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Spectral studies on inclusion complexation between 3-hydroxyflavone and 2-Hydroxypropyl-β-cyclodextrin

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ABSTRACT

3-hydroxy-2-phenylchromen-4-one (HF) is a flavonols that is formed when hypoxanthine is attached to a ribose ring (also known as a ribofuranose) via a β-N_9-glycosidic bond. Cyclodextrins are able to form host-guest complexes with hydrophobic molecules given the unique nature imparted by their structure.As a result, these molecules have found a number of applications in a wide range of fields. The inclusion complex of HF with 2HP-β-CD is prepared by various synthetic method such as physical method (PM), kneading method (KM) and co-precipitation method (CP). The solid inclusion complex is characterized by UV, luminescence spectra, Fourier transform infrared (FT-IR) spectroscopy, scanning electron microscopy (SEM) and powder X-ray diffraction (XRD). The anticancer activity of the solid complex is performed against breast cancer cell line and it is noticed that there is no much better activity than the HF alone. Both the HF and its solid complex showed the poor anticancer activity against MDA MB 231 cell line.

***Keywords*:** 3-Hydroxyflavone; 2-Hydroxypropyl-β-Cyclodextrin; Inclusion Complex; XRD; Cytotoxicity

1. Introduction

3-Hydroxyflavone is a chemical compound. It is the backbone of all flavonols, a type of flavonoid. It is a synthetic compound, which is not found naturally in plants. It serves as a model molecule as it possesses an excited-state intramolecular proton transfer (ESIPT) effect[1] to serve as a fluorescent probe to study membranes for example[2] or intermembrane proteins[3]. Although 3-hydroxyflavone is almost insoluble in water, its aqueous solubility (hence bio-availability) can be increased by encapsulation in cyclodextrin cavities[4]. Recently some of the research work is carried out in HF by some other authors. Complex formation of HF in γ-CD medium and their excited state proton transfer (ESPT).

Cyclodextrins (CDx) are cyclic oligosaccharides formed from D-glucose units that provide a relatively hydrophobic binding site for guest molecules. The most common CDx have six (α), seven (β), or eight (γ) glucose units for which the internal cavity diameter varies between 5 to 8 Å[5-7]. The CDx 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 derivatized[8]. In addition, CDx are chiral, and this property has been explored for separation technology[9] and in the study of the complexation of various guests. Guest molecules can interact with different regions of the CDx, and different inclusion modes have been observed, e.g., inclusion within the cavity or binding to the rim. Cartoon representations frequently show an inclusion mode where the guest is located deeply within the CDx cavity, a perception that probably arises from the fact that the cavity is relatively hydrophobic. The inclusion complexation of guest molecules by the host cyclodextrins (CDs) and chemically modified cyclodextrins has been extensively studied in recent years as models of biological receptor-substrate

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interactions and is currently a significant topic in chemistry and biochemistry[10-13].

2-Hydroxypropyl - β -cyclodextrin (2HP- β -CD) represents a very useful alternative to the Beta Cyclodextrin molecules with much lower toxicities and improved solubility compared to the pristine molecules which qualify them to take a leading position in pharmaceutical and food industries. All hosts have been used in pharmaceutical formulations to the availability of the active ingredients and enhance the solubility[14–20]. Moreover, these molecules were found to play a crucial role in enhancing the shelf life of many compounds by acting as a molecular capsule where the bioactive species is protected from the harsh environmental factors around them such as light, pH and temperature[21–23]. Encapsulation of antioxidants, fragrance, flavonols and other food additives into cyclodextrins to enhance the solubility and non-volatility have also attracted lots of interest[24–28].

The supramolecular chemistry gives a broad idea of intermolecular interactions has been performed by host-guest system. The cyclodextrin is mostly hopeful to form inclusion complexes, especially with various guest molecules with proper structure[29]. In supramolecular chemistry, host–guest chemistry describes complexes that are composed of two or more molecules or ions that are held together in unique structural relationships by forces other than those of full covalent bonds. Host–guest chemistry encompasses the idea of molecular recognition and interactions through non covalent bonding.

Globally breast cancer is the most frequent female malignancy. It is the most frequently diagnosed cancer and is the second leading cause of cancer death among women in India. Despite advances in cancer treatment over the past decades, the prognosis of patients with breast cancer breast cancer has improved only to a small extent. Thus, there is an urgent need to develop new and effective strategies for the prevention and treatment of this form of cancer. Breast cancer is when cancer develops from breast tissue. Signs of breast cancer may include a lump in the breast, a change in breast shape, dimpling of the skin, fluid coming from the nipple, or a red scaly patch of skin. In those with distant spread of the disease, there may be bone pain, swollen lymph nodes, shortness of breath, or yellow skin.

In our earlier results are focused the study of complexation of some organic compounds with β-CD based on proton shift effect[30-32]. Our aim of the present work is to evaluate the effect of inclusion complexation of HF with 2-HydroxyPropyl -β-Cyclodextrin in solid state. About solid inclusion complex, common used methods include UV, Fluorescence spectra, FT-IR, Powder X-ray diffraction. Many inclusion complexes of guests with CDs were studied by these methods[33-34]. In this work, we not only studied the character of HF-2HP-β-CD complex, we tried the anticancer effect of pure HF and their solid complexes against MDA MB 231 cell line.

2. Materials & methods

2.1 Materials

Hydroxy flavone (HF) and2-HydroxyPropyl -β-Cyclodextrin (2HP-β-CD) are purchased from Alfa Acer and used as received. Distilled water is 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 are obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

2.2 Cell lines and Culture medium

MDA MB 231 (Breast carcinoma) cell line is procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells are cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µ g/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells are dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures are grown in 25 cm^2 culture flasks and all experiments are carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

2.3 Preparation of solid inclusion complexes

The solid inclusion complexes of HF and 2HP-β-CD were prepared using physical mixture, kneading method and co-precipitation techniques.

2.3.1 Preparation of physical mixture of the

HF and HCD

In the physical mixture process, the accurate weight of HF and 2HP-β-CD are allowed to continuous agitation using a mortar and pestle for 10 min. Finally we obtained the homogeneous mixture of HF and 2HP-β-CD called physical mixture (PM)

2.3.2 Preparation of solid complex by kneading method

The pure HF and 2HP-β-CD substances are accurately weighed and transferred into a pestle and mortar. Then, sufficient quantity of water is added in it to make a pasty form. The whole content is further allowed for grinding up to half an hour with pestle in mortar itself under careful condition. A yellow powder is obtained as called kneading product after it is dried for 48 hours in an oven at 303K.

2.3.3 Preparation of the HF and β-CD by co-precipitation method

The inclusion complex of HF and 2HP-β-CD is prepared using the co-precipitation method. The accurate weight of 2HP-β-CD is dissolved in distilled water to become a saturated solution. Other hand accurate weight of of HF is dissolved in distilled water to get a saturated solution. The HF solution is added slowly to the 2HP-β-CD solution up to suspension is formed. The suspension is stirred continuously for 48 hours at 303K. The solution is kept in a refrigerator for 24 hours. After 24 hours, the solution became as yellow precipitate called as solid complex of HF with 2HP-β-CD.

2.4 Preparation of Test Solutions for cytotoxic study

For Cytotoxicity studies, each weighed test drugs are separately dissolved in distilled DMSO and volume is made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions are prepared from this for carrying out cytotoxic studies.

2.5 Instruments

UV-Visible spectra are recorded with UV-1800 SHIMADZU spectrophotometer while luminescence spectra are recorded between the wavelength ranges from 270 to 800nm with RF-5301PC Spectrofluorophotometer. FT-IR spectra are obtained with iD1 Thermo nicolate iS5 FT-IR spectrophotometer using KBr pellet. The range of spectra is from 500–4000 cm^{-1}. Microscopic morphological structure measurements are performed with FEI Quanta FEG 200 scanning electron microscope (SEM). Powder X-ray diffraction spectra are taken by D8 Advance X-ray instrument (BRUKER, Germany) with 2.2 KW Cu anode, 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 is carried out with TGA 4000 perkin elmer in nitrogen atmosphere at 20ml/min. The temperature range is 35-600 deg with heating rate 10 deg/min.

3. Results & discussion

3.1 Analysis of inclusion complex by UV Spectra

The UV spectra of pure HF, PM, KM and solid complex (CP) are displayed in **Figure 1** and their respective data are given in Table 1 The pure HF gives the maximum at 440.0 nm with absorbance 0.075. For PM and KM products, the maximum is observed at 442.0 and 443.5 nm with the absorbance 0.098 and 0.123, respectively. Whereas for the solid complex (CP) the absorption maximum is observed at 449.5 nm. Here we noticed that, the change in absorption maxima for the PM and KM products while compare with pure HF. This small change in maximum is due to the presence of 2HP-β-CDin it. On the other hand, red shifted maximum is observed (9.5 nm) with significance enhancement in absorbance for solid complex when comparing with pure HF, PM and KM. This change further supports the formation of inclusion complex between HF and 2HP-β-CD.

Compound	**UV**		**Luminescence**	
	λ_{max}	Abs	λ_{emi}	Intensity
Pure- HF	440.0	0.075	560.0	40.0
PM- HF	442.0	0.081	560.5	100.0
KM-HF	443.5	0.095	563.0	155.3
CP-HF	449.5	0.123	568.5	285.0

Table 1. UV and Luminescence spectral maxima of HF with B-CD

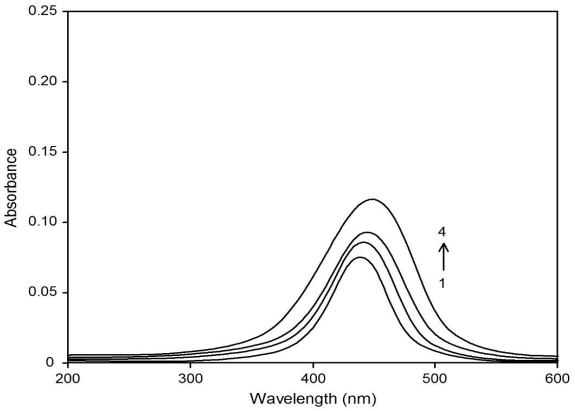


Figure 1; UV Spectra of 1) Pure HF, 2) PM, 3) KM and 4) CP product of HF: 2HP-β-CD**.**

3.2 Analysis of inclusion complex by luminescence spectra

Figure 2 depicts the luminescence spectra of pure HF, PM, KM and solid complex (CP) and the respective data are given in **Table 1**. HF showed that the emission maximum at 560.0 nm with fluorescence intensity 40.0. The emission maxima are observed at 560.5 and 563 nm with the intensity 100 and 155.3 for PM and KM products, respectively. The λ_{emi} at 568.5 nm is with intensity 285.0 is observed for solid complex. No much change is observed in emission maximum and its intensity for the PM and KM products and these are more resemblance with the fluorescence spectra of pure HF. This small change in maximum and its respective intensity is due to the presence of 2HP-β-CD role in it. On the other hand, red shifted maximum is observed (around 8.5 nm) with significant enhancement in their intensity for solid complex (CP product) when comparing it with pure HF, PM and KM. This remarkable change is attributed to the formation of inclusion complex between HF and 2HP-β-CD.

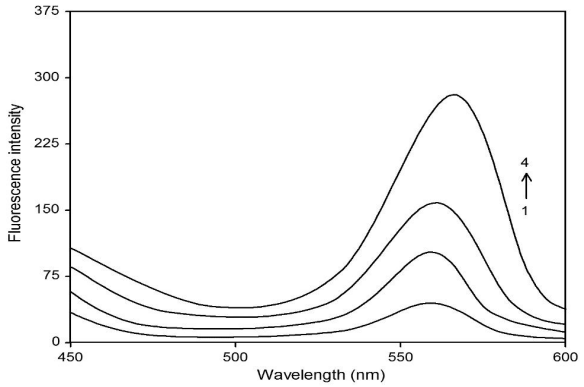


Figure 2. Fluorescence spectra of 1) Pure HF,2) PM, 3) KM and 4) CP product of HF: 2HP-β-CD (λ_{exc} = 440.0 nm)

3.3 FT-IR Study

The IR spectra of the compounds HF, 2HP-β-CD, PM, KM, CP products are shown in **Figure 3.** The formation of complex is confirmed by the comparison of pure reactants (HF and 2HP-β-CD) with its inclusion complexed products. The IR spectrum of pure HF showed their -OH, C=O, C=C, C-O-C stretching frequencies at 3469.64, 1608.75, 1559.01 and 1280.0 cm^{-1} (Table 2). Comparing the three products the PM method product showed very broad and less intense peaks (3228.27, 1607.93, 1559.45 and 1077.61 cm^{-1}) for the functional groups -OH, C=O, C=C, C-O-C, respectively. By comparing the PM and KM products, stretching frequencies are observed slightly sharp and intense peaks at 3410.29, 1608.87, 1561.67 and 1086.87 cm^{-1}. Interestingly, the CP product forms an inclusion complex which is confirmed by its strong, sharp and intense stretching frequencies observed at 3202.34 (-OH), 1664.25 (C=O), 1575.74 (C=C) and 1030.7 (C-O-C), respectively. Eventually, among the three methods (PM, KM and CP), the CP product only form the stable inclusion complex than the PM and KM products.

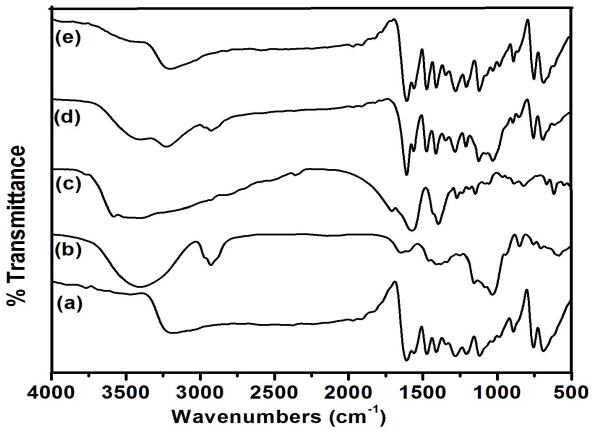


Figure 3; FT-IR spectra of a) HF, b) 2HP-β-CD, c) PM, d) KM and e) CP products**.**

Stretching Frequencies	Pure HF	PM Products	KM Products	CP Products
-OH	3469.64	3228.27	3410.29	3202.34
C = O	1608.75	1607.93	1608.87	1664.25
C=C	1559.01	1559.45	1561.67	1575.74
C – O – C	1280.00	1077.61	1086.87	1030.17

Table 2. FT-IR frequencies for various stretching in HF and their solid complexes

3.4 SEM image analysis

Scanning electron microscopy (SEM) is a qualitative method used to study the structural aspects of raw materials, i.e., CDs and guest or the products obtained by different methods of preparation, such as physical method, kneading method and Co-Precipitation method. The SEM photographs of 2HP-β-CD, HF, its inclusion complexes are shown in **Figure 4.** Pure HF showed that the clear rock and needle structure

(Figures 4a and4b) and 2HP-β-CD crystallized as a spherical crystal with cavity structures. The PM and KM product of HF: 2HP-β-CD revealed some similarities with the crystals of the free molecules and showed both crystalline components (Figures 4c and 4d & Figures 4e and 4f). In contrast, the HF: 2HP-β-CD displayed in the form of compact and homogeneous plate-like structures with crystal particles in which the original morphology of both components disappeared (Figures 4g and4h). The shape and sizes of HF and 2HP-β-CD particles are different from those of the inclusion complex, which may indicate inclusion complexation from the processing of HF and 2HP-β-CD by co-precipitation method which indicates strong indication of inclusion complex formation by CP method.

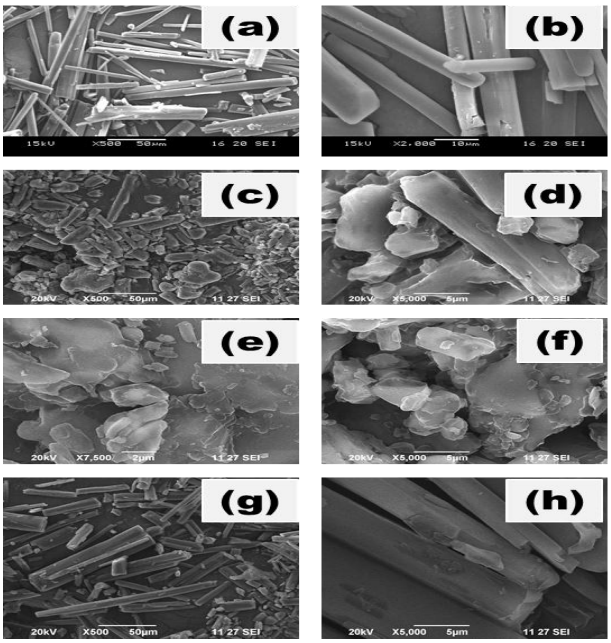


Figure 4; SEM photographs of a) HF (x500), b) HF (x2000) c) PM (x500),d) PM (x5000), e) KM (x7500), f) KM (x5000), g) CP product (x500) and h) CP product (x5000)**.**

3.5 Powder XRD Study

The XRD patterns of pure HF, 2HP-β-CD, inclusion complexes obtained by PM, KM and CP methods are depicted in **Figure 5.** The characteristic diffraction peaks of HF are observed at 2θ 11.59, 16.15, 18.24, 25.87 and 32.86º for assuming it is in crystalline nature.

The diffraction peaks of solid complex of HF with 2HP-β-CD showed peaks at 2θ 10.88, 11.44, 12.36, 15.12, 16.28, 18.24, 21.5, 24.56 and 27.76 º. The more intense peaks of pure HF at 2θ 11.59, 16.15, 18.24, 25.87, and 32.86º are reduced and some of the peaks are disappeared significantly in solid inclusion complex by CP method.

This showed that the crystalline nature is reduced gradually and increases amorphous nature. The formation of amorphous nature complex confirmed the stable complex is obtained between HF and 2HP-β-CD. In addition, the presence of new intense peak showed the changes in HF: 2HP-β-CD environment after inclusion complex formation.

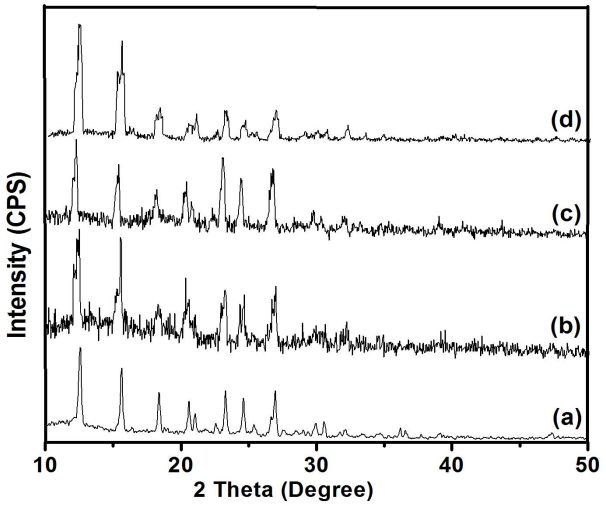
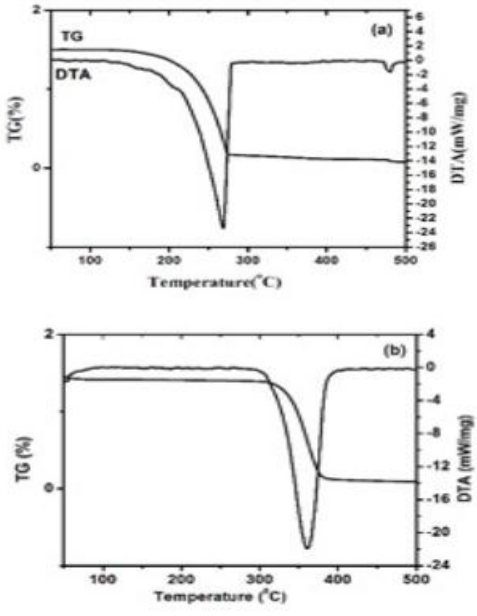


Figure 5. Powder XRD patterns of a) HF, b) PM, c) KM and d) CP Product

3.6 TG/DTA analysis of solid complex

Figure 6 showed the TG/DTA curves for pure HF, PM, KM and CP products. The TG/DTA analysis of pure 2HP-β-CD showed only one endothermic peak at 355ºC whereas the pure HF showed an endothermic peak at 268.9ºC which is assigned to the melting point of the corresponding compounds respectively. For PM and KM method, the same endothermic peak is observed at 251ºC and 260ºC respectively whereas in CP method it is observed at 275ºC. Based on the TG/DTA analysis of PM and KM products, there is a small interaction obtained in between HF and 2HP-β-CD. But, there is a strong interaction obtained in CP method product. Eventually, the TG/DTA analysis confirmed the formation of inclusion complex.



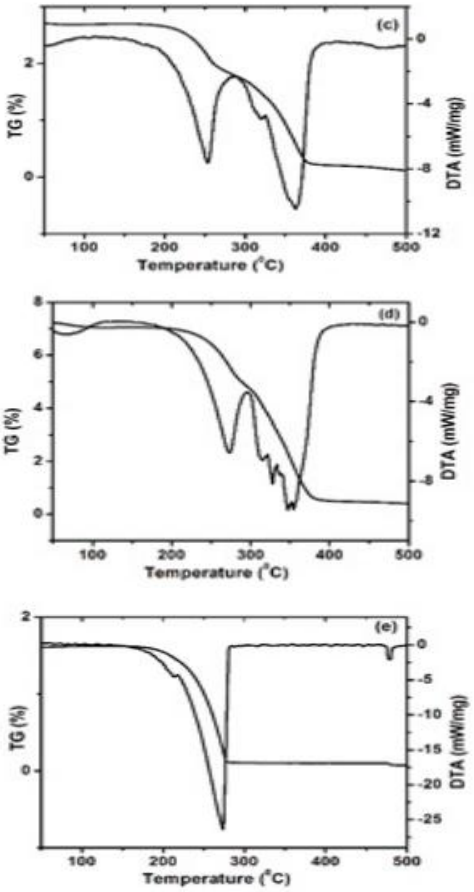


Figure 6; TG/DTA of a) HF, b) 2HP-β-CD c) PM, d) KM and e) CP Products**.**

3.7 FT Raman Analysis

FT-Raman spectra of pure HF and CP products are depicted in **Figure 7.** The stretching frequency observed at 3477.24cm^{-1} is responsible for the -OH group of pure HF whereas the frequency for this stretching is completely disappeared for the CP product.

The C-H group shows a frequency at 3153.17cm^{-1}and 3072.45 cm^{-1}likely to be for the CP product (Table 3). Peaks observed at 2380.95cm^{-1} and 1970.73 cm^{-1} meant for C=C group and observed the absence in CP. The C=O group appeared at 1611.8 cm^{-1} and for CP is observed at 616.92cm^{-1}. The major changes are observed in -OH, aromatic C-H, -C=O frequencies for CP Products. The other stretching frequencies such as C=C, and etc., are also changed while interacting with 2HP-β-CD. The FT-IR spectra are more resembled with FT-Raman spectra. No much difference between FT-Raman and FT-IR in analyzing the HF and its solid complex. Both the spectral analysis seems to the same conclusion. Hence, the part of the HF molecule has been included into the 2HP-β-CD cavity.

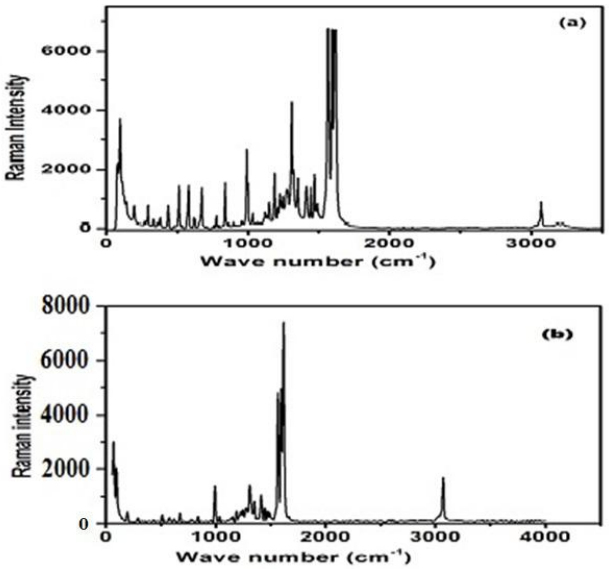


Figure 7; FT-Raman Spectra of a) Pure HF and b) CPproduct with 2HP-β-CD**.**

Pure HF (cm^{-1})	**CP product (cm^{-1})**	**Group Frequency**
3477.24	-	-OH stretching frequency
3153.17	3072.45	Aromatic C-H
2380.95, 1970.73	-	C=C shoulders and overtones
1611.85	1616.92	C=O
1562.30	1595.30	C=C Skeletal vibration of aromatic
1471.24, 1414.11	1488.64, 1412.64	C-H asymmetric bendin g mode
1353.85	1350.47	C-H in plane bending vibration
1276.63, 1120.84, 992.28	1246.99, 1032.64, 992.21	C-O and C-O-C Stretching vibration
898.11	837.44,	O-H Out of plane bending vibration
690.09	673.09	C-H out of plane bending vibration

Table 3. FT-Raman frequency data of HF and its CP products with 2HP-β-CD

3.7 In-vitro cytotoxic effect of HF against MDA MB 231 cell line

An in-vitro comparison of cytotoxicity of free HF, 2HP-β-CD and inclusion complex are assessed to verify its safe application in pharmaceutical formulations. The cytotoxic effect of pure HF and their solid complex with 2HP-β-CD are tested with MDA MB 231 cell line. Various concentrations of pure HF and their solid complex are prepared in the order of 62.5, 125, 250, 500 and 1000µm/ml separately. For each concentration, the cytotoxic effect is tested. The percentage of inhibition and corresponding CTC_{50} value for each concentration is given in Table 4. By increasing the concentrations of HF, the percentage of inhibition is gradually increased (**Figure 8**). At a higher concentration of HF (1000mg/ml) it gives 22.22% and CTC_{50} value exceeding 1000. The solid complex of HF with 2HP-β-CD is shown that the same kind of trend in percentage of inhibition (15.86%) and CTC_{50} value is exceeded to 1000. Both the substances (HF and solid complex) showed only a little effect against MDA MB 231 cell line (**Figure 9**). Since, the CTC_{50} should not exceed 1000; it's suitable for cytotoxic effect. Unfortunately, the CTC_{50} value is not in control and it exceeds 1000. The cytotoxic effect of pure HF has not reached the level which we expected.

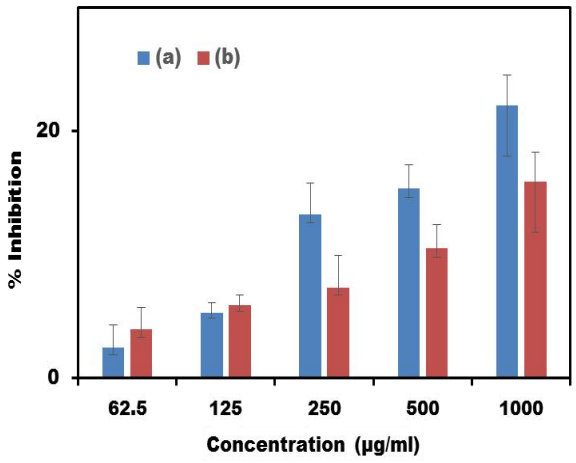


Figure 8; Cytotoxic effect of (a) HF and (b) their solid complex against MDA MB 231 Cell line**.**

Sl. No	Name of the sample	Concentrations (µg/ml)	Percentage of inhibition	CTC_{50} (µg/ml)
1	Pure HF	1000 500 250 125 62.5	22.08±2.4 15.38±1.9 13.21±2.6 5.32±0.8 2.51±1.8	>1000
2	HF: 2HP-β-CD	1000 500 250 125 62.5	15.86±4.1 10.54±0.8 7.33±0.6 5.92±0.5 3.92±0.6	>1000

Table 4. Cytotoxic effect of HF and its complex against MDA MB 231 cell line

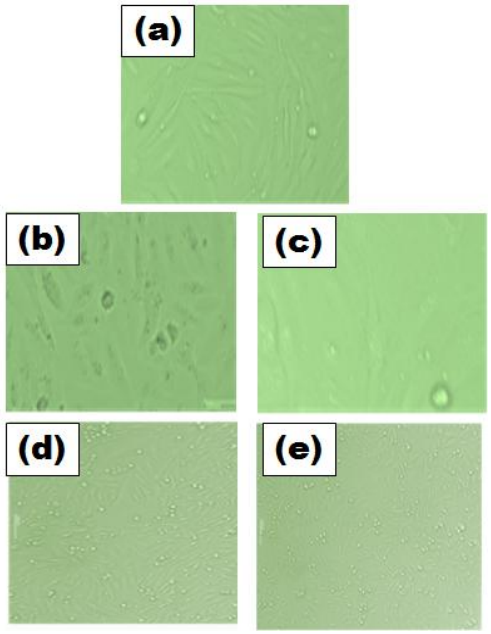


Figure 9; Photos of (a) MDA MB 231 Control, (b) HF Pure-1000C, (c) HF Pure -500C, (d) Inclusion Complex (HF: 2HP-β-CD) -1000C, (e) Inclusion Complex (HF: 2HP-β-CD) -500C**.**

4. Conclusions

HF forms stable inclusion complex with 2HP-β-CD. Solid complex is verified by various analytical methods like UV and Fluorescence techniques. It showed that the remarkable changes in absorbance and fluorescence intensity with red shifted maximum. As the stretching frequencies of HF in aromatic C=C, aromatic C-H, –OH, C-O-C- and C=O are significantly changed for those in solid complex. This part only entrapped in CD cavity. Hence no change in PM and KM. SEM and XRD analysis supports the formation of inclusion complex in solid state. HF and their complex showed that the poor anticancer activity against MDA MB 231 cell line. In addition, the toxicity profile of the inclusion complex (HF: 2HP-β-CD) was also investigated. The inclusion complex of HF/ 2HP-β-CD was found to be nontoxic against MDA MB 231 cells even at high concentration. The approach of making use of inclusion complexes may help in the design and development of HCD associated pharmaceutical formulations.

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