

Host-guest complexation between 3-hydroxyflavone and β -cyclodextrin: Preparation, characterization and cytotoxicity studies

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The inclusion complex of HF with β -CD has been prepared by various synthetic method such as physical method (PM), kneading method (KM) and co-precipitation method (CP) and characterized by UV, luminescence spectra, Fourier transform infrared spectroscopy, scanning electron microscopy and powder X-ray diffraction. Investigations into the anticancer activity of the solid complex against breast cancer cell line show that it is not much better than that of the HF alone. Both the HF and its solid complex show the poor anticancer activity against MDA MB 231 cell line.

Keywords: Host-guest complexation, Hydroxyflavones, Cytotoxicity, Inclusion complexes, Cyclodextrin

3-Hydroxyflavone is the backbone of all flavonols, and is a model molecule as it possesses an excited-state intramolecular proton transfer (ESIPT) effect¹ to serve as a fluorescent probe to study membranes² and intermembrane proteins³. Although 3-hydroxyflavone is almost insoluble in water, its aqueous solubility (hence bio-availability) can be increased by encapsulation in cyclodextrin cavities⁴. Cyclodextrins (CD) are cyclic oligosaccharides formed from D-glucose units that provide a relatively hydrophobic binding site for guest molecules. The most common CDs have six α , seven β , or eight γ glucose units for which the internal cavity diameter varies between 5 and 8 Å (refs 5, 6). The CDs have been widely employed

as host molecules in supramolecular chemistry since the size of their cavities can be systematically varied and the hydroxyl groups at both rims can be derivatized⁷. In addition, CD are chiral, and this property has been explored for separation technology⁷ and the complexation of various guests. Guest molecules can interact with different regions of the CD, and different inclusion modes have been observed, e.g., inclusion within the cavity or binding to the rim. The inclusion complexation of guest molecules by the host cyclodextrins and chemically modified cyclodextrins has been extensively studied in recent years as models of biological receptor-substrate interactions and is currently a significant topic in chemistry and biochemistry⁸⁻¹¹. β -Cyclodextrin is widely used because it is readily available and its cavity size is suitable for many guest molecules¹². The core structure of β -cyclodextrin is well known with the hydrophilic outer surface and a hydrophobic inner cavity¹³. β -CD with inclusion complex properties has been widely used in different areas, such as the medicine¹⁴⁻¹⁷, chemistry¹⁸⁻¹⁹, agriculture²⁰⁻²¹, etc.

We have earlier reported complexation of some organic compounds with β -CD based on proton shift effect²²⁻²⁴. Herein, we have evaluated the effect of inclusion complexation of HF with β -cyclodextrin in the solid state. The inclusion complex has been characterized by UV, fluorescence spectra, FT-IR and Powder X-ray diffraction patterns. Herein, we have also investigated the anticancer effect of pure HF and its solid complexes against MDA MB 231 cell line.

Experimental

Hydroxyflavone (HF) and β -cyclodextrin (β -CD) were purchased from Alfa Acer and used as received. Distilled water was used throughout the study. 3-(4,5-Dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), Phosphate buffered saline (PBS), Dulbecco's modified eagle's medium (DMEM) and trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, glucose and antibiotics were procured from Hi-Media Laboratories Ltd., Mumbai, while dimethyl sulfoxide (DMSO) and propanol were from E. Merck Ltd., Mumbai, India.

MDA MB 231 (Breast carcinoma) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL) and amphotericin B (5 µg/mL) in a humidified atmosphere of 5% CO₂ at 37 °C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cc culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

The solid inclusion complexes of HF and β-CD were prepared in the molar ratio of 1:1 using the physical mixture, kneading method and co-precipitation techniques. In the physical mixture process, accurately weighed HF and β-CD (molar ratio: 1:1) were kept under continuous agitation using a mortar and pestle for 10 min. The homogeneous mixture of HF and β-CD thus obtained was designated as physical mixture (PM)

In the kneading method, the pure HF and β-CD were accurately weighed at a molar ratio of 1:1 and transferred to a pestle and mortar. Then, sufficient quantity of water was added to make a pasty form, which was ground further up to half an hour with pestle and mortar. The yellow powder obtained after drying for 48 h in an oven at 303 K was designated as kneading products.

In the coprecipitation method, the inclusion complex of HF and β-CD at 1:1 molar ratio was prepared by dissolving accurately weighed amount (0.75 g) of β-CD in distilled water to obtain a saturated solution. Separately, accurately weighed amount of (0.75 g) HF was dissolved in distilled water to get a saturated solution. The HF solution was added slowly to the β-CD solution till a suspension was formed. The suspension was stirred continuously for 48 h at 303 K and kept in a refrigerator for 24 h. After 24 h, a yellow precipitate was obtained which was designated as solid complex of HF with β-CD.

For cytotoxicity studies, weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/mL concentration. Serial two-fold dilutions were prepared from these stock solutions for cytotoxic studies.

UV-visible spectra are recorded with UV-1800 Shimadzu spectrophotometer, while fluorescence

spectra were recorded in the wavelength range of 270-800 nm with RF-5301PC spectrofluorophotometer. FT-IR spectra were obtained with iD1 Thermo nicolite iS5 FT-IR spectrophotometer using KBr pellet, in the range of 500-4000 cm⁻¹. Microscopic morphological structures were recorded with FEI Quanta FEG 200 scanning electron microscope. Powder X-ray diffraction spectra were recorded on D8 Advance X-ray instrument (Bruker, Germany) with 2.2 KW Cu anode and ceramic X-ray tube as the source, Lynx Eye (Silicon strip detector technology) as the detector, Ni filter as the beta filter and zero back ground sample holder (PMMA sample holder). TG/DTA analysis was carried out with TGA 4000 Perkin Elmer in nitrogen atmosphere at 20mL/min, in the temperature range of 35-600 °C at a heating rate of 10 °C/min.

Results and discussion

UV-visible spectra of pure HF, PM, KM and solid complex (CP) are given in Fig. 1. The pure HF shows the maximum at 440.0 nm, while for PM and KM products the maxima are seen at 443.0 and 444.0 nm, respectively. The absorption maximum for the solid complex is seen at at 450.0 nm. Here we observed a change in absorption maximum for PM and KM, as compared with that of pure HF. This small change is due to the presence of β-CD. On the other hand, red shifted maximum is observed (10 nm) with significance enhancement in absorbance for solid complex in comparison with that of pure HF, PM and KM. This change is attributed to the formation of inclusion complex between HF and β-CD.

Figure 2 depicts the fluorescence spectra of pure HF, PM, KM and solid complex (CP). HF shows emission maximum at 560.0 nm with fluorescence intensity of 40.0, while the emission maxima are

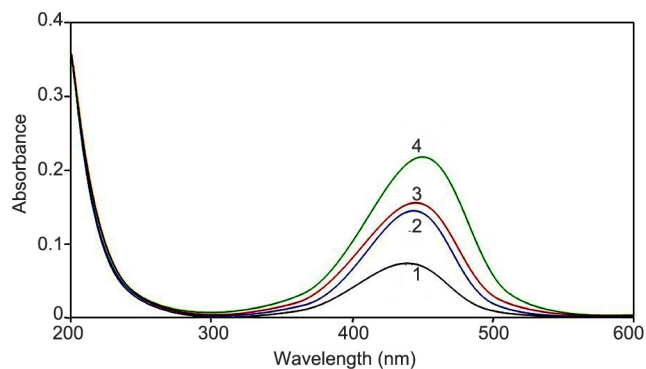


Fig. 1 – UV-vis spectra of pure HF (1), physical mixture (2), kneading method (3), and, inclusion complex of HF: β-CD (4).

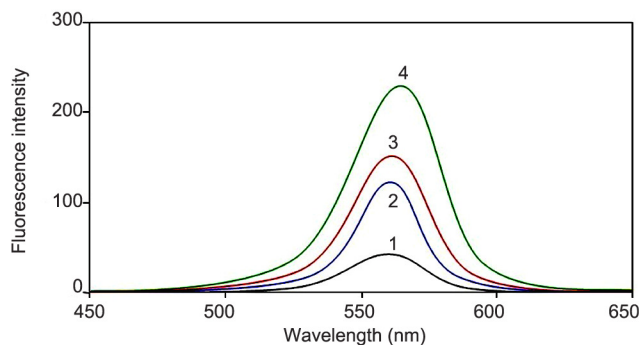


Fig. 2 – Fluorescence spectra of pure HF(1), physical mixture (2), kneading method (3), and, inclusion complex of HF: β -CD (4).

observed at 561.0 and 563.0 nm with the intensity of 125 and 145.0 for PM and KM products, respectively. The $\lambda_{\text{emission}}$ at 567.0 nm is observed with intensity of 220.0 for the solid complex. Not much change is observed in the emission maximum and its intensity for the PM and KM products which closely resembles the fluorescence spectra of pure HF. This small change in maximum and its respective intensity is only due to the presence of β -CD role in it. On the other hand, red shift is observed (around 7.0 nm) with significant enhancement in intensity for the solid complex (CP product) as compared to pure HF, PM and KM. This significant change is attributed to the formation of the inclusion complex between HF and β -CD.

The formation of inclusion complex was further confirmed by FT-IR spectral study by comparison of the guest molecule with the solid complex. IR spectra of the physical mixture, KM, and CP products are represented in Fig. 3. The FT-IR band of β -CD is observed at 3385.23 cm^{-1} due to symmetric stretching of $\nu(\text{OH})$. Also, in the IR spectrum of β -CD, the absorption band with maximum at 2924.70 cm^{-1} is observed, which is attributed to the valence vibration of the C-H bonds in the CH and CH_2 groups. Absorption peaks are observed at 1157.30 cm^{-1} , 1079.98 cm^{-1} and 1028 cm^{-1} which correspond to the symmetrical $\nu(\text{C-C})$, $\nu(\text{C-O-C})$ and bending vibration of $\nu(\text{OH})$, respectively. The absorption bands in the region $950\text{-}700\text{ cm}^{-1}$ belong to the deformation vibration of the C-H bonds and the pulsation vibration in glucopyranose cycle.

Among all the possible stretching frequencies of HF, we have selected some important stretching frequencies for discussion. In the IR spectrum of HF a strong absorption peak is seen at 1608.51 cm^{-1} for aromatic C=C. The stretching frequencies for HF

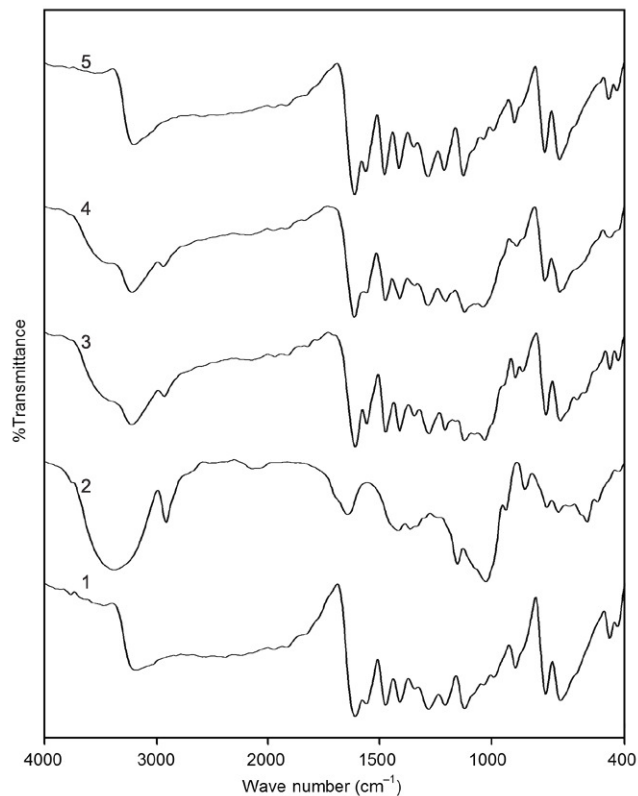


Fig. 3 – FT-IR spectra of pure HF (1), β -CD (2), physical mixture (3), kneading mixture (4), and, inclusion complex of HF: β -CD (5).

observed at 3178 , 3469.64 and 1280 cm^{-1} which corresponds to the presence of aromatic C-H, -OH and C-O-C, respectively.

The IR spectrum of the solid complex of HF with β -CD (Fig. 3) differs from the IR spectra of free β -CD and HF. The band of the OH functional group of HF and β -CD is shifted to lower wave number at 3540 cm^{-1} . At the same time, the vibration band and $\nu(\text{C-O-C})$ is shifted to higher wave number at 1289.72 cm^{-1} with 22% transmittance. The decrease in the $\nu(\text{OH})$ between the inclusion complex of HF and β -CD, and HF is due to the changes in the microenvironment which leads to the formation of hydrogen bonding and the presence of the van der Waals forces during their interaction to form the inclusion complex. On the other hand, the FT-IR spectra of PM and KM products almost imitates the characteristic peaks of free β -CD and HF, which can be regarded as a simple superimposition of free β -CD and HF molecules. Thus, the FT-IR spectrum significantly proves the formation of the HF- β -CD inclusion complex.

Scanning electron microscopy (SEM) has been employed to investigate the microstructures of the inclusion complexes in the solid state. Figure 4(a-d) shows the SEM images of HF, β -CD, PM, KM products and solid complex at X500 magnification, while Fig. 4(a'-d') shows the same at 2000X magnification to visualize the size and shape of each category. Clear rock and the needle structure are seen for pure HF for both magnifications. The complexed form of HF also shows the needle image, but it has quite different from that of HF alone. The PM and KM products are prepared by simple mixing of HF with β -CD. Hence, the images appear as such without

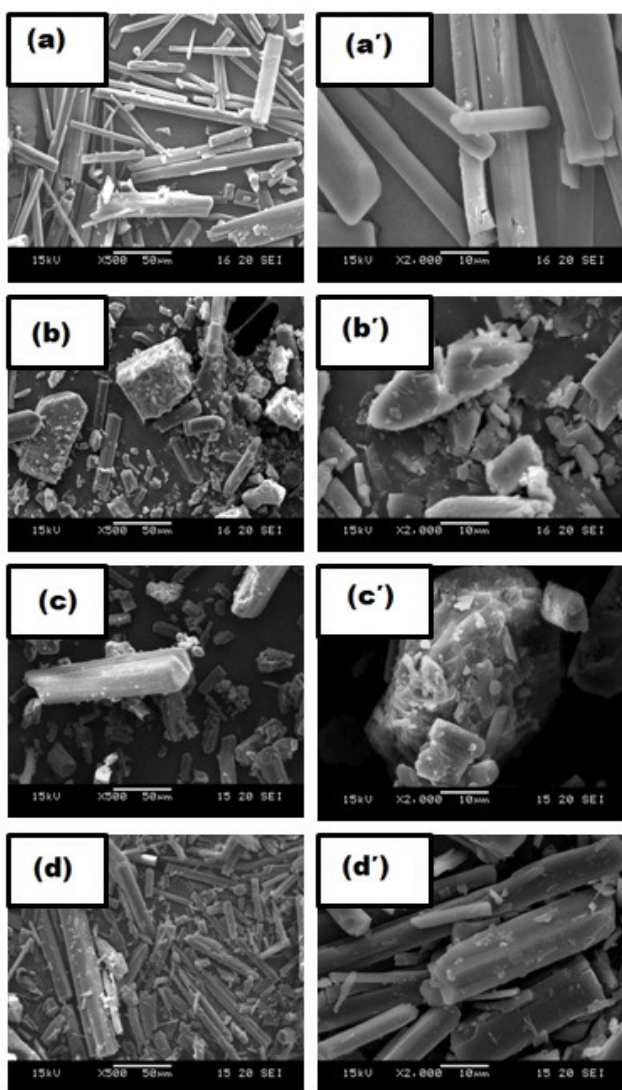


Fig. 4 – SEM photographs of the compounds at 500X magnification. (a) pure HF, (b) physical method, (c) kneading method and (d) HF: β -CD inclusion complex. [(a'-d')] are the corresponding images at 2000X magnification].

any differences (Fig. 4b and 4c). Further, SEM images also show that the shape and size of the inclusion complex are completely different from those of free HF and β -CD. A comparison of SEM images shows the complex formation between HF and β -CD in the solid state.

Generally the crystalline nature of the guest molecule is reduced and the number of amorphous structures is increased in the solid inclusion complex²⁵. Hence, the solid complexes usually reveal a smaller number and less intense peaks. Figure 5 shows the XRD patterns of pure HF, β -CD, inclusion complexes prepared by CP method, as well as PM and KM products. The characteristic diffraction peaks of HF are observed at 2θ 11.59°, 16.15°, 18.24°, 25.87° and 32.86° (Fig. 5, curve 1). Some sharp peaks at 2θ 10.75°, 12.67°, 15.43°, 19.62° and 22.82° are present in the β -CD powder (Fig. 5, curve 2). The diffraction patterns of solid complex of HF show peaks at 10.62°, 15.17°, 17.94°, 24.38° and 30.75° (Fig. 5, curve 5). The intensity peaks at 2θ 11.59°, 16.15°, 18.24°, 25.87° and 32.86° of HF are reduced significantly in diffraction patterns of HF: β -CD inclusion complex, which suggests the reduction in the crystalline nature of HF on addition of β -CD. The crystalline nature of HF is lost during the formation of the stable complex with β -CD. Moreover, the change in diffraction patterns of solid complex is only due to the inclusion of HF molecule into the cavity of β -CD. Further, new intense diffraction peaks are also observed

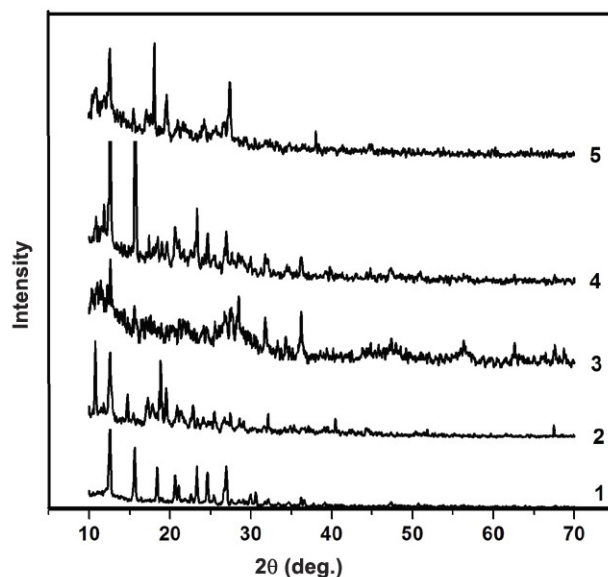


Fig. 5 – Powder XRD patterns of pure HF (1), β -CD (2), physical mixture (3), kneading mixture (4), and, inclusion complex of HF: β -CD (5).

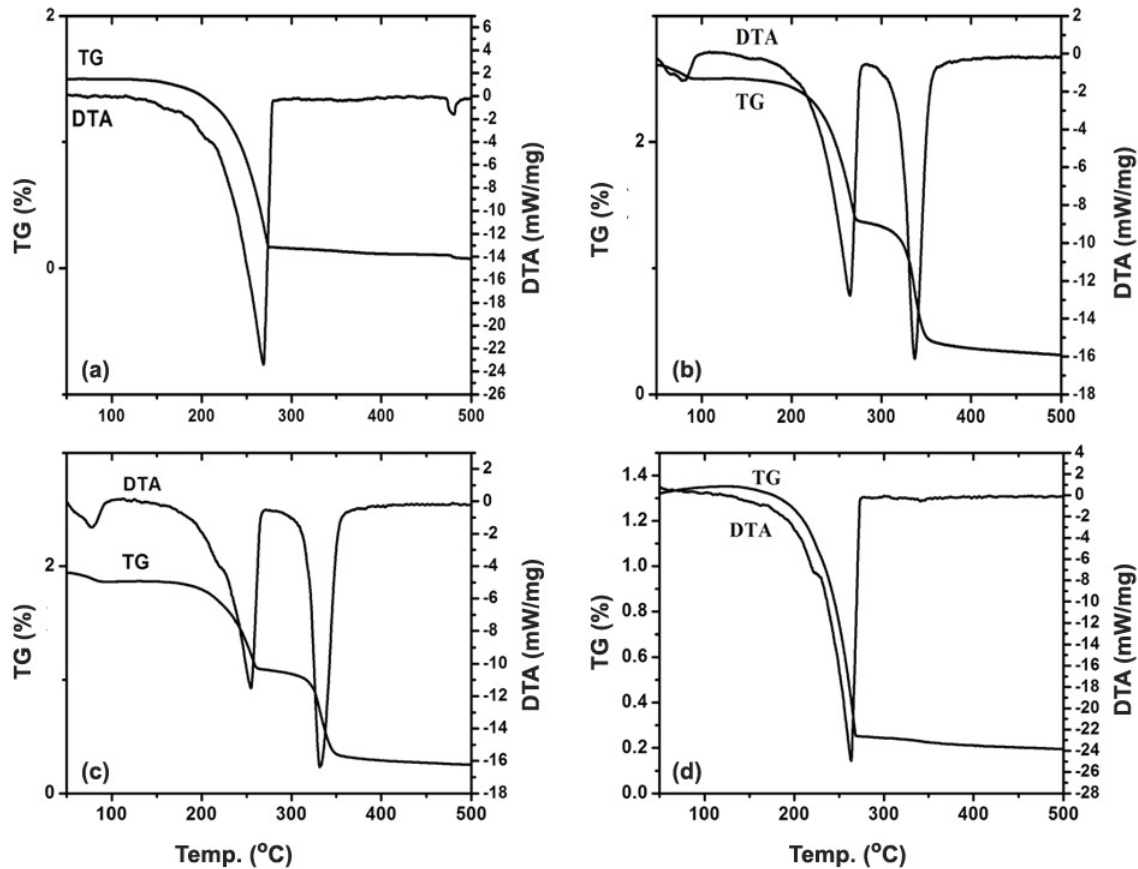


Fig. 6 – TG/DTA of (a) pure HF, (b) physical mixture, (c) kneading method, and, (d) inclusion complex of HF: β -CD.

for HF- β -CD inclusion complex, which indicates a change in HF- β -CD environment after inclusion complex formation.

The pattern of the PM product is similar to that of β -CD, while the pattern of KM product (Fig. 5, curves 3 and 4) is similar to that of pure HF. Not much difference is noted for PM and KM products of HF by the addition of β -CD. Only a slight decrease in peak intensity is noted. Hence, No inclusion complex is obtained in the case of PM and KM.

The TG/DTA curves obtained for the pure HF, PM, KM and CP products are presented in Fig. 6. The TG/DTA curve of β -CD shows three endothermic peaks. The first endothermic peak between 69.5 °C and 118 °C with 12% weight loss corresponds to the dehydration of β -CDx. There were nine water molecules per β -CD molecule. The second small endothermic peak appears at 225 °C, while no weight loss is seen in the TG curve, indicating a reversible *trans* conformation²⁶. TG/DTA curve of pure HF exhibits an endothermic peak at 268.89 °C assigned to the melting point of the pure HF molecule.

For physical mixture (Fig. 6b) this peak is seen at 263.12 °C and for the KM product, the endothermic peak is at 251.34 °C. The minor change observed for PM and KM is due to the small interaction between HF and β -CD. On the other hand, the endothermic peak at 262.65 °C for the solid complex (Fig. 6d) is due to the strong influence of β -CD on the HF molecule. From the thermal analysis, the formation of solid complex is confirmed.

In vitro comparison of cytotoxicity of free HF, β -CD and inclusion complex was assessed to verify its safe application in pharmaceutical formulations. The cytotoxic effects of pure HF and its solid complex with β -CD were tested with MDA MB 231 cell line, at varying concentrations of pure HF and its solid complex in the order of 62.5, 125, 250, 500 and 1000 μ m/mL individually. The percentage of inhibition and corresponding CTC₅₀ value for each concentration is given in Table 1. On increasing the concentration of HF, the percentage of inhibition is gradually increased. At higher concentration of HF (1000 mg/mL) it is 22.22% with the CTC₅₀ value exceeding 1000. The

Table 1 – Cytotoxic activity of HF and its inclusion complex against MDA MB 231 cell line

No.	Comp.	Conc (µg/mL)	%Inhibition	CTC ₅₀ (µg/mL)
1	Pure HF	1000	22.08±2.4	>1000
		500	15.38±1.9	
		250	13.21±2.6	
		125	5.32±0.8	
		62.5	2.51±1.8	
2	HF-β-CD complex	1000	19.33±2.6	>1000
		500	17.98±3.0	
		250	11.16±2.3	
		125	6.39±2.0	
		62.5	4.50±2.1	

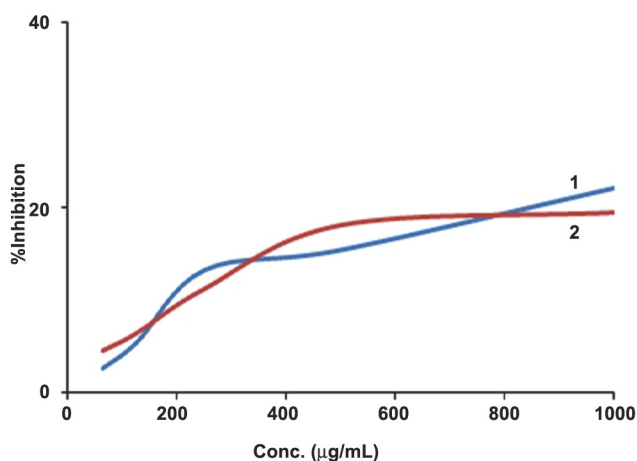


Fig. 7 – Cytotoxic activity of pure HF (1), and, solid inclusion complex (2) against MDA MB 231 cell line.

solid complex of HF with β-CD showed the same trend with percentage of inhibition of 19.33% and CTC₅₀ value exceeding 1000. Both the compounds showed only a minor effect against MDA MB 231 cell line (≈20% inhibition). When the CTC₅₀ value is less than 1000, it is considered safe for cytotoxic activity. However, in this case we observed CTC₅₀ value exceeding 1000. Hence, the cytotoxic effect of pure HF and its complex did not reach the expected level.

Figure 7 shows that there is no major difference between the cytotoxic activities of pure HF and solid complex. β-CD inclusion does not induce or increase toxicity of HF. Hence, cyclodextrin based approach

for design of new formulations of HF would be an interesting strategy and may have potential application in the design and development of β-CD associated pharmaceutical formulations.

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