 2. It demonstrates that CUR solubility increased linearly with increasing β-CD concentration with a slope of less than unit. Figure 2 represents an

A_L-type diagram stoichiometric ratio of 1:1. An A_L-type phase solubility diagram is obtained when inclusion complex is first order with respect to ligand and first or higher order with respect to the substrate. The amount of stability constant, $K_s(K_{1:1})$ is estimated through phase solubility diagram.

3.2. NMR investigations

Compared to other techniques such as DSC and FTIR, ^{1}H-NMR is the most powerful mean to study the inclusion complexes. The NMR spectra give an obvious distinction between inclusion complexes and other possible interactions. When the guest molecule is placed inside the β-CD cavity, the internal protons of β-CD (H-3, H-5) are involved in a chemical shift change. Fig. 3 shows the ^{1}H-NMR results for β-CD, CUR and their inclusion complex. The comparison of Fig. 3A and 3B reveals the chemical shift of H-3 and H-5 protons of β-CD in presence of curcumin. Fig. 3C and 3D also show that after inclusion complex formation, the specific peaks of drug between 6.50 to 7.60 ppm (ascribed to aromatic rings) are completely eliminated which confirms the internalization of aromatic rings of CUR inside β-CD cavity.

Table 1 shows the chemical shift changes of β-CD and its inclusion complex with CUR based on ^{1}H-NMR investigations. According to the table, all the β-CD peaks exist in inclusion complex spectrum but due to inclusion of drug their signals are shifted. It is shown that the chemical shift changes for H-3 and H-5 protons are higher than other protons of β-CD, which declare the formation of inclusion complex. The slight marginal shift of protons H-2 and H-4 suggest that CUR has interactions only with the internal hole of β-CD and side interactions are not involved in solubility enhancement of CUR. The resonance corresponding to the anomeric proton of glucose in the β-CD-CUR complex was shifted downfield from the corresponding resonance in free β-CD. This downfield displacement indicates that the anomeric proton of β-CD is close to an electronegative atom. It is clear that the methoxy groups (OCH3) of a CUR molecule are strong electron withdrawing groups because of the high electronegativity of the oxygen atoms. This effect supports our conclusion that the CUR aromatic rings are indeed complexed inside the β-CD cavity. Moreover, the relatively large shift of the anomeric proton confirms that CUR molecule enters from the wider end of the CD where the secondary hydroxyls are located.

2D ROESY NMR is one of the most effective techniques to study the internal interactions of host-guests especially those of inclusion complexes. The ^{1}H-NMR results which indirectly merely shows the presence of the

guest molecule inside β-CD cavity, is verified and confirmed by the 2D ROESY techniques. In fact the cross-peak caused by NOE between the protons of the guest molecule and β-CD in less than 4 A^o distances, gives useful information on the structure of the inclusion complex.

Fig. 4a shows the 2D ROESY pattern for CUR-β-CD inclusion complex. By showing the chemical shift change in the internal protons (H-3 and H-5), the ^{1}H-NMR results prove the formation of complex. Fig. 4a shows a strong cross-peak formed between H-3 proton of β-CD and the aromatic ring group of protons (H-9', H-12' and H-13') from CUR. This cross-peak is demonstrated by a green arrow. This suggests that the formation of inclusion complex is from the hydrophobic aromatic ring side of the CUR which in turn sheds light on the mechanism by which β-CD enhances the aqueous solubility of drug.

According to the results of ^{1}H-NMR and 2D ROESY, a model similar to one demonstrated in Fig. 4b can be proposed for the topology of the inclusion complex of CUR and β-CD. Of course this model is for the state when a single molecule of CUR forms a complex with one molecule of β-CD but it is obvious that CUR enters the β-CD cavity from both its aromatic ring.

3.3. Molecular modeling results

Molecular modeling studies are usually correlated with NMR studies since they demonstrate a supplementary method to make experimental information rationalized. In the present study, the molecular modeling was conducted to determine a reasonable topology for the inclusion complex and to correlate these findings with the NMR results. The distances between CUR and β-CD protons were calculated using molecular modeling results. By performing docking studies of CUR in β-CD cavity and the result of NMR, it was observed that CUR interacts with β-CD in 1:2 ratio. Also, it was shown that in a stable complex two aromatic rings of CUR interact with two β-CDs through hydrogen bonds. It shows that the shortest distance between CUR and H-3 and H-5 are 1.78489 and 3.51773 A^o for one side and 1.90202 and 2.59 A^o for the other. It is consistent with the result of 2D NMR. The enthalpy of formation for this complex is -15.8961 $^{kJ}/_{mol}$ based on molecular modeling calculations. Negative enthalpy shows that the complex formation process has been exothermic. Low enthalpy value justifies the involvement of low-energy interactions such as displacement of water molecules from the cavity of β-CD Due to the

high concentration of curcumin molecules, hydrophobic-hydrophobic interaction and formation of hydrogen bonds (Karathanos, Mourtzinos, Yannakopoulou, & Andrikopoulos, 2007; Tommasini, Raneri, Ficarra, Calabrò, Stancanelli, & Ficarra, 2004). According to the model (Fig. 5), a CUR sticks to a β-CD from each side with hydrogen bonds. This finding is in good accordance with NMR results.

Conclusion

The inclusion complex formation between CUR and β-CD was studied by phase solubility diagram, ^{1}H and 2D ROESY NMR experiments and molecular modeling. It was shown that the CUR solubility increased linearly with increasing β-CD concentration. The stoichiometry of 1:1 or 1:2 of the inclusion complex was determined by phase solubility diagram as well as the association constant of complexes, 167 M^{-1}. According to the ^{1}H NMR spectra, chemical shift of interior protons of β-CD (H-3 and H-5) have been changed after inclusion complexation which indicated that the aromatic rings of the CUR have entered into the β-CD cavity. 2D ROESY NMR showed a cross-peak between H-3 proton of β-CD and the aromatic rings of curcumin, which confirmed the results of ^{1}H NMR and clarified the mechanism by which β-CD enhances the aqueous solubility of CUR. Finally, the possible structure of β-CD:CUR inclusion complex was estimated by molecular modeling showing that it is the hydrophobic aromatic rings of curcumin that have been covered by the cavity of β-CD. This study proves that NMR spectroscopy is a useful tool for studying inclusion complexes of CDs with water insoluble drugs.

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Figure Captions:

Fig. 1. Chemical structure of (a) Curcumin; (b) β-Cyclodextrin

Fig. 2. Phase solubility diagram

Fig. 3. Proton nuclear magnetic resonance (^{1}H-NMR) spectra of β-CD in the absence and presence of CUR in DMSO-d_6 at 25 ◦C. (A): βCD, (B): Inclusion complex of βCD and CUR, (C): CUR and (D): Full spectra of inclusion complex of βCD and CUR.

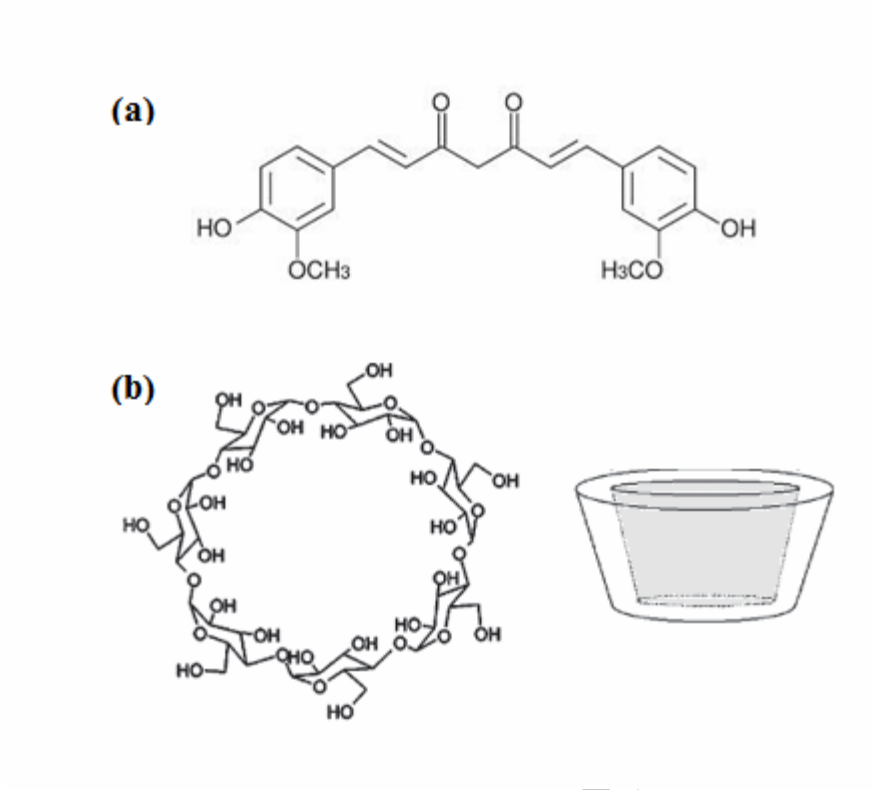
Fig. 4. (a) Rotating overhauser effect spectroscopy (ROESY) pattern in DMSO-d_6 at 25 ◦C of the inclusion complex of β-CD and CUR. The cross-peaks have shown the interaction between aromatic ring of CUR and H3 proton inside the cavity of β-CD; (b) Possible topology of CUR-β-CD inclusion complex in aqueous solution

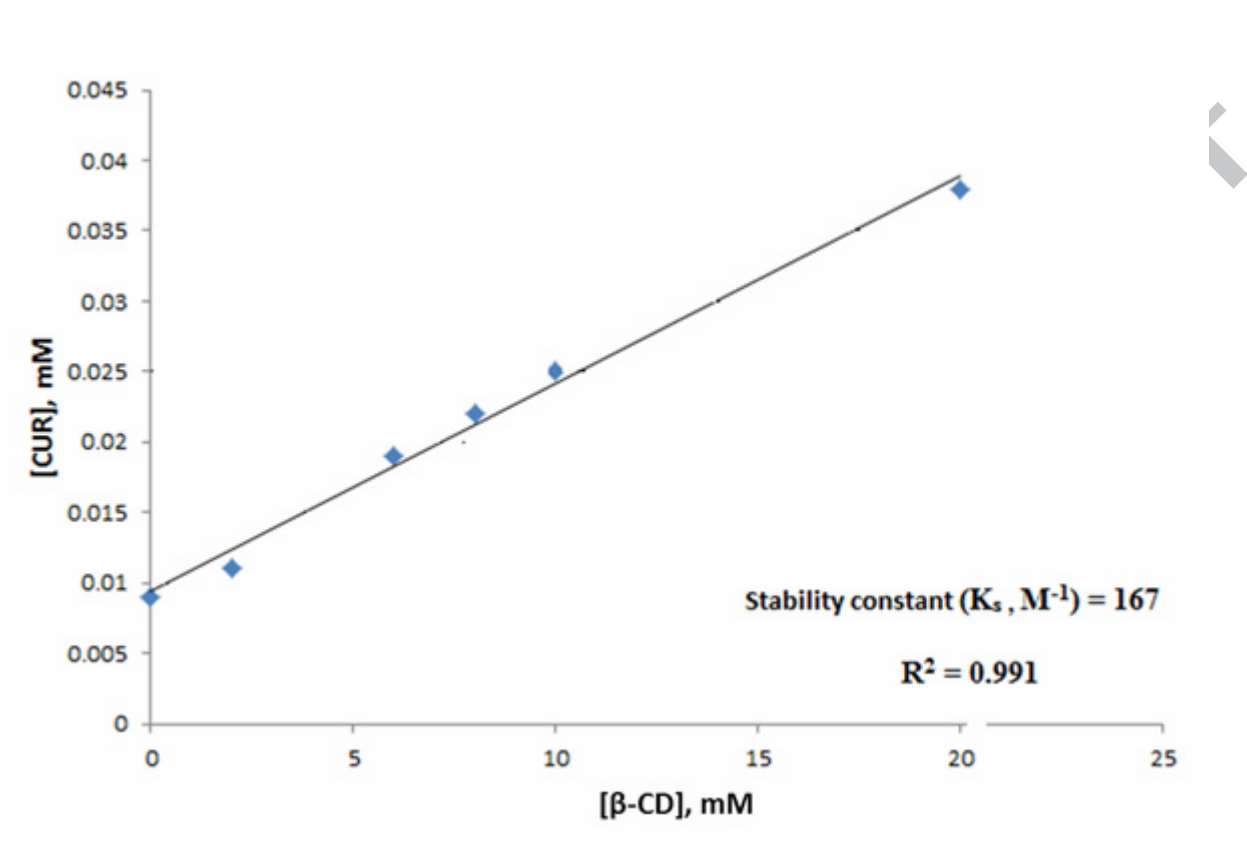
Fig. 5. (a): 3D structure that shows hydrogen bonds between CUR and β-CD, (b): 2D structure of CUR that shows hydrogen donors and acceptors. Different sides of view of complex of two β-CD and one CUR (c) from side and (d) from top.

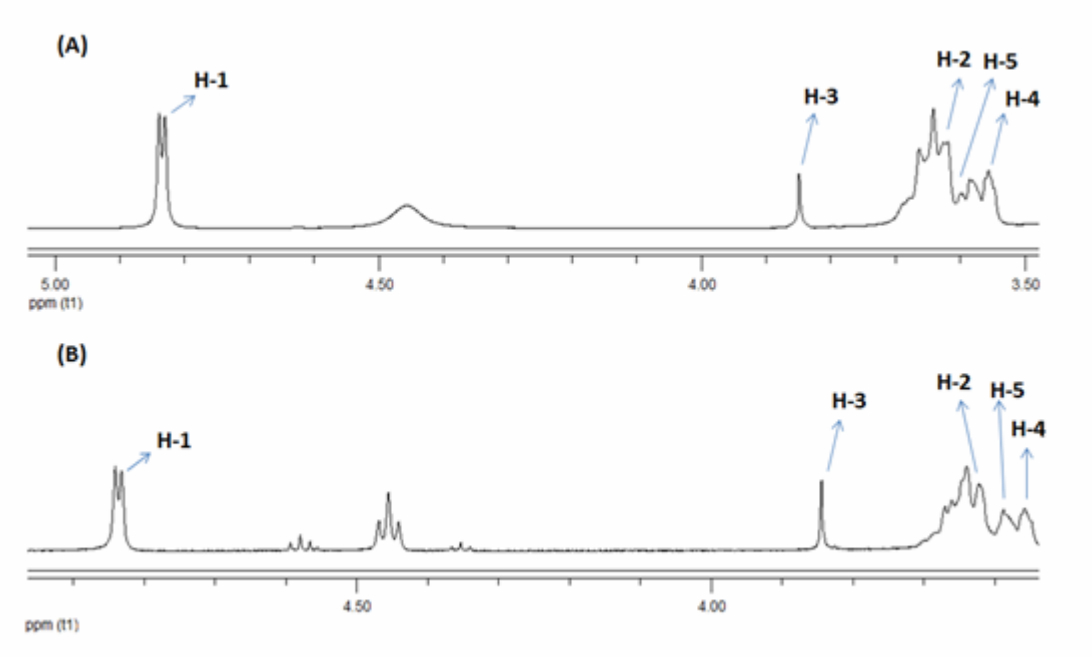
Table 1

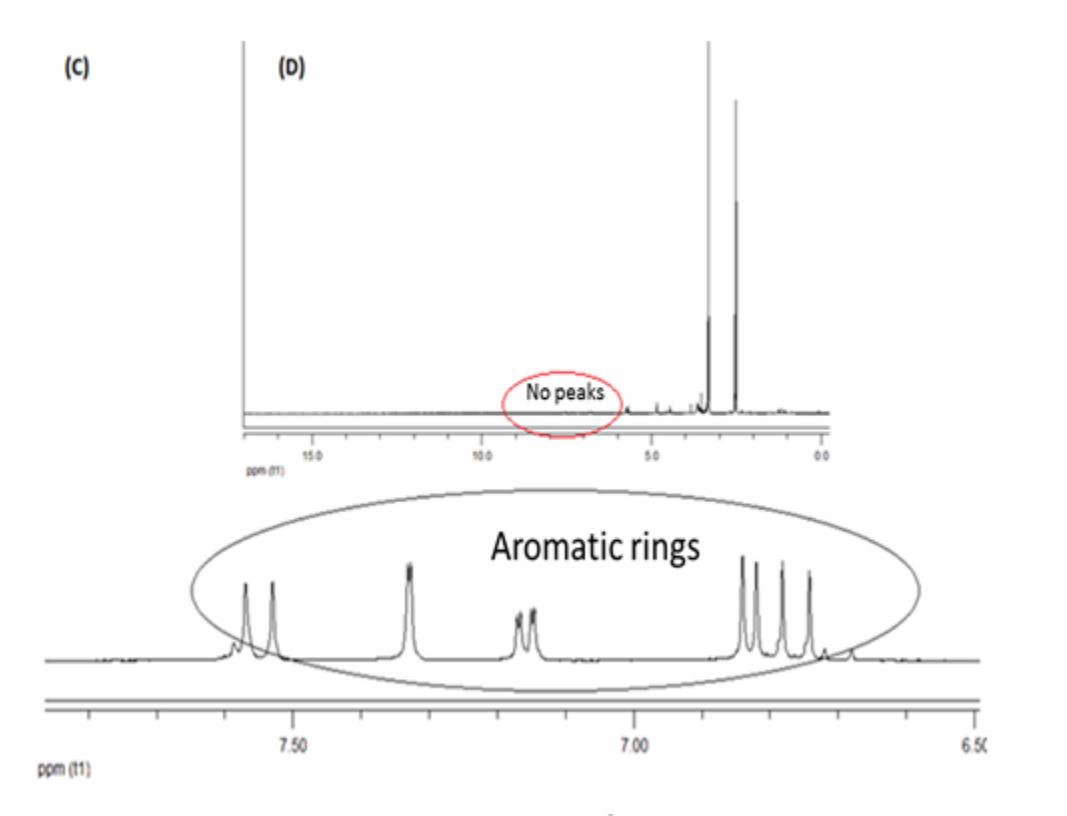
^{1}H-chemical shifts (δ) corresponding to β-CD in the presence and absence of CUR

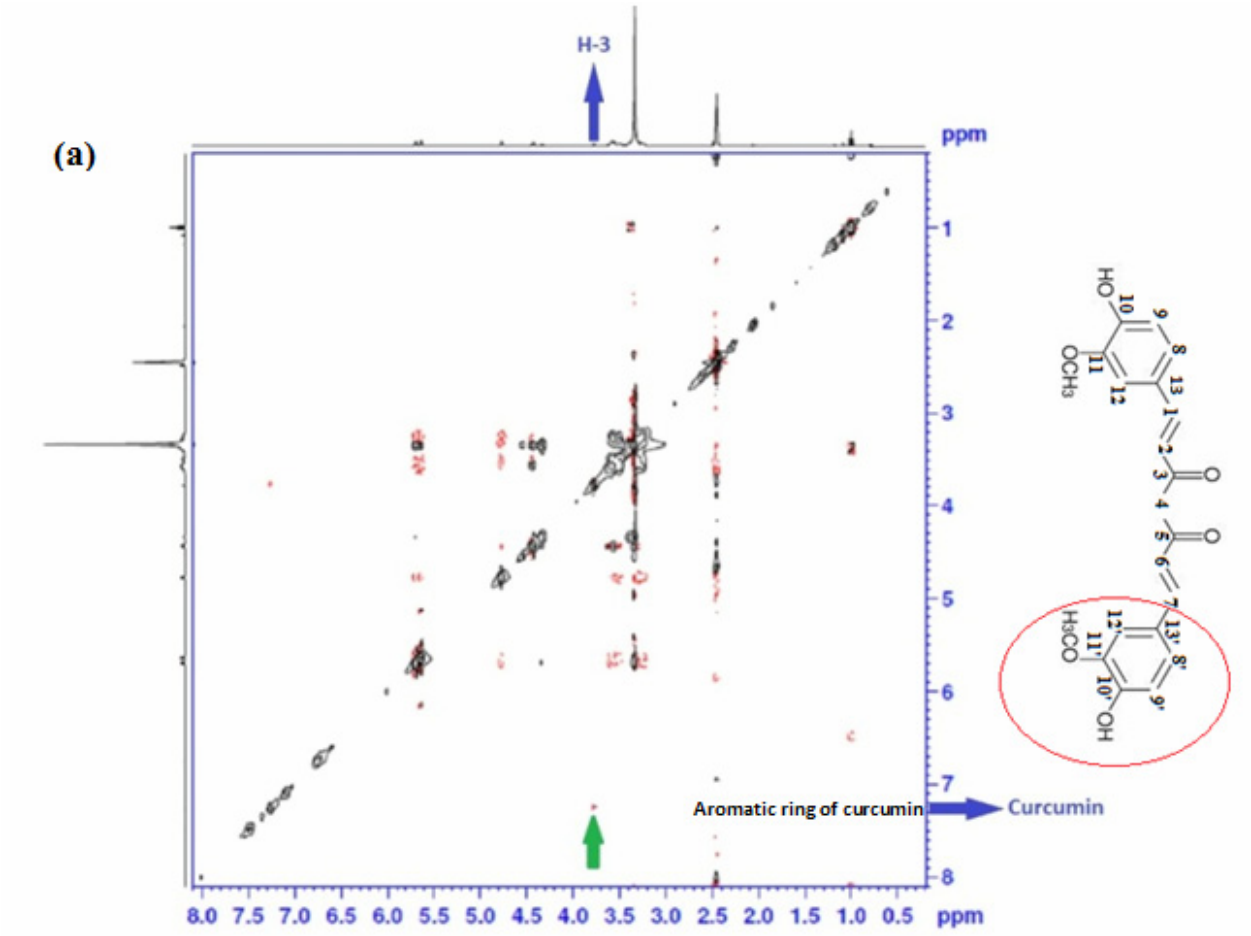
β-CD protons	δ_{free} (ppm)	$\delta_{Complex}$ (ppm)	$\Delta\delta$ (ppm)
H_1	4.830	4.841	0.011
H_2	3.625	3.622	-0.003
H_3	3.830	3.844	0.011
H_4	3.556	3.550	-0.006
H_5	3.599	3.601	0.011
2-OH	5.723	5.723	0
6-OH	4.457	4.455	-0.002



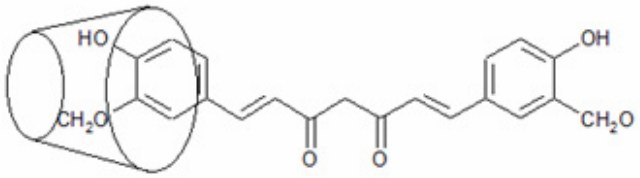







(a)
H-3
ppm
Aromatic ring of curcumin
Curcumin
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
HO
OCH3
H3CO
OH

(b)


HO
CH2O
OH
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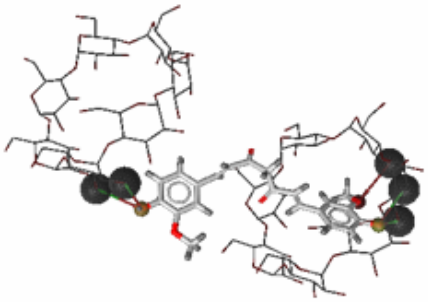
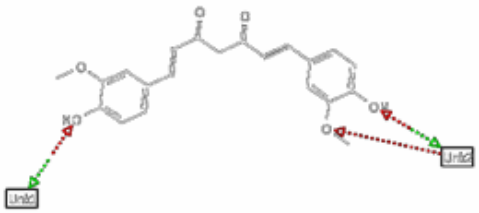
O
O

a



b



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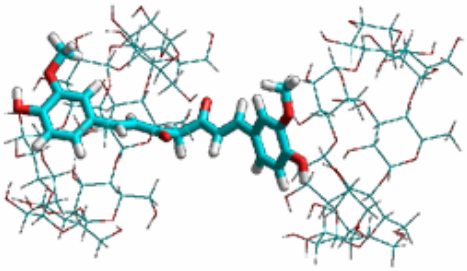

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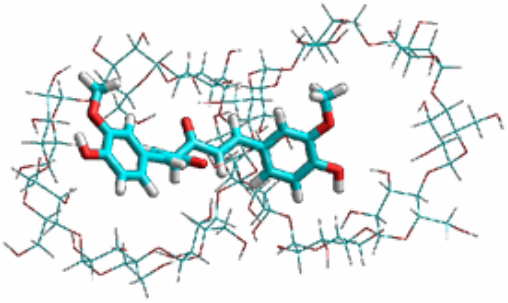

Highlights

- Curcumin as Nutraceutical is insoluble in water
- Cyclodextrins are capable of enhancing curcumin aqueous solubility
- Curcumin solubility increased linearly with increasing β-CD concentration
- Shielding aromatic groups of the drug is responsible for solubility enhancement
- The stoichiometry of 1:1 or 1:2 of the inclusion complex was determined