A Non-Covalent Bonding Interaction Between Amitriptyline And Beta-Cyclodextrin: Comparisons Of *In-Vitro* Cytotoxic Activity Against Breast Cancer Cell Line

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Abstract
The formation of supramolecular host-guest inclusion complex of amitriptyline [AP] and β-Cyclodextrin [β-CD] have been studied in solution state. The stoichiometric ratio of the inclusion complexes between AP and β-CD in solution state is confirmed by the absorption and fluorescence spectroscopic tehniques. The binding constant value has been calculated for the 1:1 complex by using Benesi-Hildebrand equation. An in-vitro cytotoxic performance also checked for pure AP and its inclusion complex with β-CD and the inclusion complex clearly had shown that the no change after making AP towards inclusion complex with β-CD.
Keywords: AP; Cyclodextrin; Fluorescence; in-vitro cytotoxicity.

INTRODUCTION

Tricyclic antidepressants (TCAs) are heterocyclic compounds used primarily as antidepressants. AP belongs to tricyclic dibenzocycloheptadiene derivatives and chemically AP is 3-(10, 11-dihydro-5H-dibenzo [a, d] cycloheptene-5-ylidene)-*N*, *N* diethyl-1-propanamine hydrochloride [1]. The value of angle of AP molecules plays an important role, if it is more planar the greater the neuroleptic activity [2]. It is assumed that amitriptyline acts by blocking the receptors of neurotransmitters, naradrenaline in the central nervous system which results an increase of concentration of both molecules with a subsequent-enhancement of their antidepressant patency [3]. AP is mainly used to treat the mental/mood problems. It can help to develop mood and feelings of happiness, relieve nervousness and stress, help to sleep and increase the energy level. Amitriptyline suffers from several drawbacks like that antiarrhytmic, anticholinergic, cardiovascular and hyperthermia side effect [4]. Cyclodextrins (CDs) are macro cyclic oligosaccharides of six; seven or eight linked α, 1, 4-linked D-glucopyranose units, denoted α-, β- and γ-CDs, respectively. The β-Cyclodextrin is the widely used because the lowest price, however, its solubility in water is relatively low and the toxicity of β-cyclodextrin limits its further application in pharmaceutical formulations [5]. The β-Cyclodextrin, a family of cyclic amylose-derived oligomers with a hydrophilic outer surface and a lipophilic central cavity, are well known for its abilities to form inclusion complexes with various drug molecules. Generally the formation of host-guest inclusion complex has the capability to alter the physical, chemical and

biological properties of drug moieties. Supramolecular system deals with β-Cyclodextrin form an important building block, as they can form inclusion complexes with hydrophobic drug molecules. This attitude has been utilized for various applications such as drug delivery **[6],** nano-structures **[7],** supramolecular polymers **[8],** self-healing materials **[9],** amphiphiles **[10],** and bioactive materials **[11]** or in the solubilization of hydrophobic monomers or RAFT agents **[12, 13].** Now a day, breast cancer is one of the major diseases in worldwide and it affects all human body. It occurs, 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 this paper, we tried our research to pharmaceutical field, particularly in breast-cancer research. We strongly believed that this article most hopeful for anticancer research in future.

In our earlier results we mainly focused the interaction of some organic compounds with β-CD and their proton shifts in liquid medium **[14-16].** Our aim of the present work is to evaluate the complex formation between AP with β-CD in aqueous solution. Here, we report the complex formation between AP and β-CD in liquid as well as solid state too with the accordance of characterization of the solid complex. Furthermore, we performed the In vitro CT effect of pure AP and their solid complex against MCF-7 (Human Breast carcinoma) cell line.

1. MATERIALS AND METHODS

2.1. Materials

AP and β-CD are purchased from Sigma-Aldrich chemical company. The all experimental solutions are prepared by using double distilled water. All other chemicals and solvents are used the Spectrograde. The molarity of AP solution (3×10^{-4} M) and the different concentration of β-CD solutions ($0-1.2\times10^{-3}$ M) are prepared by using double distilled water. The solutions are prepared just before taking UV and fluorescence measurements. 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 Instruments

The UV–visible spectra and fluorescence spectra measurements are recorded on KJENA model SPECORD 200 PLUS double-beam UV Visible spectrophotometer and Perkin Elmer spectro fluorometer. The pH of the solutions is measured on Elico pH meter L1–120.

2.3. Preparation of inclusion complexes in solutions

Double distilled water is used for the preparation of aqueous solutions. The concentration of stock solution of the AP is 3×10^{-4} mol dm^{-3}. It is made up to the following various concentration of β-CD solutions 0, 0.002, 0.004, 0.006, 0.008, 0.010 and 0.012 mol dm^{-3}, respectively. About 0.3 ml of AP solution is added to all the β-CD concentration to reach the final concentration of AP in each flask is 9.0×10^{-4} mol dm^{-3}. The experiments are carried out at room temperature 303K.

2.4. Preparations of solid inclusion complexes

The solid inclusion complexes of AP and β-CD are prepared as follows.

2.4.1 Preparation of PM of the AP and β-CD

Accurately weighed 0.50 g of AP and 0.50g of β-CD are exactly weighed. The PM of AP and β-CD with 1:1 molar ratio is prepared by grinding in a ceramic mortar.

2.4.2 Preparation of the AP and β-CD by Co- Precipitation method

The inclusion complex of AP and β-CD is prepared by co-precipitation method. Accurately weighed (0.50g) of β-CD is dissolved in double distilled water to become clear solution. Accurately weighed

0.50 g of AP is put into a 50 ml beaker and 20 ml distilled water added, yielding a clear white color solution. Then the AP solution is slowly poured into the β-CD solution. The above mixture is allowed to constantly stirred for 48 hours at room temperature. The reaction mixture is kept in a refrigerator for 24 hours. After 24 hours, the solution becomes as a white precipitate and then it is slowly changing to a clear solution. It is subjected to Freeze drying method which is known as lyophilisation. Then it gives the white product called as solid inclusion complex between AP and β-CD.

2.5. Preparation of the 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 0.1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions are prepared for carrying out the cytotoxic studies.

The monolayer cell culture is trypsinized and the cell count is adjusted to 1.0×10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) is added. After 24 h, when a partial monolayer is formed, the supernatant is flicked off, ished the monolayer once with medium and 100 μl of different test concentrations of test drugs are added on to the partial monolayer in microtitre plates. The plates are then incubated at 37^0C for 3 days in 5% CO_2 atmosphere, and microscopic examination is carried out and observations are noted every 24 h intervals. After 72 h, the drug solutions in the wells are discarded and 50μl of MTT in PBS is added to each well. The plates are gently shaken and incubated for 3 h at 37^0C in 5% CO_2 atmosphere. The supernatant is removed and 100 μl of propanol is added and the plates are gently shaken to solubilize the formed formazan. The absorbance is measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition is calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{an OD of control group}} \times 100$$

3   RESULTS AND DISCUSSION

3.1 Interaction of AP with β-CD in solution state

Figure 1 shows the absorption spectra of AP with various concentrations of β-CD at pH 6.5. Upon gradual increasing the concentration of β-CD, the absorption maxima is red shifted and hyperchromic shift is observed in spectral bands of AP, which is resulted due to host-guest interaction between AP and β-CD. The absorption spectral maxima of AP with increase concentrations of β-CD is given in Table 1. This result indicates that the improved dissolution of the guest molecule by inclusion complexation reaction [17] through the hydrophobic interaction of AP with β-CD. The binding constant and stoichiometry of inclusion complex formation can be determined from the changes in absorption maxima and its absorbance of AP by the addition of β-CD using Benesi-Hildebrand equation [BH]. The BH equation for inclusion complex of AP in with β-CD medium is assumed as 1:1 and it is given in equation (1)

$$1/(A-Ao) = 1/\Delta\varepsilon + 1/K[AP]o\, \Delta\varepsilon[\beta\text{-CD}]_0 \tag{1}$$

Where, (A-Ao) is the difference between the absorbance of AP, in the presence and absence of β-CD. Δε is the difference between the molar extinction co-efficients of AP in the presence and the absence of β-CD. [AP] o and [β-CD] o are the initial concentrations of AP and β-CD, respectively.

A good linear plot ($R^2 = 0.99$) is obtained, when 1/ (A-Ao) is plotted against 1/ [β-CD] shown in **Inset figure 1**. This result shows that the inclusion complex between AP and β-CD is found to be 1:1 stoichiometry. The binding constant value 'K' is determined from the slope of the linear plot, according to the equation (2) **[18-20]** and it is found to be 87.30M^{-1} at 303K

$$K = 1/ \text{Slope} [A'-Ao] \tag{2}$$

Table 1 Absorption and fluorescence spectral maximum of AP with different concentrations of β-CD at pH 6.5

S.No	Conc. of β-CD (M)	Absorbance Spectrum		Fluorescence Spectrum	
		λ_{max} (nm)	Absorbance	λ_{flu} (nm)	Intensity
1	0	240	0.38	348.5	2.618
2	0.002	240	0.40	348.5	3.629
3	0.004	240	0.44	348.5	4.793
4	0.006	240	0.47	348.5	5.487
5	0.008	240	0.48	348.5	5.687
6	0.010	240	0.55	348.5	6.409
7	0.012	240	0.66	348.5	8.450
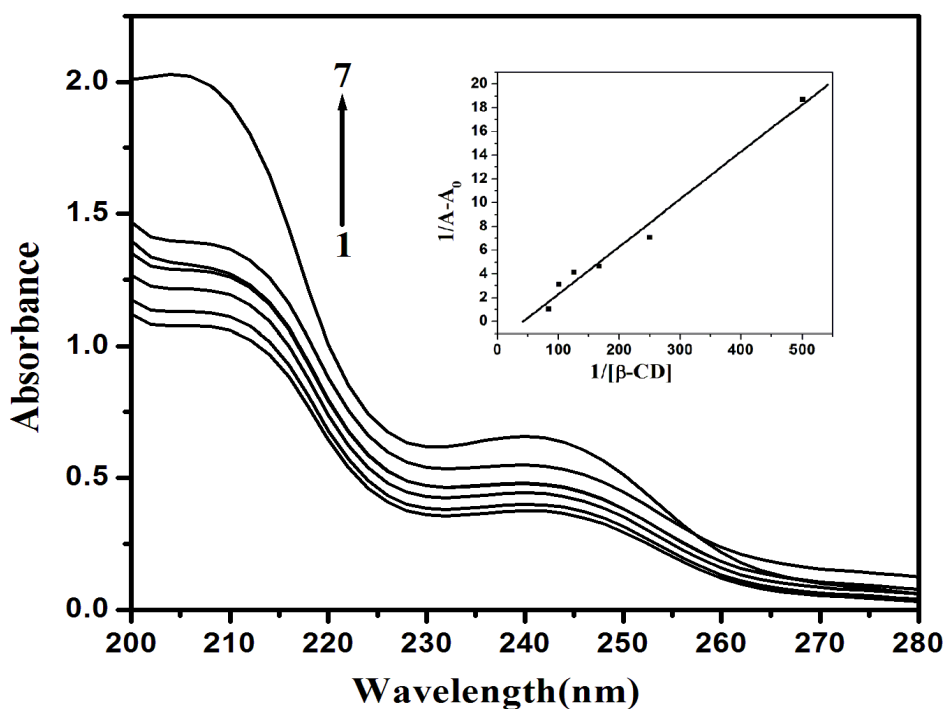
Fig. 1 Fluorescence spectra of AP in different β-CD concentrations (M): (1) 0, (2) 0.002, (3) 0.004, (4) 0.006, (5) 0.008, (6)0.010 and (7) 0.012. **Inset figure:** Benesi–Hildebrand fluorescence plot for 1:1 complexation of INS with β-CD

The fluorescence spectra of AP in aqueous solution containing various concentrations of β-CD are shown in **Figure 2**. The enhancement of fluorescence intensity with red shift in fluorescence maximum of AP while increasing concentrations of β-CD up to 1.2×10^{-3}M results the formation of the inclusion complex between AP and β-CD (**Table 1**). If increase the fluorescence intensity with remarkable shift in spectral maxima of the guest molecule by the addition of β-CD, indicated that the formation of the inclusion complex **[21-23].** The interaction of AP with β-CD can be analyzed by BH equations for 1:1 **[18-20]** stoichiometric ratio following equation (3).

$$1/I-I_o = 1/I'-I_o + 1/K \ (I'-I_o) \ [\beta\text{-CD}] \tag{3}$$

Where 'K' is binding constant, I_o is the intensity of fluorescence of AP in the absence of β-CD. I is the intensity of certain concentrations of β-CD and I' is the fluorescence intensity of AP with the highest concentration of β-CD. A good linear plot is observed, when $1/I-I_o$ is plotted against $1/[\beta\text{-CD}]$ (**Inset.Fig.2**). From the linear plot, the complex has stoichiometry. The binding constant 'K' is calculated from the slop of the linear plot by using the equation (4) and it is found to be $93.32M^{-1}$

$$K = 1/ \ Slope \ [I'-I_o] \tag{4}$$

The Gibbs free energy change (ΔG) for this inclusion process has been calculated from equation (5) at 303K.

$$\Delta G = - \ 2.303 \ RT \ log \ K \tag{5}$$

Where, ΔG is the Gibbs free energy change, R is the gas constant [J/mol.K], T is the temperature (K) and "K" is the binding constant in mol^{-1}. The ΔG is calculated from the "K" value obtained from absorption and fluorescence data are -11.26 and -11.43 KJ mol^{-1}.KJ mol^{-1}, respectively. The ΔG values are negative, which indicates that the inclusion complexation proceeds spontaneously at 303 K.
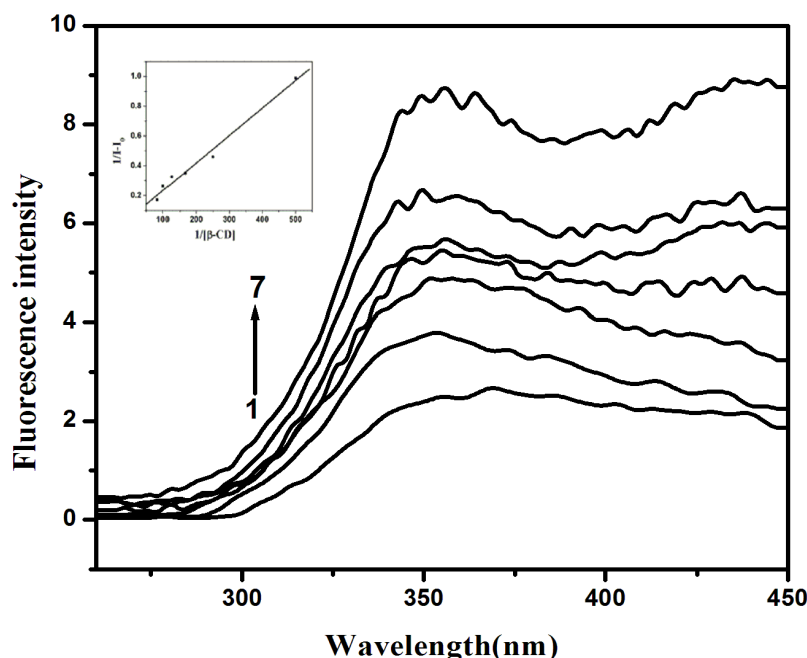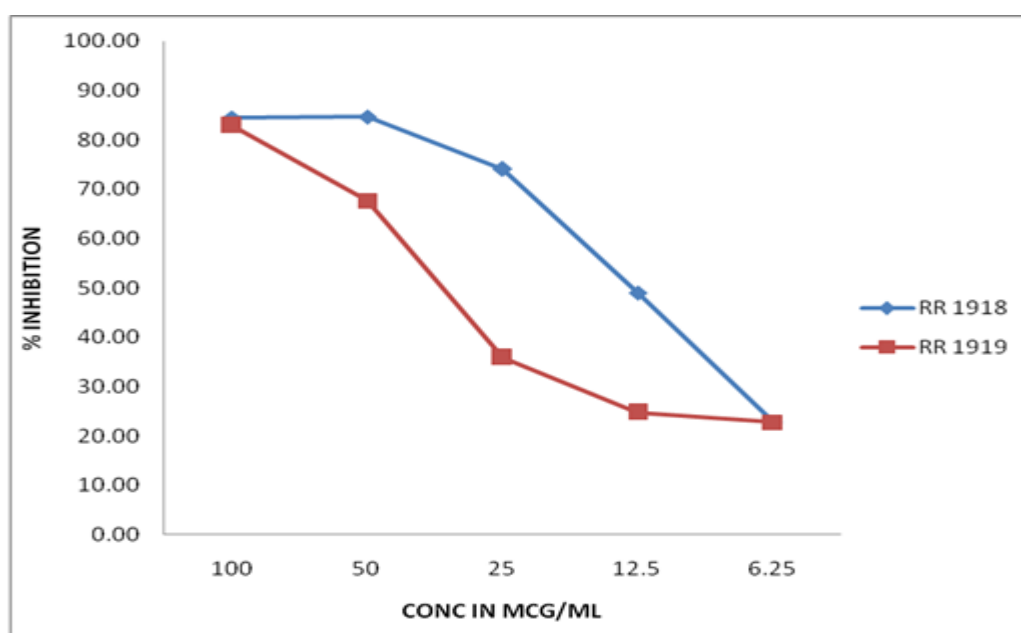

Fig. 2 Fluorescence spectra of AP in different β-CD concentrations (M): (1) 0, (2) 0.002, (3) 0.004, (4) 0.006, (5) 0.008, (6)0.010 and (7) 0.012. **Inset figure:** Benesi–Hildebrand fluorescence plot for 1:1 complexation of INS with β-CD

3.2. In-Vitro Cytotoxic Ability

In this study, the cytotoxic (CT) effect of AP and their solid complex is examined. These two samples are tested against the MCF-7 cell line at a range of 6.25 to 100 µg/ml using MTT assay. The various concentrations of pure AP and their solid complexes are prepared as 6.25, 12.5, 25, 50 and 100 µg/ml. CT effect of pure AP and their solid complex are presented in Table 2. At lowest concentration, the percentage of inhibition is around 5% for pure AP. The gradual increases in the percentage inhibition of pure AP while increasing their concentration. Around 60% of CT effect is observed at the highest concentration of pure AP (100 µg/ml). For solid complex, the percentage of inhibition is almost equal with pure AP up to their concentration reached 25µg/ml. The sudden increase in percentage of inhibition is noted at 50 mg/ml for solid complex. Around 40% higher inhibitions are observed in solid complex than that of pure AP. A further increase in the concentration of solid complex, the CT effect is again increasing up to 20%. The graphical representation of CT effect is displayed in Figure 3. The related photos are also presented in Figure 4. Generally the substance is suitable for CT effect, if their CTC_{50} value is less than 100. The lowest CTC_{50} value is adopted for the activity of against cancer cell lines. Hence CTC_{50} value is directly proportional to the percentage of inhibition. Here, we observed the CTC_{50} value is 13.00 for AP pure while it is 36.00 for the solid complex. Hence, β-CD encapsulation enhances the CT effect of pure AP.

Table 2 Cytotoxic Properties Of Test Drugs Against MCF-7 Cell Line

Sl. No	Name of the sample	Test Conc. (%)	% Cytotoxicity	CTC_{50} (µg/ml)
1	Pure AP compound	100 50 25 12.5 6.25	84.47±0.2 84.70±0.9 74.06±0.9 48.98±0.8 22.75±4.3	13.00±0.5
2	Inclusion complex	100 50 25 12.5 6.25	82.88±0.5 67.69±5.1 35.89±2.4 24.80±4.8 22.75±5.8	36.00±2.6

Fig. 3 Cytotoxic effect of the sample RR 1918 (AP), RR 1919 (complex) on MCF-7 Cell line
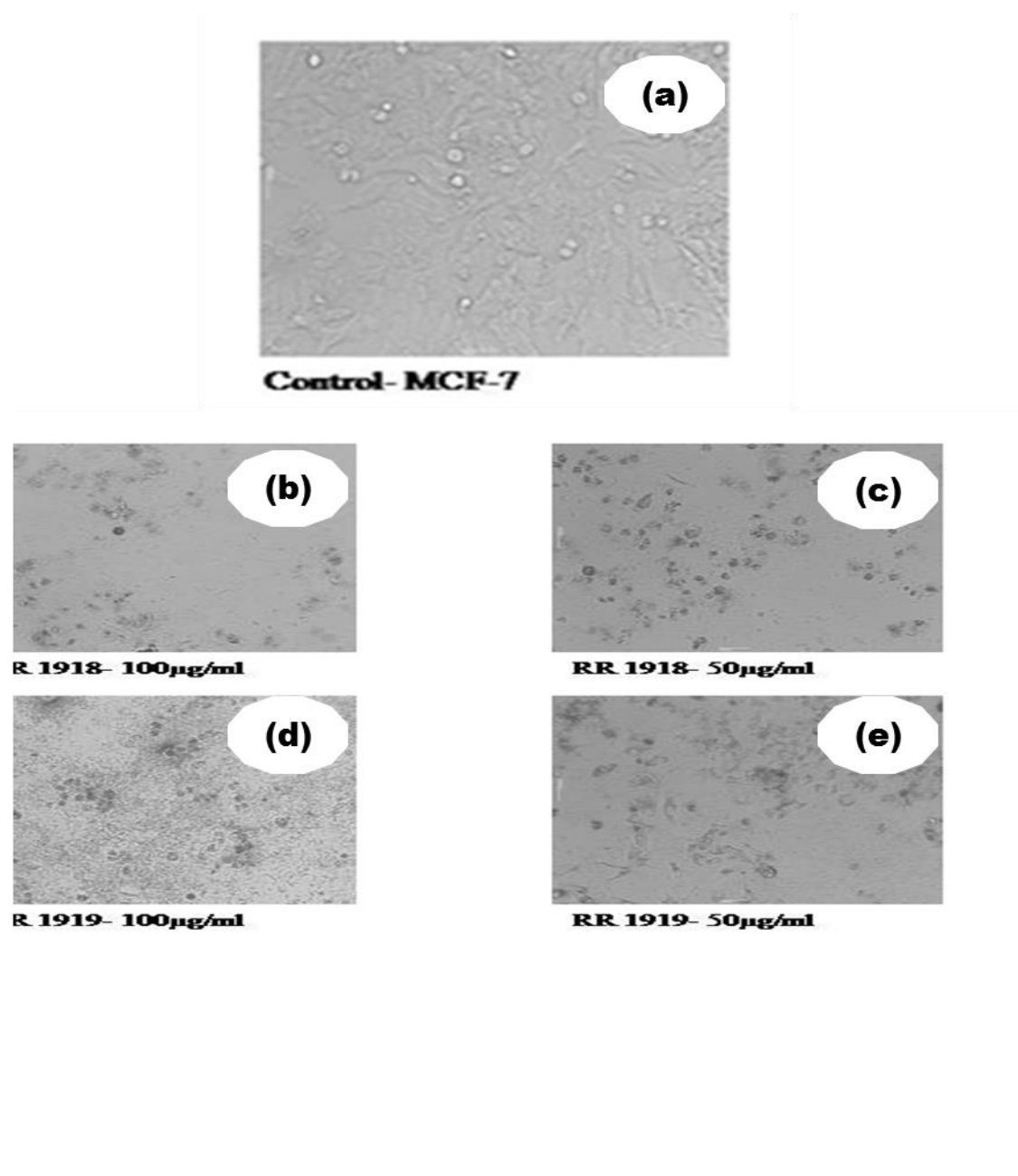
Fig. 4 Photos of (a) MCF-7 Control, (b) AP Pure-100μg/ml, (c) AP Pure -50 μg/ml, (d) Solid inclusion Complex -100 μg/ml, (e) Solid inclusion Complex -50 μg/ml

4. CONCLUSIONS

AP molecule forms stable inclusion complex with β-CD in liquid state. The enhancement of absorbance and intensity of AP by the addition of β-CD confirms the formation of the inclusion complex. The inclusion complex is formed as a 1:1 stoichiometry of AP with good binding constant value. Furthermore, in-vitro cytotoxic behavior also performed for pure AP and their solid complex against the MCF-7 cell line and resulted that the retaining the ability for both the samples.

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