Research Article

Structural Characterization of Inclusion Complex of Stigmasterol with Alpha-Cyclodextrin using Spectroscopy and Molecular Modeling

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Abstract

Background: Stigmasterol possesses numerous physiological effects and is used as food supplements and behaves as a pharmaceutical agent. It exhibits anticancer effects against various cancers. The usefulness of the stigmasterol is restricted due to its poor solubility. To overcome this and enhance the solubility and bioavailability of this phytosterol, molecular encapsulation is utilized to augment the desirable properties of stigmasterol. **Aim:** This research work aims to investigate the interaction between stigmasterol and alpha-cyclodextrin (α-CD) in aqueous solution as well as in solid state and experimentally examined by spectral techniques. **Methods:** The liquid complexes are characterized by ultraviolet (UV)-visible spectroscopy and solid inclusion complexes are characterized by Fourier transformer infrared resonance and ^{1}H nuclear magnetic resonance spectroscopy. The thermal behavior of the complex is analyzed by differential scanning calorimeter. Phase solubility studies are done to learn the solubility of the newly synthesized complex. **Results:** Formation constant from UV-visible analysis is found to be 569 M^{-1} by Benesi–Hildebrand equation. The solubility constant is calculated to be 52 M^{-1}. The results obtained prove the inclusion which is confirmed through molecular docking studies. **Conclusion:** The newly synthesized inclusion complex is a potent pharmaceutical agent in drug formulation as stigmasterol solubility is enhanced when included in the cavity of α-CD.

Keywords: Alpha-cyclodextrin, formation constant, inclusion complex, stigmasterol

Introduction

Stigmasterol, known as stigmasterin found in various medicinal plants, is an unsaturated phytosterol resembling cholesterol in both structure and function. The molecule constitutes a rigid tetracyclic backbone (6-6-6-5) with one secondary hydroxyl group at one end and one C_{10} branched hydrocarbon chain at the other end.[1] It is a secondary metabolite used in health-enhancing constituents of natural food.[2] According to Song *et al*.,[3] stigmasterol possesses pharmacological properties such as cytotoxicity, antioxidant, anti-inflammatory, antimutagenic, hypoglycemic, antiosteoarthritic, and antitumor activity. Despite a wide range of potential attractiveness, stigmasterol is poorly used by the pharmaceutical industry due to its low solubility, high melting point, and chalky taste.[4] To overcome this problem, stigmasterol may be complexed with different compounds, which would enhance their physicochemical properties.[5] One such is to form an inclusion complex with alpha-cyclodextrin (α-CD). CDs are water-soluble, nonreducing, and macrocyclic oligosaccharides that have glucose units formed by an α-1,4 linkage with a lipophilic central cavity and a hydrophilic outer surface.[6] CDs enhance the delivery of low water-soluble and chemically unstable drugs to the body through biological membranes by improving the bioavailability of drug molecules. In this research, we evaluate the interaction between stigmasterol and

Access this article online

Quick Response Code:

Website: www.bmbtrj.org

DOI: 10.4103/bbrj.bbrj_228_21

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How to cite this article: Francy K, Kumari JP, Roselet SL. Structural characterization of inclusion complex of stigmasterol with alpha-cyclodextrin using spectroscopy and molecular modeling. Biomed Biotechnol Res J 2021;XX:XX-XX.

Submitted: 13-Sep-2021; **Revised:** 23-sep-2021;
Accepted: 23-Sep-2021; **Published:** ***

α-CD in the solid and liquid state and assess their potentiality in drug delivery system.

Materials and Methods

Chemicals

Stigmasterol and ethanol were purchased from Himedia (India). α-CD was purchased from Sigma Aldrich. Both were used as purchased. Double-distilled water was used.

Preparation of solid inclusion complex of stigmasterol and alpha-cyclodextrin

About 0.1238 g of stigmasterol was accurately weighed and dissolved in 30 ml ethanol. About 0.2919 g of α-CD was dissolved separately in 30 ml double-distilled water. Both the solutions were mixed in the beaker and put over an electromagnetic stirrer to stir continuously for 48 h at room temperature. The precipitate obtained after evaporation was dried and used for characterization.[7]

Preparation of liquid inclusion complexes

About 0.1650 g of stigmasterol was dissolved in 10 ml of ethanol and about 0.2918 g of α-CD was dissolved in 30 ml double-distilled water in a beaker. Liquid inclusion complexes were synthesized by varying the concentration of α-CD from 2×10^{-3} M to 1×10^{-3} M. The reactions were carried out at room temperature.[8]

Characterization techniques

Ultraviolet visible spectroscopy

Absorbance values were recorded for the liquid inclusion complexes using an ultraviolet (UV)-1800, (Shimadzu) spectrophotometer.[9]

Fourier transform infrared study of inclusion complexes

Fourier transform infrared (FTIR) for the solid inclusion complexes were recorded by FTIR Schmidz spectrometer by KBr pellet method. The samples were ground gently with anhydrous KBr and compressed to a pellet form. Spectrum was recorded in the range of 400–4000 cm^{-1} at 298 K.[4]

Nuclear magnetic resonance analysis

^{1}H nuclear magnetic resonance (NMR) spectroscopic studies of the solid inclusion complexes were recorded in Bruker 400 MHz FTNMR spectrometer. For all the samples, $CDCl_3$ was used as solvent and tetramethylsilane (TMS) as an internal reference. The chemical shifts were reported in ppm (δ) relative to TMS at 298 K.[10]

Phase solubility studies

Phase solubility studies of stigmasterol with different concentrations of α-CD were determined by the method proposed by Higuchi and Connors at room temperature.[11] The excess amount of stigmasterol was dissolved in 60 ml of ethanol and then added to 10 ml of double-distilled water containing various concentrations of α-CD and taken in stoppered conical flasks, and the mixture was placed on a rotary flask shaker for 72 h, with continuous shaking. The suspensions were filtered through Whatman filter paper. UV-1800 (Shimadzu) spectrophotometer was used at 269–227 nm. The solubility constant (Ks) was calculated from the slope of the linear portion of the phase solubility diagram.[12]

$$K_s = \text{slope}/S_0 (1 - \text{slope})$$

where S_0 is the aqueous solubility of stigmasterol.

Differential scanning calorimetry

Differential scanning calorimetric (DSC) analysis for the solid inclusion complexes was carried out on NETZCH DSC 204 calorimeter. A sample of approximately 1.4 mg was weighed in aluminum pans. These samples were heated over a range of 25°C–300°C at a constant rate of 10°C/min in a nitrogen purge of 50 ml/min. An empty aluminum pan is used as a reference.[6]

Molecular docking

Molecules required for the molecular modeling studies were retrieved from PubChem database and drawn using ChemSketch tool. Before the analysis, molecules were prepared and hydrogen atoms were added by Chimera software. Then, it was converted as pdb format to molecular docking and inclusion. Initially, Patchdock server was utilized to process the docking and reveal the grid values. Autodock Vina was utilized to study the host–guest interaction. The grid values were adjusted and executed for molecular docking between stigmasterol and α-CD. Complex files were analyzed and modeled for major forces by Pymol and Chimera tools.[13]

Results and Discussion

Absorption spectral studies

It is important to characterize the formulation of the inclusion complex in solution state since the administration of the drug is carried out in solution form.[6] The liquid inclusion complex is analyzed by UV-visible spectroscopy.[9] The absorption maxima of stigmasterol in varying concentrations of α-CD

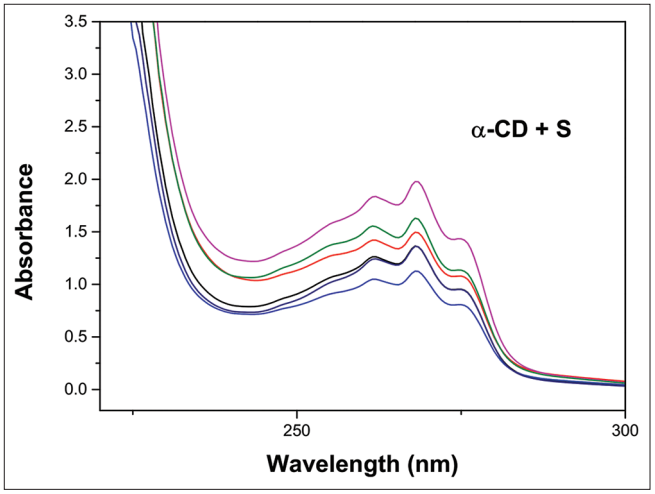

Figure 1: Absorption spectra of stigmasterol at various concentration of alpha-cyclodextrin

are shown in Figure 1. The absorbance and intensities are listed in Table 1. From Figure 1 and Table 1, it is clear that the absorption maxima shift toward a higher wavelength and the intensity of the peaks rises on increasing the concentration of α-CD. The shifting absorption may be due to the fact that complex is stabilized within the cavity of α-CD. The absorption maxima exhibit a redshift in stigmasterol with λ_{abs} Σ262 to λ_{abs} Σ268 nm on varying the concentration of α-CD complexes. The shifting of absorption peak position confirms the complex formation between stigmasterol and α-CD which is in line with the report of Panda and Nayak.[9]

The stoichiometric ratio for the inclusion complex is found to be 1:1. This theory was confirmed by the linear relationship procured from the reciprocal plot of $\frac{1}{A - A0}$ versus $\frac{1}{[\alpha\text{-CD}]}$ based on Benesi–Hilderbrand equation[14] for 1:1 complex as shown in Figure 2.

$$\frac{1}{A - A0} = \frac{1}{A' - A0} + \frac{1}{K[A - A0][\alpha\text{-CD}]}$$

where A_0 is the initial absorption intensity, A′ is the absorption intensity of the stigmasterol α-CD inclusion complex, A is the observed absorption intensity, and K is the formation constant. From the results obtained, the formation constant is found to be 560 M^{-1} for the complex. The high formation constant value indicates the stability of the complex formed.[15]

Fourier transform infrared analysis

FTIR analytical technique discovers the vibrational frequency of chemical bonds, the decrease in intensity or absence of characteristic bands in the compound under investigation. These changes reveal the interaction between the host and the guest in CD complexes. FTIR spectra of α-CD–stigmasterol complex are studied in KBr pelleting to evaluate the host–guest interaction in the inclusion complex. Williams *et al*.[16] employed FTIR to prove the complex formation of HP-α-CD and cholesterol in solution. Figure 3 shows the FTIR spectrum of stigmasterol, α-CD, and inclusion complex. It is important to note that few significant changes occur in the FTIR spectrum of the complex compared to the spectrum of stigmasterol. The bands at 1463 cm^{-1}[17] corresponding to C-H stretching are shifted to a lower wavelength at 1315 cm^{-1}. A week band at 1249 cm^{-1}[18] denoting the presence of $CH_2(CH_3)_2$ is shifted to 1251 cm^{-1}. The aliphatic CH stretching narrow band at 2920 cm^{-1}[17] is shifted to 2933 cm^{-1}. The broad hydroxyl band of free α-CD is found to be narrowed in the complex spectrum. All these changes in the FTIR spectrum prove that the hydrocarbon tail of stigmasterol enters into the cavity of α-CD. The frequency corresponding to sp3-hybridized C-H stretching at 2368, 2366, and 2333 cm^{-1} remains the same in both the guest and the inclusion complex. This shows that cyclohexane rings lie outside the cavity.

The notable changes in IR spectral characteristics may be attributed to the restriction of the compounds for undergoing vibration within α-CD cavity due to the van der Waal forces, weak interaction such as H-bonding, and hydrophobic interactions.[8] This observation serves as proof for the

Table 1: Absorption spectral data of stigmasterol with alpha-cyclodextrin

Concentration of α-CD	λ_{abs}	Absorbance	1/A-A_0	Log ε	1/[α-CD]
0	262	1.0494			
0.002	263.5	1.2430	5.16	4.427	500
0.004	264	1.2633	4.67	4.501	250
0.006	265	1.4208	2.69	4.508	166.6
0.008	266	1.5547	1.97	4.559	125
0.010	268	1.8363	1.27	4.670	100

α-CD: Alpha-cyclodextrin

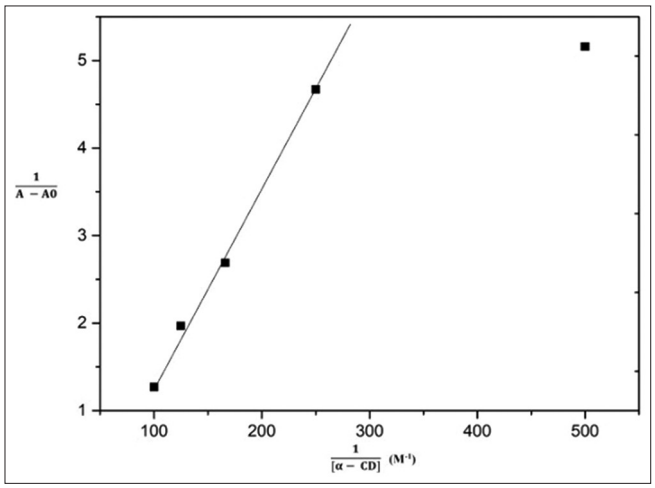

Figure 2: Bensi–Hilderbrand plot of stigmasterol–alpha-cyclodextrin complex

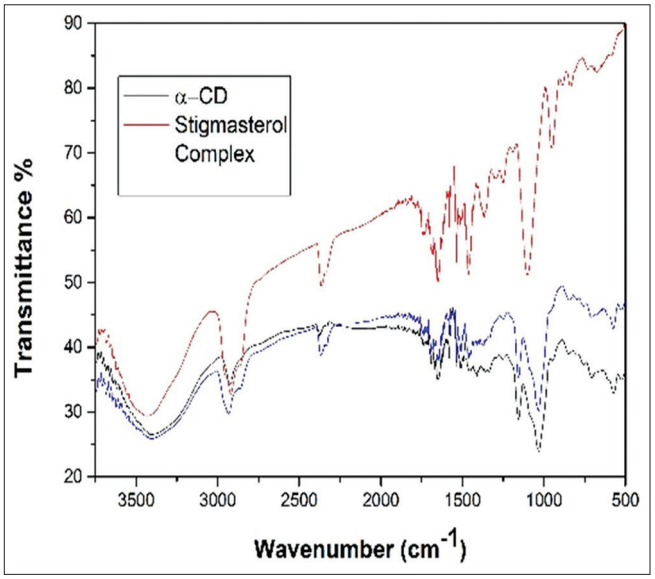

Figure 3: Fourier transform infrared spectra of alpha-cyclodextrin, stigmasterol, and alpha-cyclodextrin–stigmasterol complex

transference of stigmasterol from a more protic environment to a less protic environment. The interaction between stigmasterol and α-CD leading to an inclusion complex formation is further supported by ^{1}H NMR data.

^{1}H nuclear magnetic resonance spectra analysis

^{1}H NMR can provide evidence for the inclusion of guest molecules within the cavity of α-CD. Generally, when a guest molecule is encapsulated within the CD cavity, the hydrogen atoms present on the inner surface of the cavity (H-3 and H-5) will be shielded considerably[19] by the outer surface (H-1, H-2, H-4) and will be unaffected by the formation of inclusion complex.[7] The formation of inclusion complexes was studied by the changes in chemical shift caused by the guest and host to each other. The chemical shift change is defined as the difference in the chemical shift between the host and the host–guest complex.[20] A positive sign indicates a downfield shift, and negative sign indicates an upfield shift.[21] From Figure 4a-c and Tables 2 and 3, it is clear that in the case of α-CD–stigmasterol complex, the H3 and H5 protons are shielded and experience upfield shift than H2 protons. Further, the chemical shift in H3 (δ-0.010) protons is more magnitude than of H5 (δ-0.005) protons. There are few signals that disappeared in the complex spectrum. The fact that the α-CD: stigmasterol peak did not split into multiple peaks also indicates that there are no multiple binding sites. These changes indicate the partial inclusion of stigmasterol into the cavity of α-CD and confirm the formation of α-CD–stigmasterol complex, confirming the insights that emerged from FTIR spectroscopy.

Differential scanning analysis

DSC analysis measures the existence of an interaction between guest and host in the inclusion complex and is used to characterize CD inclusion complexes by considering the variation in peak temperature or intensity.[5] When the drug is encapsulated into the cavity of CD, their boiling point and melting point shift to different temperatures or disappear within the temperature range at which CD is decomposed ascribes to the formation of an inclusion complex.[22]

DSC thermograms of α-CD, stigmasterol, and inclusion complex are shown in Figure 5. DSC thermogram of stigmasterol exhibits a sharp exothermic peak at 169.6°C corresponding to its melting point; however, in the case of

Table 2: ^{1}H-chemical shifts (ppm) of the protons of alpha-cyclodextrin and alpha-cyclodextrin:stigmasterol in the free and complex states

Proton α-CD	δ_0 α-CD (free)	δ_c (complex) α-CD:S	$\Delta\delta=\delta_c-\delta_0$
H-1	4.00	4.00	0
H-2	3.517	3.515	−0.002
H-3	3.936	3.926	−0.010
H-4	3.560	3.560	0
H-5	3.605	3.600	−0.005
H-6	3.914	3.914	0

α-CD: Alpha-cyclodextrin, δ_c: Chemical shift of complex, δ_0: Chemical shift of pure α-CD, $\Delta\delta$: Difference in chemical shift between pure α-CD and complex, S: Stigmasterol

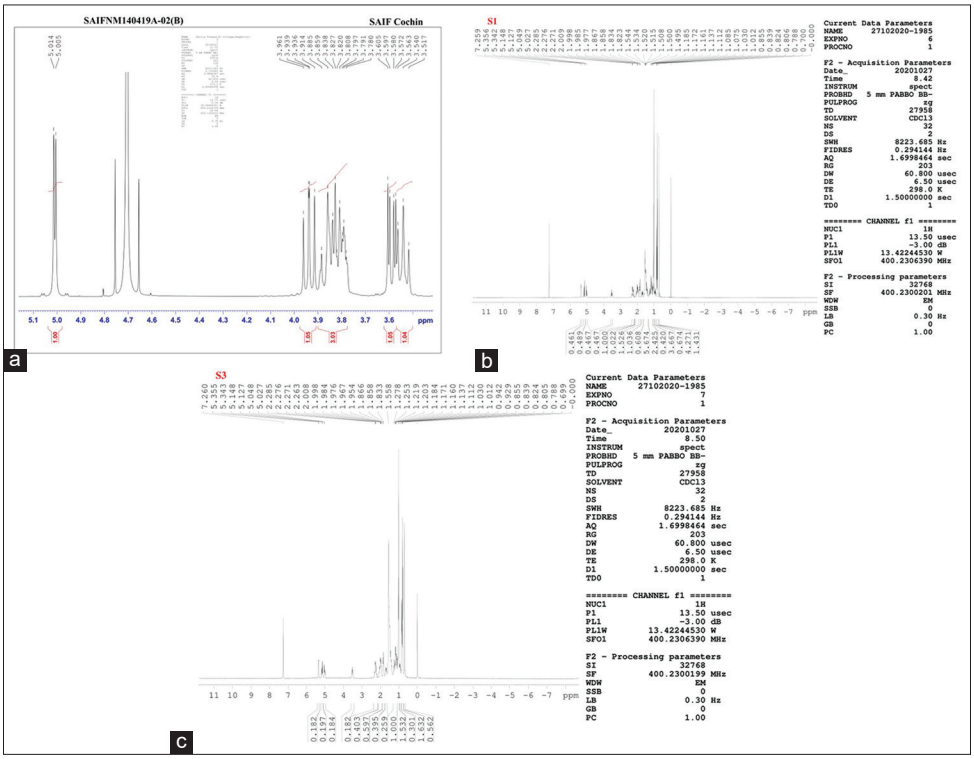

Figure 4: (a) Nuclear magnetic resonance spectrum of alpha-cyclodextrin. (b) ^{1}H nuclear magnetic resonance of stigmasterol. (c) ^{1}H nuclear magnetic resonance of alpha-cyclodextrin –stigmasterol complex

α-CD–stigmasterol inclusion complex, a shift in the exothermic peak to the left at 167.2°C occurs. Similarly, the peaks of α-CD at 77.7, 102.6, and 108.6 are shifted to the right in the DSC thermogram of α-CD–stigmasterol inclusion complex. These visible shifts in the thermograms of the complex compared to stigmasterol and α-CD serve as evidence for the partial encapsulation of stigmasterol in the α-CD cavity.

Phase solubility studies

The complexation of stigmasterol with α-CD, the effect of α-CD on the solubility of stigmasterol, the type of phase solubility, and the stability constants (Ks) are determined by phase solubility studies. Figure 6 depicts the phase solubility diagram for inclusion complex. It is visible that the phase solubility diagram obtained can be classified as AL type, according to Higuchi and Connors.[11] The aqueous solubility of stigmasterol increases linearly as a function of the concentration of α-CD. It is assumed that the increase in solubility observed is due to the formation of a 1:1 inclusion complex between stigmasterol and α-CD. The solubility constant (Ks) for α-CD–stigmasterol inclusion complex is 52 M^{-1} indicating weak interactions between the guest and the host molecules.

Molecular modeling

Docking has been utilized to perform virtual screening of compounds and propose structural hypotheses of how the drug binds with CD with lead optimization. Stigmasterol is docked with α-CD, after optimizing their structures. The optimized structures of the inclusion complex are shown in Figure 7 a and b, respectively. From Figure 7a and b, it is clear that stigmasterol is bound to α-CD and the aliphatic hydrocarbon tail has entered through the wider rim of the α-CD cavity. As the results of NMR confirm a single binding site, docking studies also support the data obtained experimentally.

Table 3: ^{1}H-chemical shifts (ppm) of the protons of stigmasterol and alpha-cyclodextrin: stigmasterol in the free and complex states

Position of hydrogen (S)	δ_0 of S	δ_c of the complex α-CD: S	$\Delta^\delta = \delta_c - \delta_0$ ($^\delta$S−$^\delta$α-CD: S)
C3-OH	2.285	2.285	0
C6-H	5.027	5.027	0
C18	0.839	0.839	0
C19	0.788	0.788	0
C21	0.855	0.840	−0.015
C22	5.127	5.027	−0.100
C23	5.148	5.048	−0.100
C24	2.271	2.263	−0.008
C25	2.009	2.008	−0.001
C26	1.520	1.510	−0.010
C27	1.495	1.490	−0.005
C28	1.998	1.984	−0.014
C29	1.823	1.731	−0.092

α-CD: Alpha-cyclodextrin, $^\delta_c$: Chemical shift of complex, $^\delta_0$: Chemical shift of pure α-CD, Δ^δ: Difference in chemical shift between pure α-CD and complex, S: Stigmasterol

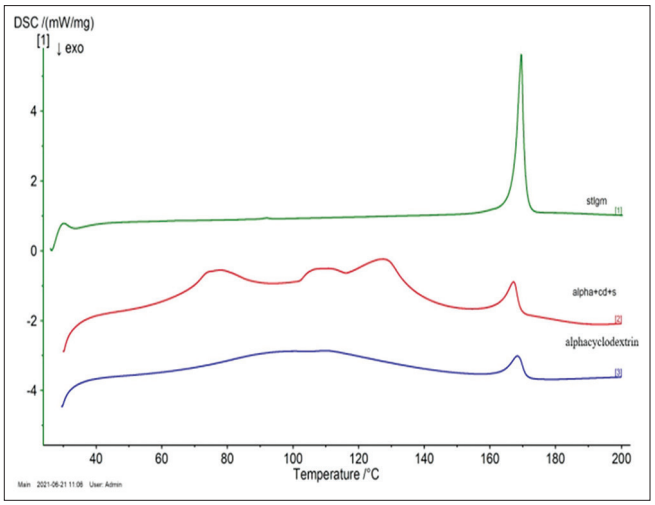

Figure 5: Differential scanning calorimetric thermograms of stigmasterol, alpha-cyclodextrin: stigmasterol, and alpha-cyclodextrin

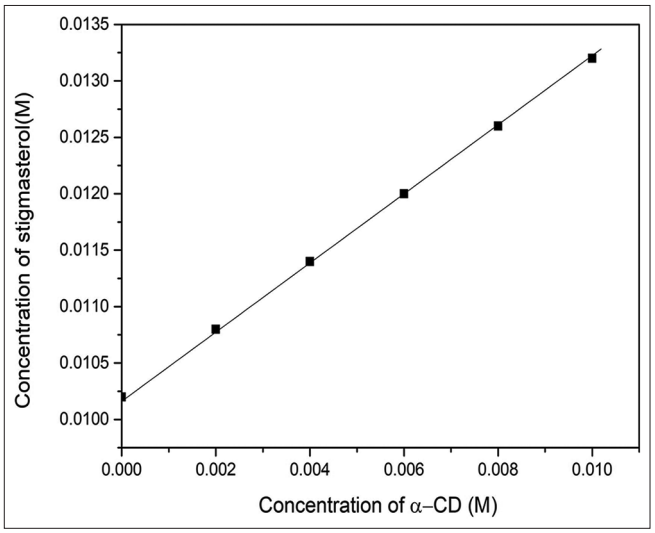

Figure 6: Effect of alpha-cyclodextrin on solubility of stigmasterol

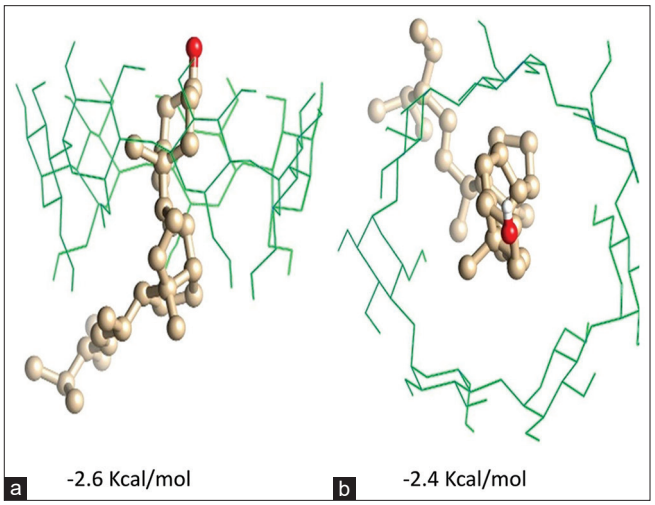

Figure 7: (a and b) The most stable-optimized geometry of the inclusion complex at different views and positions

Conclusion

The present research work demonstrates the inclusion of stigmasterol within the α-CD cavity. The absorption maxima are redshifted with the formation constant 560 M^{-1} indicating the formation of inclusion complex. Phase solubility studies explain the formation of 1:1 complex, and the solubility of stigmasterol is enhanced on complexation with α-CD. DSC studies confirm the partial inclusion of stigmasterol into the α-CD cavity. The results obtained from ^{1}H NMR and FTIR show that the possibility of the aliphatic tail of stigmasterol enters into the wider rim of α-CD which is substantiated by the stable-optimized structures obtained through molecular docking.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Ali H, Dixit S, Ali D, Alqahtani SM, Alkahtani S, Alarifi S. Isolation and evaluation of anticancer efficacy of stigmasterol in a mouse model of DMBA-induced skin carcinoma. Drug Des Devel Ther 2015;9:2793-800.
2. Peshin T, Kar HK. Isolation and characterization of β–sitosterol-3-O- β-D-glucoside from the extract of the flowers of *Viola odorata*. Br J Pharm Res 2017;16:1-8.
3. Bae H, Song G, Lim W. Stigmasterol causes ovarian cancer cell apoptosis by inducing endoplasmic reticulum and mitochondrial dysfunction. Pharmaceutics 2020;12:E488.
4. Meng X, Pan Q, Yun L. Preparation and properties of phytosterols with hydroxypropyl β–cyclodextrin inclusion complexes. J Eur Food Res Technol 2012;235:1039-47.
5. de Almeida Magalhães TS, de Oliveira Macedo PC, Kawashima Pacheco SY, Silva SS, Barbosa EG, Pereira RR, *et al*. Development and evaluation of antimicrobial and modulatory activity of inclusion complex of *Euterpe oleracea* mart oil and β-cyclodextrin or HP-β-cyclodextrin. Int J Mol Sci 2020;21:E942.
6. Cowins J, Abimbola O, Ananaba G, Wang XQ, Khan I. Preparation and characterization of β–sitosterol/β-cyclodextrin crystalline inclusion complexes. J Incl Phenom Macrocycl Chem 2015;83:141-8.
7. Roselet LS, Kumari JP. 1H NMR-A validation tool for supramolecular complexes of α-cyclodextrin with antidiabetic drugs. Materials Today: Proceedings 2021;37:88-93.
8. Udrescu L, Sbarcea L, Fulias A, Ledeti I, Vlase G, Barvinschi P, *et al*. Physicochemical analysis and molecular modeling of the fosinopril β–cyclodextrin inclusion complex. J.spectroscopy 2014:748468.
9. Panda S, Nayak S. Studies on absorption and emission characteristics of inclusion complexes of some 4-arylidenamino-5-phenyl-4H-1, 2, 4-triazole-3-thiols. J Fluoresc 2016;26:413-25.
10. Cruz JR, Becker BA, Morris KF, Larive CK. NMR characterization of the host-guest inclusion complex between beta-cyclodextrin and doxepin. Magn Reson Chem 2008;46:838-45.
11. Higuchi T, Connors KA. Phase solubility techniques. Adv Anal Chem Instrum 1965;4:117-212.
12. dos Santos C, Buera MP, Mazzobre MF. Phase solubility studies and stability of cholesterol/β-cyclodextrin inclusion complexes. J Sci Food Agric 2011;91:2551-7.
13. Jennifer SH. Structural characterization of the Brokker's merocyanine/β-cyclodextrin Complex using NMR spectroscopy and molecular modeling. J Mol Struct 2010;965:31-8.
14. Benesi HA, Hilderbrand JH. A spectrophotometric investigation of the interaction of the iodine with aromatic hydrocarbons. J Chem Soc 1949;71:2703-7.
15. Saha S, Roy A, Roy K, Roy MN. Study to explore the mechanism to form inclusion complexes of β-cyclodextrin with vitamin molecules. Sci Rep 2016;6:35764.
16. Williams RO 3rd, Mahaguna V, Sriwongjanya M. Characterization of an inclusion complex of cholesterol and hydroxypropyl-beta-cyclodextrin. Eur J Pharm Biopharm 1998;46:355-60.
17. Edilu A, Adane L, Woyessa D. *In vitro* antibacterial activities of compounds isolated from roots of *Caylusea abyssinica*. Ann Clin Microbiol Antimicrob 2015;14:15.
18. Pratheema P, Gurupriya S, Sahira, BK, Cathrine L. Isolation and characterization of stigmasterol from methanolic extract of rhizomes of *Alpinia calcarata*. World J Pharm Pharma Sci 2017;6:1872-85.
19. Upadhyay SK, Ali SM. Molecular recognition of flunarizine dihydrochloride and β-cyclodextrin inclusion complex by NMR and computational approaches. Chem Cent J 2018;12:33.
20. Goswami S, Sarkar M. Fluorescence, FTIR and 1H NMR study of the inclusion complexes of the painkiller lornoxicam with β,γ–cyclodextrins and their hydroxyl propyl derivatives in aqueous solution at different pH and in solid state. New J Chem 2018;42:15146-52.
21. Zhao R, Sandström C, Zhang H, Tan T. NMR study on the inclusion complexes of β-cyclodextrin with isoflavones. Molecules 2016;21:372.
22. Roselet LS, Kumari PJ. Inclusion studies on α–cyclodextrin complexes of glipizide and gliclazide with effect of pH. Asian J Pharma Clin Res 2017;10:273-80.