

A Unique Polymeric Nano-carrier for Anti-tuberculosis Therapy

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Experimental section:

Materials:

Exo-oxabicyclo-[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride were prepared following the reported procedure. All reagents Furan, Maleic anhydride, dicyclohexyl carbodiimide (DCC), 4-dimethylamino-pyridine(DMAP), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, 1-hydroxybenzotriazole (HOBT), tri fluoroacetic acid (TFA), retinal, and tert-butyl carbazate were purchased from Sigma Aldrich and used as received without further purification. Amino benzoic acid, 3-amino benzaldehyde ethylene acetal, mono methyl ether poly (ethylene oxide) (PEO-OH, Mn = 1100) from Alfa Aeser and Acros Organics and used without further purification. Dichloromethane (DCM) and dimethyl formamide (DMF) were dried with calcium chloride (CaCl₂) and calcium hydride CaH₂ respectively and then distilled prior to use. All deuterated solvents CDCl₃, D₂O and DMSO-d₆ were purchased from Chembridge Isotope Laboratories. All other solvents and reagents of synthesis and analytical grade were used as received unless otherwise mentioned.

For Cell Studies: Dulbecco's modified Eagle's medium (DMEM), minimal essential medium (MEM), penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Invitrogen. 3-(4, 5 dimethyl-2-thiazolyl)-2, 5-diphenyl-2H- tetrazolium bromide (MTT) were purchased from USB (Cleveland, OH). Vectashield mounting medium with DAPI (Vector Laboratories).

Characterization:

Gel Permeation Chromatography (GPC). Molecular weights and PDIs were measured by Waters gel permeation chromatography in THF relative to PMMA and PS standards on systems equipped with Waters Model 515 HPLC pump and Waters Model 2414 Refractive Index Detector at 35 °C with a flow rate of 1 mL/min. HRMS analyses were performed with Q-TOF YA263 high resolution (Waters Corporation) instruments by +ve mode electrospray ionization.

Fluorometry. Fluorescence emission spectra were recorded on a Fluorescence spectrometer (Horiba Jobin Yvon, Fluoromax-3, Xe-150 W, 250-900 nm).

Nuclear Magnetic Resonance (NMR). The ¹H NMR spectroscopy was carried out on a Bruker 500 MHz spectrometer using CDCl₃, D₂O and DMSO-d₆ as a solvent. ¹H NMR spectra of solutions in CDCl₃, D₂O and DMSO-d₆ were calibrated to tetramethylsilane as internal standard (δH 0.00).

Fourier Transform Infra Red (FT-IR). FT-IR spectra were obtained on FT-IR Perkin-Elmer spectrometer at a nominal resolution of 2 cm⁻¹.

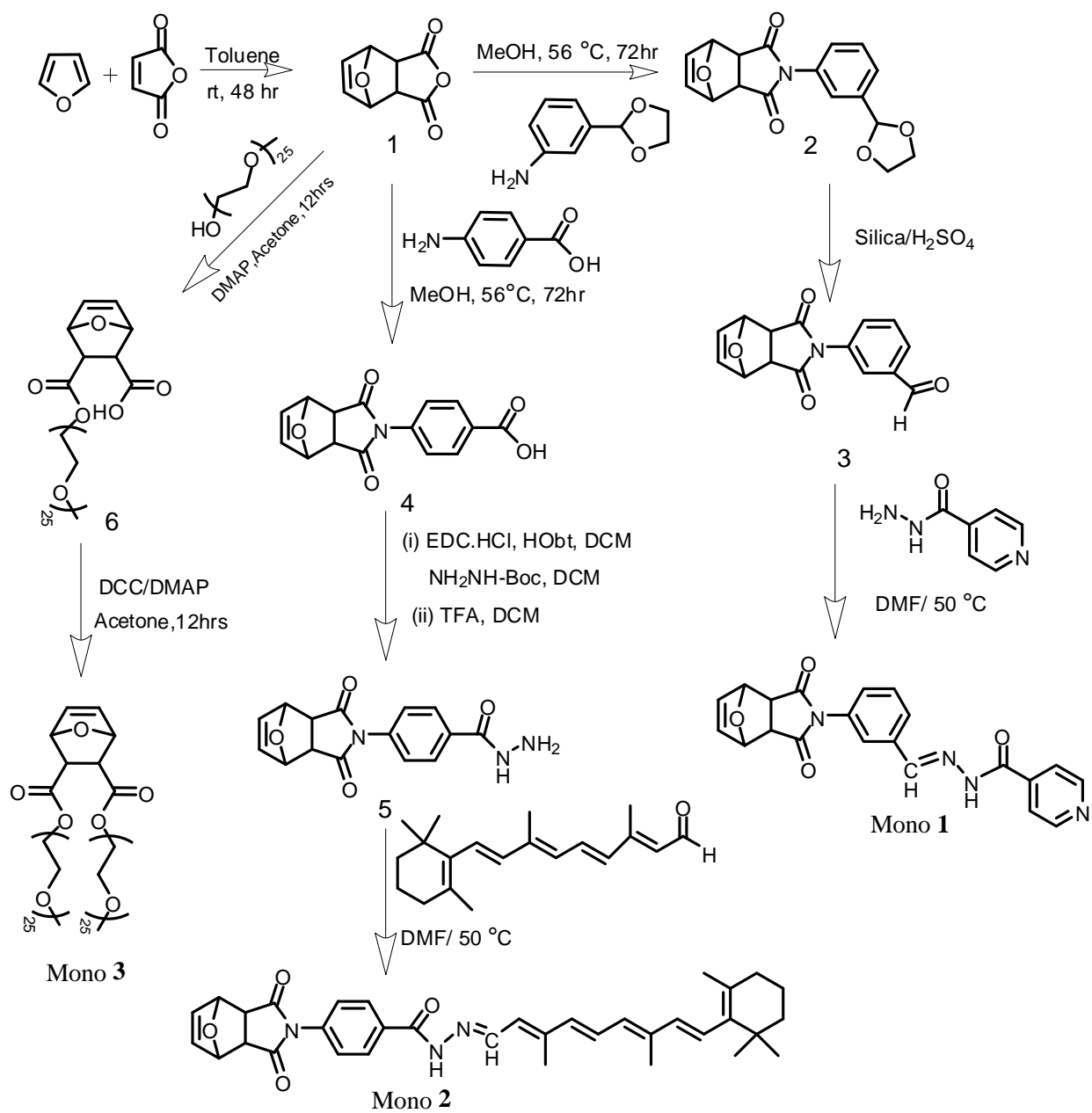
Ultra Violet (UV) Spectroscopy. UV-visible absorption measurements were carried out on U-4100 spectrophotometer HITACHI UV-vis spectrometer, with a scan rate of 500 nm/min.

Dynamic Light Scattering (DLS). Particle size of QDs were measured by dynamic light scattering (DLS), using a Malvern Zetasizer Nano equipped with a 4.0 mW He-Ne laser operating at $\lambda = 633$ nm. All samples were measured in aqueous as well as methanol at room temperature and a scattering angle of 173° .

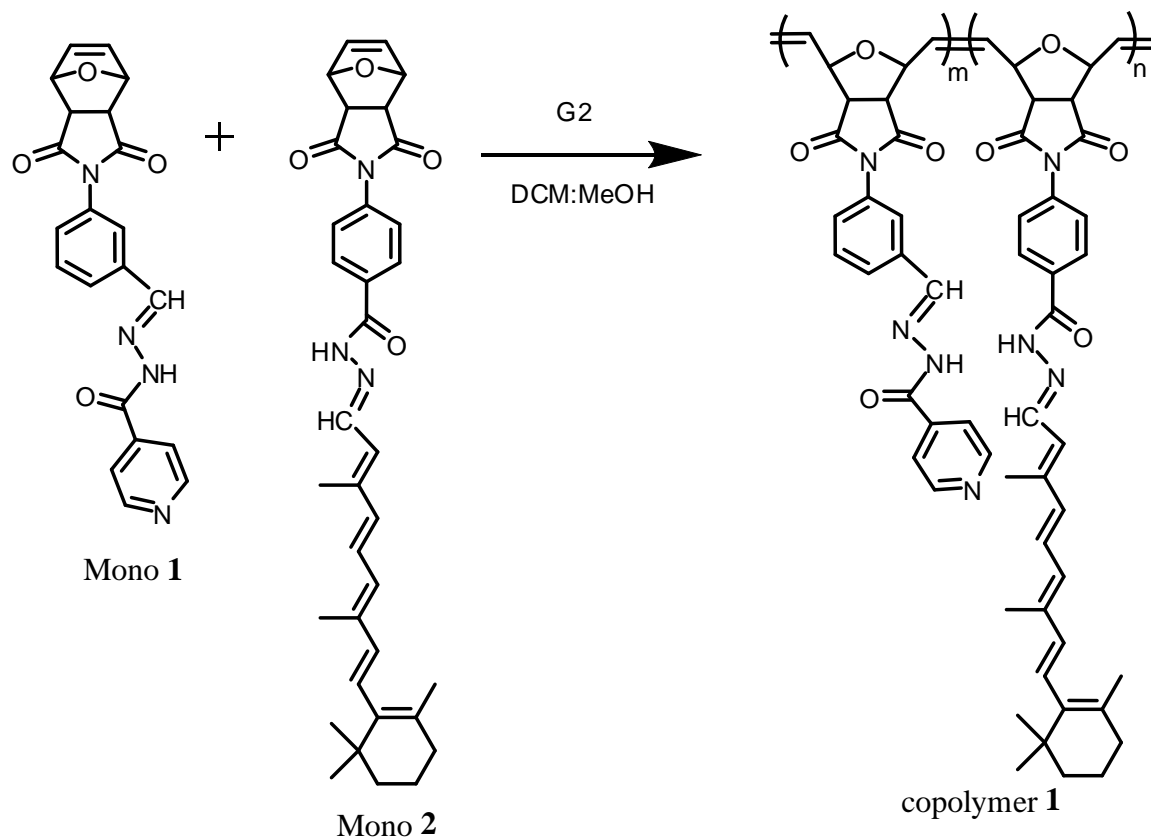
Transmission Electron Microscopy (TEM). Low resolution transmission electron microscopy (TEM) was performed on a JEOL 200 CX microscope. TEM grids were purchased from Ted Pella, Inc. and consisted of 3-4 nm amorphous carbon film supported on a 400-mesh copper grid.

Atomic Force Microscopy (AFM). The morphologies of the polymer was investigated from NT-MDT micro-40 AFM instrument using a semicontact mode at a scan rate of 1 Hz.

Synthesis Scheme 1: Monomers synthesis.



Scheme 2: Synthesis of CP 1.



Synthetic procedure:

Synthesis of compound 1 (SI Scheme1): A 10 g (102 mmol) of maleic anhydride was charged in a two neck round bottom flask. Charged 80 mL of toluene, stirred for 30 min and filtered. In filtrate 8.32 g (122.4 mmol) of furan was added. This solution was stirred for 48 h at room temperature. White colour solid was precipitated and filtered the solid and washed with cold toluene (90 % yield). ^1H NMR (500 MHz, CDCl_3): δ 6.56 (s, 2 H), 5.44 (s, 2H), 3.17(s, 2H); ^{13}C NMR (125 MHz, dmsO-d_6): δ 171.54, 137.20, 81.63, 49.08; MS (ESI) calculated for $\text{C}_8\text{H}_6\text{O}_4$ ($\text{M}^+\text{+H}$) 166.027, observed 166.

Synthesis of compound 2 (SI Scheme1): 1 g (6.024 mmol) of compound 1 and 0.993 g (6.024 mmol) of 3-amino benzaldehyde ethylene acetal was charged in two necks round bottom flask. Charged 25 mL of methanol. This solution was stirred at 56 °C for 3days and concentrated on under vacuum. Charged dichloromethane followed by water to the reaction mixture. Organic layer was washed with 2 x 30 mL of water followed by sodium bicarbonate wash. Finally organic layer was washed with brine solution. Organic layer concentrated under vacuum to yield a solid product. (700 mg, 70% yield). ^1H NMR (500 MHz, dmsO-d_6): δ 7.30-7.50 (d, 2H), 7.10-7.22 (d, 2H), 6.60 (s, 2 H), 5.43(s,1H), 5.25 (s, 2H), 3.23(m,4H), 3.07(s, 2H); ^{13}C NMR (100 MHz, dmsO-d_6): δ 176.68, 136.61, 134.65, 128.84, 126.80, 126.58, 101.89, 80.76, 64.85, 47.50; MS (ESI) calculated for $\text{C}_{17}\text{H}_{15}\text{NO}_5$ ($\text{M}^+\text{+H}$) 314.074, observed 314.

Synthesis of compound 3 (SI Scheme1): 1 g (3.19 mmol) of compound **2** was dissolved in 10 mL dichloromethane. Charged 1 g of H₂SO₄-silica and stirred for 3h at 40 °C. The reaction mixture was filtered. The filtrate was washed successively with Na₂S₂O₃ (2x20mL), saturated NaHCO₃ (2x20mL) and brine (20mL). The organic layer was separated, dried over Na₂SO₄ and evaporated under vacuum to yield greenish solid (80 % yield). ¹H NMR (400 MHz, dmsO-d₆) : δ 10.08(s, 1H), 7.98 (d, 1H), 7.76 (d, 2H), 7.58(d, 1H), 6.62 (s, 2 H), 5.27 (s, 2H), 3.12(s, 2H); ¹³C NMR (100 MHz, dmsO-d₆) : δ 192.54, 175.58, 136.89, 132.61, 130.06, 129.69, 127.00, 80.84, 47.65; MS (ESI) calculated for C₁₅H₁₁NO₄ (M⁺+H) 269.074, observed 269.61.

Synthesis of compound 4 (SI Scheme1): Exo - oxabicyclo- [2.2.1] hept-5-ene-2, 3 - dicarboxylic anhydride (compound **1**), 1.914 g (11.5 mmol) was charged in two neck reaction flask. Charged 35 mL of acetone and heated until it became clear solution. To this solution, charged 4- amino benzoic acid 1.605 g (11.5 mmol) with stirring. After fifteen minutes heating was stopped and reaction mixture was allowed to stir for about 30 minutes. The solid was filtered and dried under oven at 55 °C under vacuum. The dried intermediate was then dissolved in 30 mL of dimethyl formamide and heated to 50 °C. Acetic anhydride 15 mL (158.97 mmol) and sodium acetate 0.635 g (7.743 mmol) were charged under stirring. The reaction mixture was allowed to stir for three hours at 55 °C. After 3 h the reaction mixture was poured into 500 mL of water acidified by addition of 5 mL concentrated HCl. White colour solid was precipitated immediately and filtered the solid and washed with water and dried at 90 °C, under vacuum (80 % yield). ¹H NMR (DMSO-d₆, 400 MHz): δ 13.03 (bs, 1H), 8.0 - 8.2 (m, 2H), 7.4 - 7.5 (m, 2H), 6.6 (s, 2H), 3.1 (s, 2H). ¹³C NMR (DMSO-d₆, 400 MHz): δ 175.43, 166.59, 136.65, 135.78, 130.0, 126.79, 80.86, 47.58. IR (KBr, cm⁻¹): 3236, 2635, 2073, 1954, 1826, 1780, 1729, 1698, 1607, 1515, 1418, 1218, 1144, 1125, 1020, 975, 950, 912, 883, 878, 804, 726, 672, 633, 598, 541, 521. MS (ESI) calculated for C₈H₁₀O₂Na [M⁺ + H] 285.05, observed 284.95.

Synthesis of compound 5 (SI Scheme1): 1 g (6.92 mmol) of compound **4** was dissolved in 10 mL of dimethyl formamide. 0.85 g (4.46 mmol) of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC.HCl) and 1-hydroxybenzotriazole (HOBT) 0.62 g (4.46 mmol) in to the reaction mixture. Reaction mixture allowed to stir for 15 h at room temperature. Reaction mixture was cooled to 0-5 °C. Tertiary butyl carbazate was dissolved in dimethyl formamide and this solution was added to the reaction mixture at 0-5 °C. Reaction mixture was stirred for another 30 minutes at 0-5 °C. Charged ethyl acetate followed by water to the reaction mixture. Organic layer was washed with 2 x 30 mL of water followed by sodium bicarbonate wash. Finally organic layer was washed with brine solution. Organic layer concentrated under vacuum to yield a white colour solid. 500 mg (1.754 mmol) of this product was dissolved in 5 mL of dichloromethane at room temperature. Trifluoroacetic acid 6 mL was charged in to the reaction mixture. Reaction mixture stirred for 1 h at room temperature. Reaction mixture concentrated to pasty mass, and charged diethyl ether resultant white product was collected by suction filtration,

washed with 10 mL diethyl ether and dried at 40 °C under vacuum (420 mg, 84 % yield). ¹H NMR (500 MHz, dms_o-d₆): δ 10.92(s, 1H), 7.97 (d, 2H), 7.42(d, 2H), 6.66 (s, 2 H), 5.30 (s, 2H), 3.7 (bs, 2H), 3.12(s, 2H); ¹³C NMR (125 MHz, dms_o-d₆): δ 172.01, 169.20, 137.31, 132.01, 128.01, 82.10, 49.53; MS (ESI) calculated for C₁₅H₁₃N₃O₄ (M⁺+H) 299.091, observed 299.

Synthesis of Mono 1 (SI Scheme1): 0.2 g (0.743 mmol) of compound **3** was taken in two necks round bottom flask. Charged 5 mL of dry DMF followed by 0.102 g (0.743 mmol) of isoniazid and 0.1 mL triethyl amine. The reaction mixture was stirred for 24 h at 45 °C. Charged dichloromethane followed by water to the reaction mixture. Organic layer was washed with 2 x 30 mL of water followed by sodium bicarbonate and brine solution wash. Finally organic layer was dried over Sodium sulphate concentrated under vacuum to yield brownish solid (70 % yield). ¹H NMR (400 MHz, dms_o-d₆): δ 12.29(s, 1H), 8.90(s, 2H), 8.48 (s, 1H), 7.83 (d, 2H), 7.75(d, 1H), 7.68(s, 1H), 7.60(t, 1H), 7.31(d, 1H), 6.62 (s, 2 H), 5.29 (s, 2H), 3.11(s, 2H); ¹³C NMR (125 MHz, dms_o-d₆): δ 175.75, 165.59, 150.41, 147.87, 140.39, 136.70, 134.80, 132.74, 130.15, 129.74, 128.72, 121.62, 80.84, 47.66, MS (ESI) calculated for C₂₁H₁₆N₄O₄ (M⁺+H) 389.121, observed 389.

Synthesis of Mono 2 (SI Scheme1): 0.1 g (0.3344 mmol) of compound **5** was taken in two necks round bottom flask. Then 5 mL of dry DMF was charged, followed by 0.094 g (0.3344 mmol) of retinal and 0.1 mL triethyl amine was charged. Reaction mixture was stirred for 24 h at 45 °C. Dichloromethane was added to reaction mixture and washed with water (3x20 mL). Finally organic layer was dried over sodium sulphate and concentrated under vacuum to yield dark brown solid (70 % yield). ¹H NMR (500 MHz, dms_o-d₆): δ 10.35(s, 1H), 7.97 (d, 2H), 7.42(d, 2H), 7.28(d, 1H), 6.66 (s, 2 H), 6-6.42(m, 4H), 5.9-6.2(m, 2H), 5.30 (s, 2H), 3.12(s, 2H), 1.91(m, 6H), 1.5-1.7(s, 3H), 1.3-1.5(m, 6H), 1.16 (s, 6H); ¹³C NMR (125 MHz, dms_o-d₆): δ 172.01, 159.20, 139.2, 137.31, 132.01, 128.01, 124.08, 82.10, 49.53, 39.15, 33.02, 32.6, 28.18, 21.56, 21.4, 19.1, 13.3, 13.2; MS (ESI) calculated for C₃₅H₃₉N₃O₄ (M⁺+H) 565.291, found 565.872.

Synthesis of Mono 3 (SI Scheme1): 8.7 g of (7.9 mmol) of PEO-OH (M_n=1100 g/mol) was charged in a round bottom flask and heated at 120 °C for 3h. Reaction mass cooled to room temperature. 10 mL of acetone was added followed by 0.5 g (3.012 mmol) of 7-oxo-exo norbornene, 0.037 g of DMAP (10 MOL %, 0.3032 mmol) was added and the reaction was stirred for 12h at room temperature. To this reaction mixture 1 g (4.854 mmol) of DCC was charged and stirred for another 12h. Finally reaction mixture was filtered and purified by three time precipitations from diethyl ether (85 % yield). ¹H NMR (500 MHz, CDCl₃): δ 6.21(s, 2H), 4.27(m, 2H), 4.11 (m, 2H), 3.88 (m, 2H), 3.65 (s, 4H), 3.38 (s, 3H), 3.10 (s, 2H), 2.65 (s, 2H), 1.66 (d, J=1.2 Hz, 1H), 1.50(d, J=1.2 Hz, 1H). ¹³C NMR (125 MHz, CDCl₃): δ 171.54, 137.20, 81.63, 68.29, 66.11, 59.20, 49.08.

General Polymerization Procedure:

A known amount of monomers (Mono **1**: Mono **2** : Mono **3** i.e. 2:2:1) was weighed into a two neck round bottom flask, placed under an atmosphere of nitrogen, and dissolved in anhydrous dichloromethane (1mL). Into a separate flask, a required amount of second generation Grubbs' catalyst was added, flushed with nitrogen, and dissolved in a minimum amount of anhydrous dichloromethane and methanol (9:1 v/v %) solvent. The monomer was transferred to the flask containing the Grubbs' catalyst. The reaction was allowed to stir at room temperature until the polymerization is complete, after which ethyl vinyl ether (0.2mL) was added to quench the polymerization and An aliquot was taken for GPC analysis and the remaining product was precipitated from pentane.

Cytotoxicity assay of CP2. Cytotoxicity of **CP2** in Hela cell lines was quantitatively determined using MTT enzymatic and colorimetric assay. All cell lines were seeded at 1×10^4 cells /well in 96 well plates and maintained in culture for 24 hrs at 37 °C in their respective medium. After 24 hrs medium was removed, cells were washed with PBS and medium containing different concentration of **CP2** (1 μ g - 1 mg) were added to the designated wells. The whole experimental plate was incubated upto 72 hrs. Fresh 20 μ l of MTT from 5mg/ml stock solution were added to each well, followed by incubation for 4 hrs at 37 °C. After 4 hrs, medium from the wells were removed and 100 μ l of DMSO were added to each well and incubated for 15 mins to completely solubilize the cells. The absorbance of the resulting solution was measured at 515 nm, and cell survivals were determined by comparison of optical density with untreated respective control cell cultures.

Cell-growth inhibition assay by trypan blue exclusion method: Cells were seeded at 1.25×10^4 cells in 24 well tissue culture plates and the inhibition activities of cell growth and division of **CP2** polymer were quantitatively determined by visual cell counting using a haemocytometer chamber. After 24 hrs of cell plating different concentration of **CP2** (1 μ g - 1 mg) was added to the respective wells. Designated wells for control were maintained with respective medium without adding any **CP2**. Whole plate was incubated for additional 72 hrs. Cell counting was performed by trypan blue exclusion method at 24, 48 and 72 hrs. The extent of cell inhibition was determined by viable cell population counts and compared with untreated control cell culture for each time point.

Renal Clearance Experiment: 8 weeks old pathogen free C57BL/6 male mice were used from a breeding colony originally obtained from Jackson laboratory (Bar harbour, USA). All animals were maintained in accordance with protocols approved by the institutional animal ethical committee of IISER-Kolkata. The authorization to use C57Bl/6 laboratory strain mice was approved by CPCSEA, India. One mg of **CP2** was dissolved into 1 ml of demonized autoclaved H₂O-on the day of experiment. Working

dilution was 14.2 $\mu\text{g/ml}$ and from the working solution 0.5 ml was fed by stainless steel animal feeding needle to each mice of a group of 4 mice. Urine was collected just before the feeding (control) and then every one and half hrs interval of post feeding until 12 hrs continuously and then after 24, 48, and 72 hrs for fluorescence measurement.

Spectral Details:

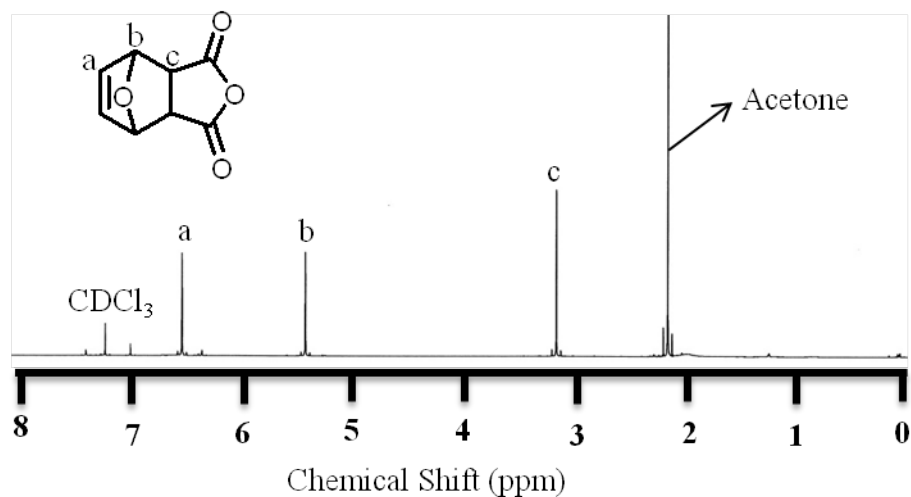


Fig S1: ^1H NMR spectrum of compound **1** in CDCl_3 .

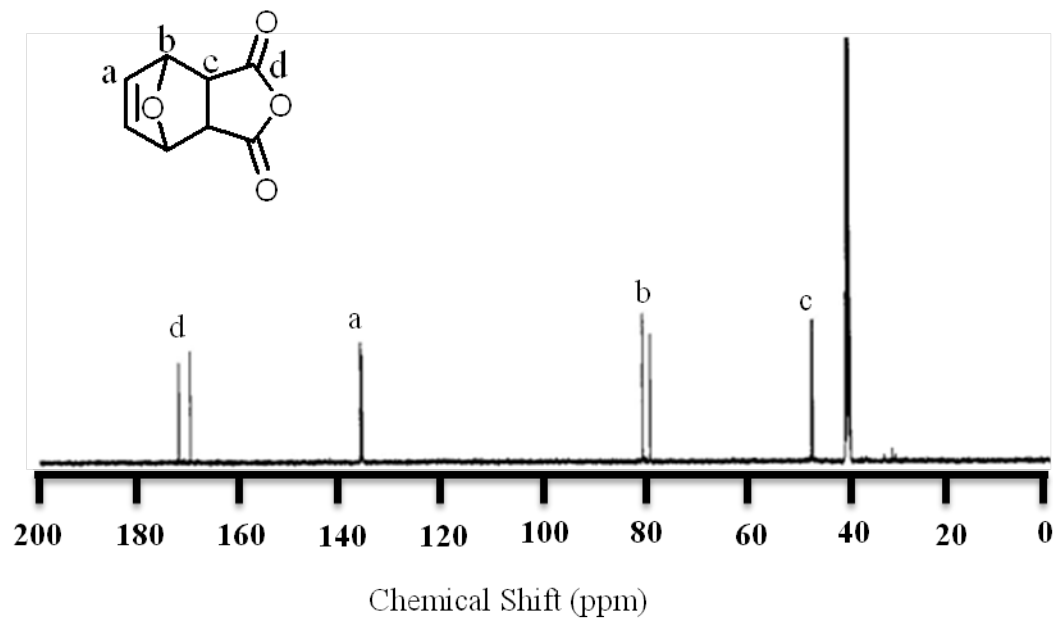


Fig S2: ^{13}C NMR spectrum of compound **1** in DMSO-d_6 .

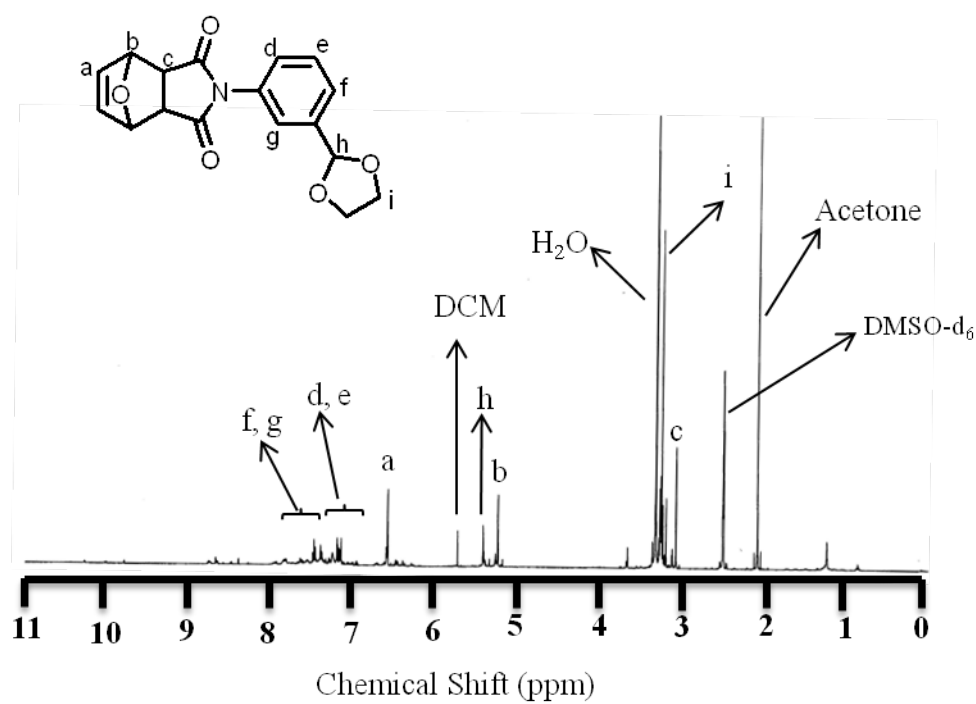


Fig S3: ^1H NMR spectrum of compound **2** in DMSO-d_6 .

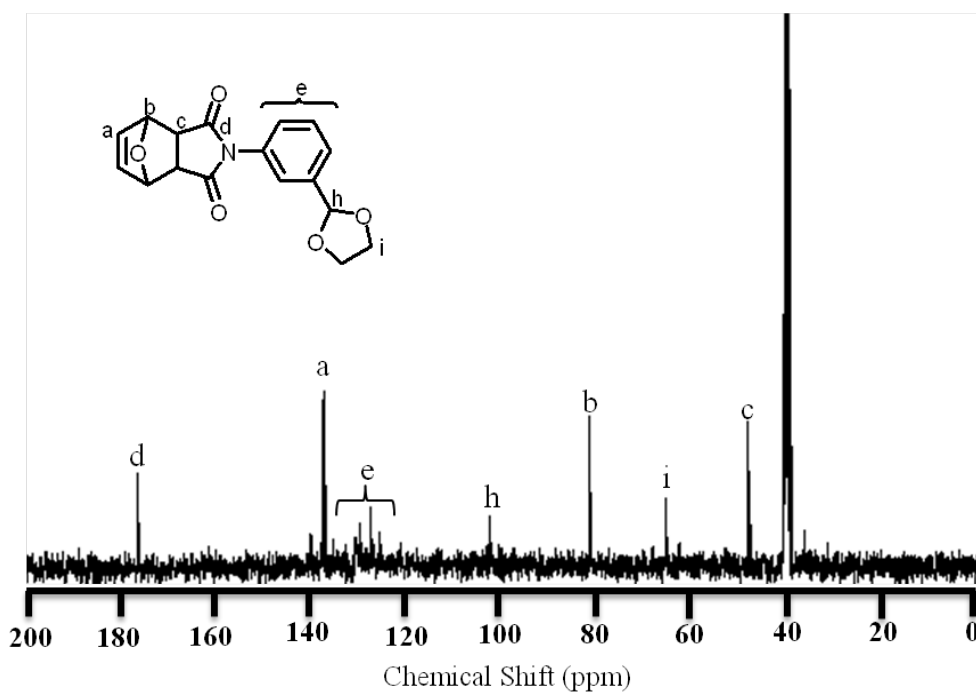


Fig S4: ^{13}C NMR spectrum of compound **2** in DMSO-d_6 .

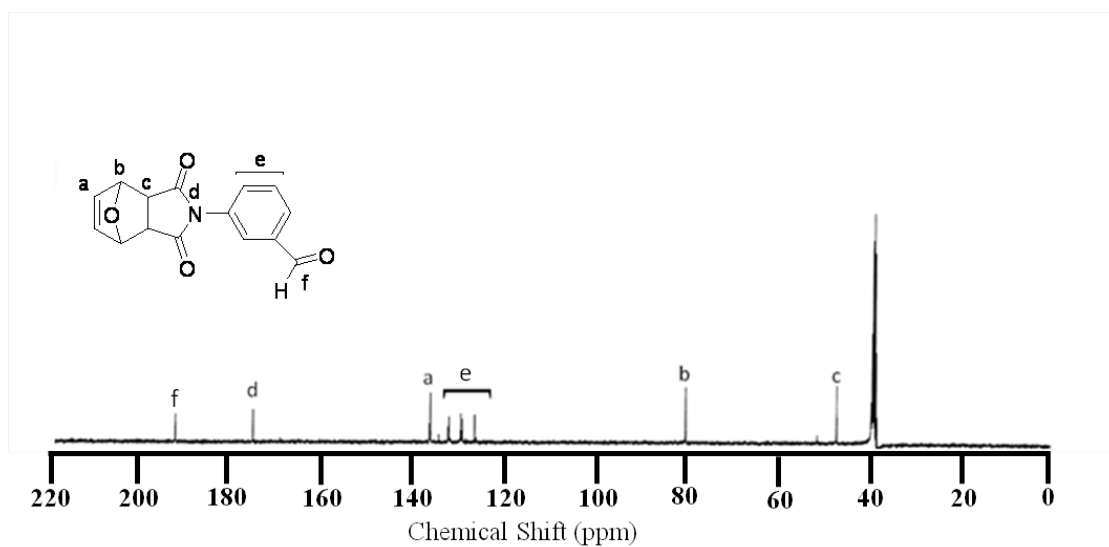


Fig S5: ^{13}C NMR spectrum of compound **3** in DMS-d_6 .

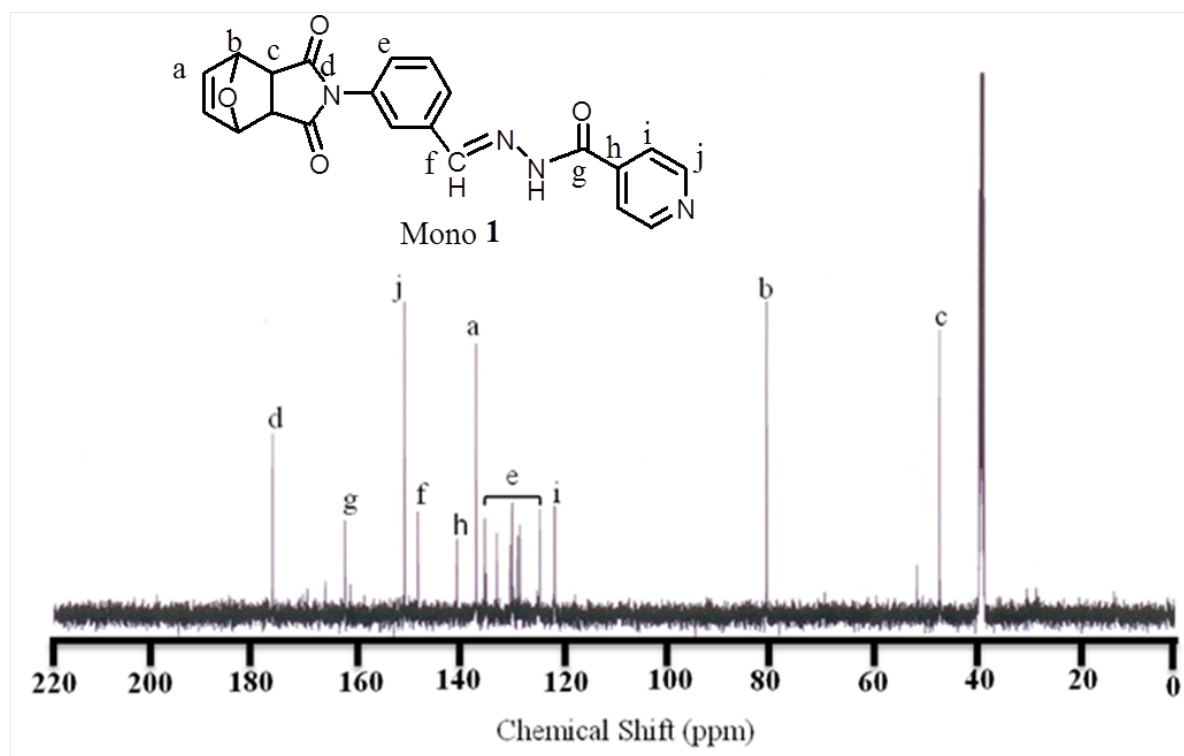


Fig S6: ^{13}C NMR spectrum of Mono **1** in DMSO-d_6 .

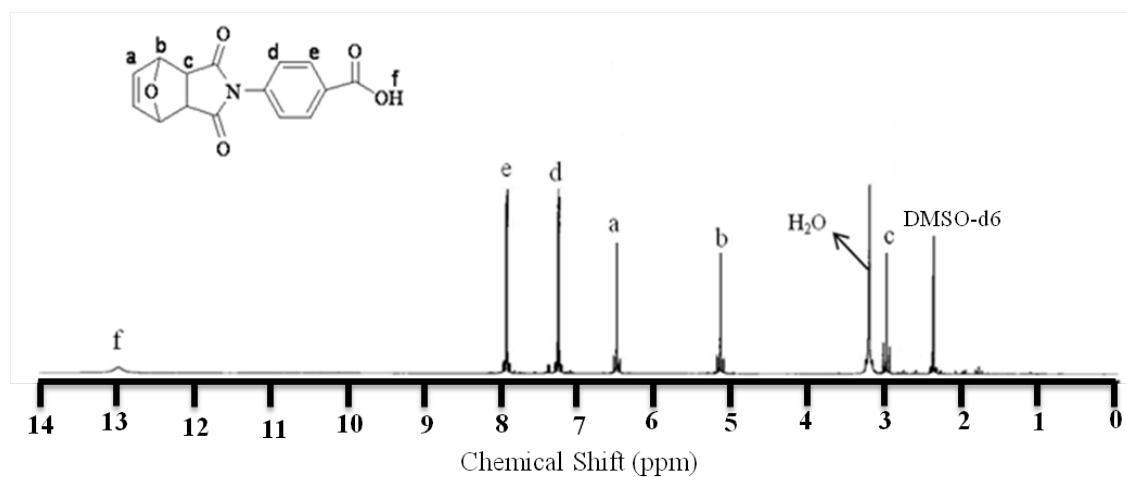


Fig S7: ^1H NMR spectrum of compound **4** in DMSO-d_6 .

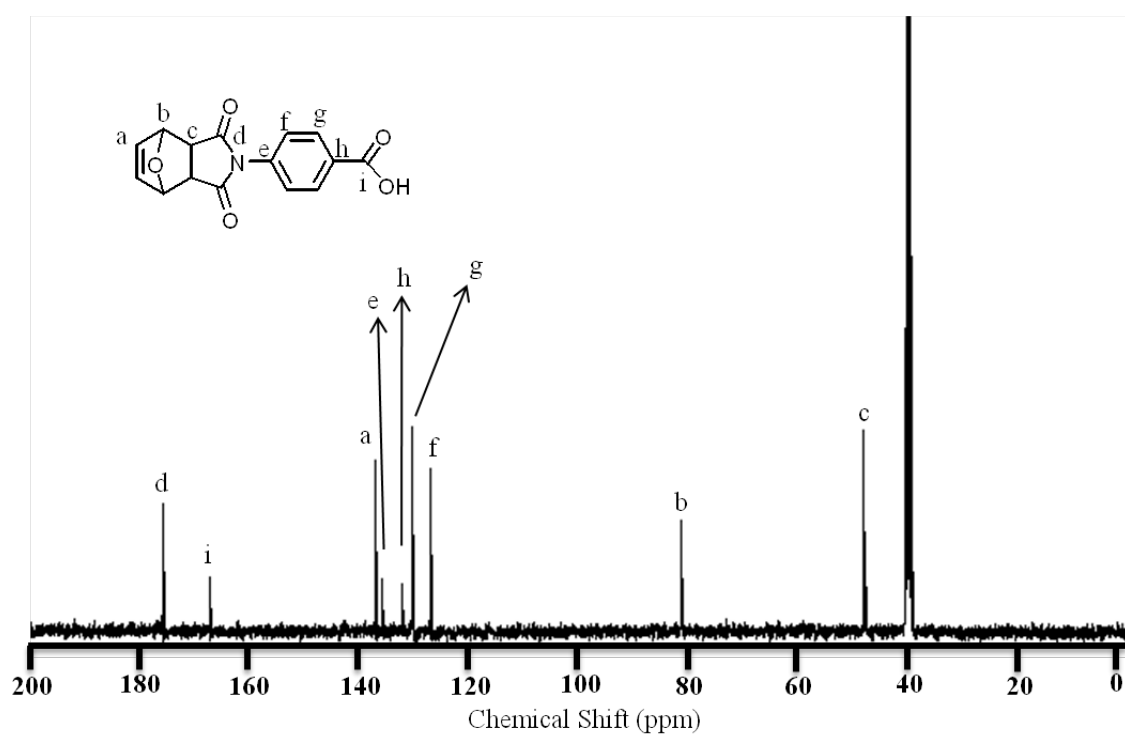


Fig S8: ^{13}C NMR spectrum of compound **4** in DMSO-d_6 .

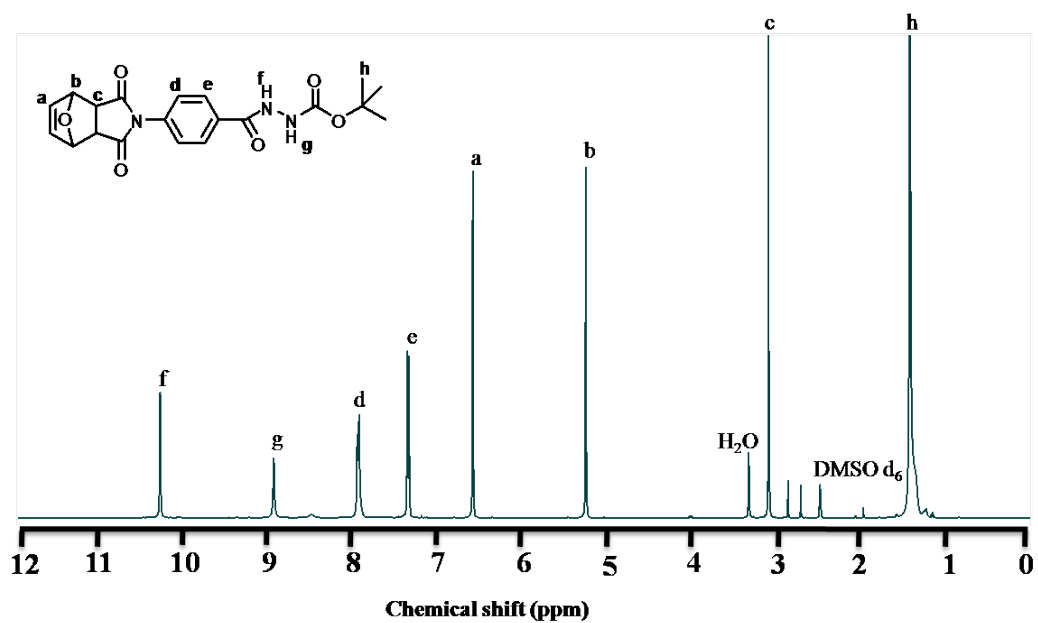


Fig S9: ¹H NMR spectra of tertiary butyl hydrazone Nadic carboxylate in DMSO-d₆.

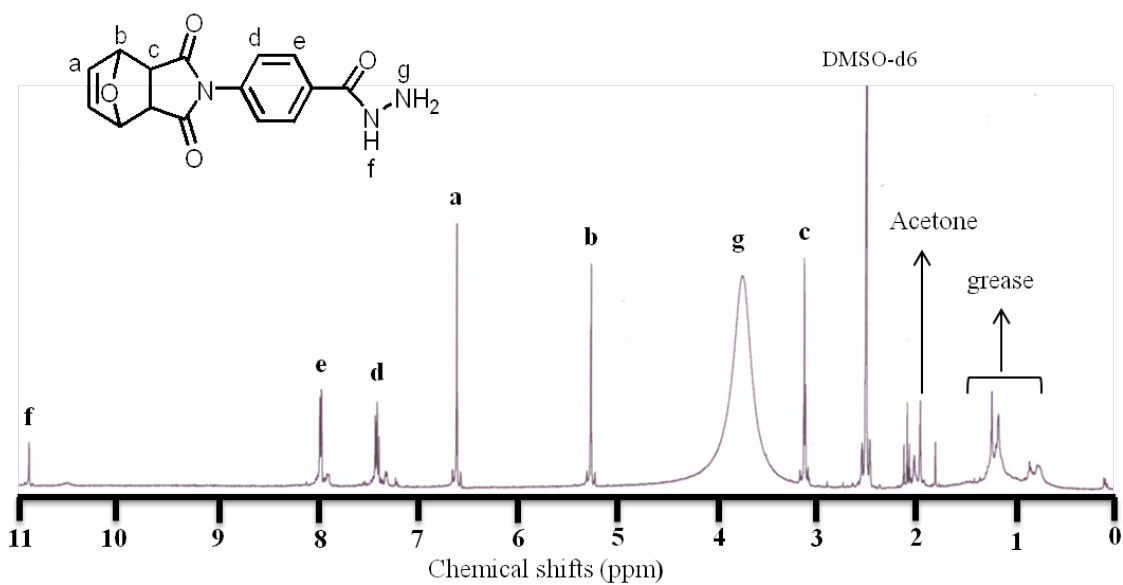


Fig S10: ¹H NMR spectrum of compound **5** in DMSO-d₆.

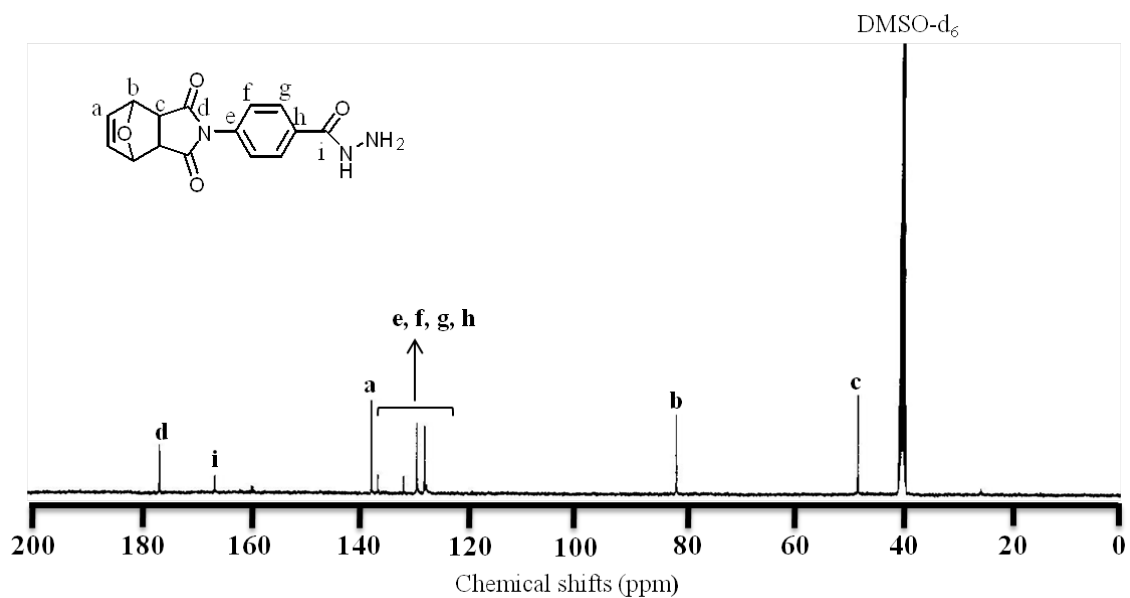


Fig S11: ¹³C NMR spectrum of compound **5** in DMSO-d₆.

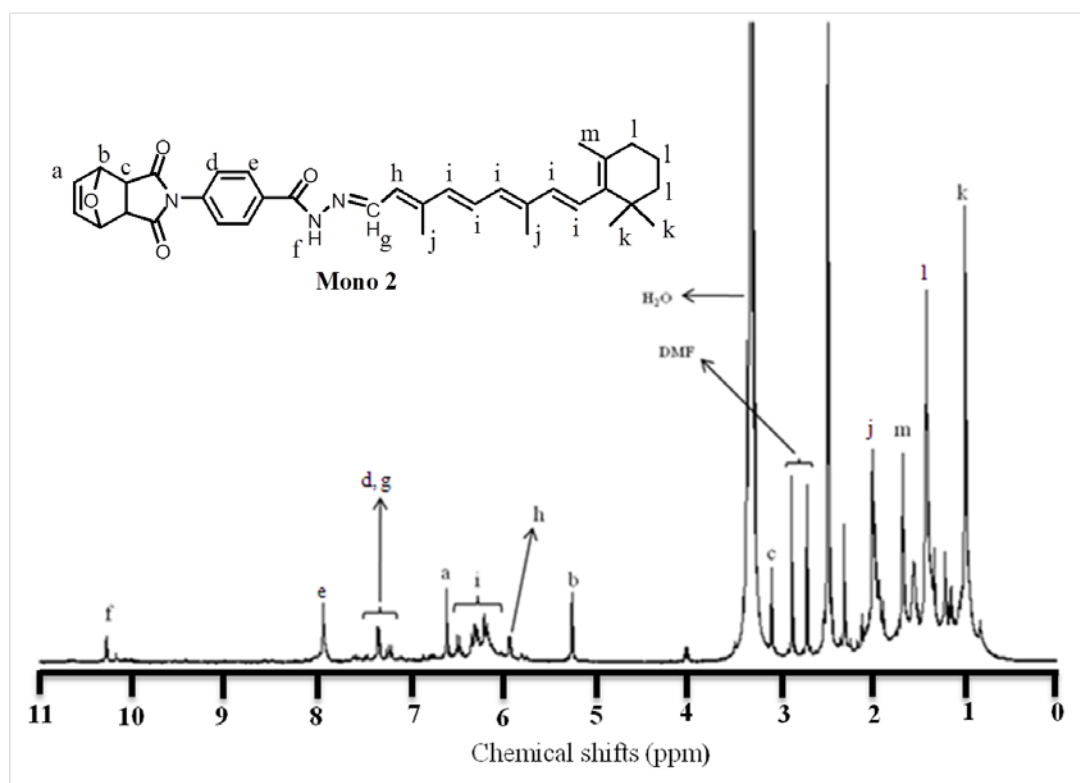


Fig S12: ¹H NMR spectrum of Mono **2** in DMSO-d₆.

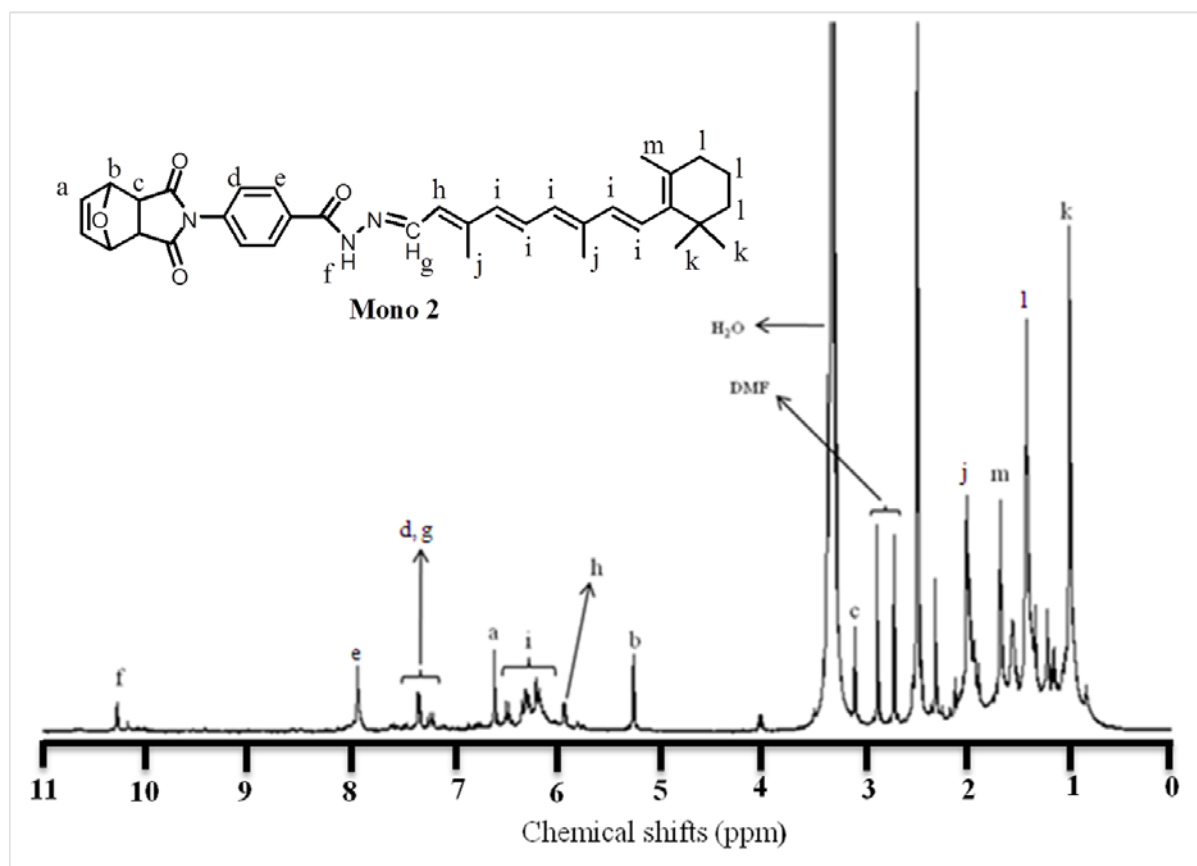


Fig S13: ¹³C NMR spectrum of Mono 2 in CDCl₃.

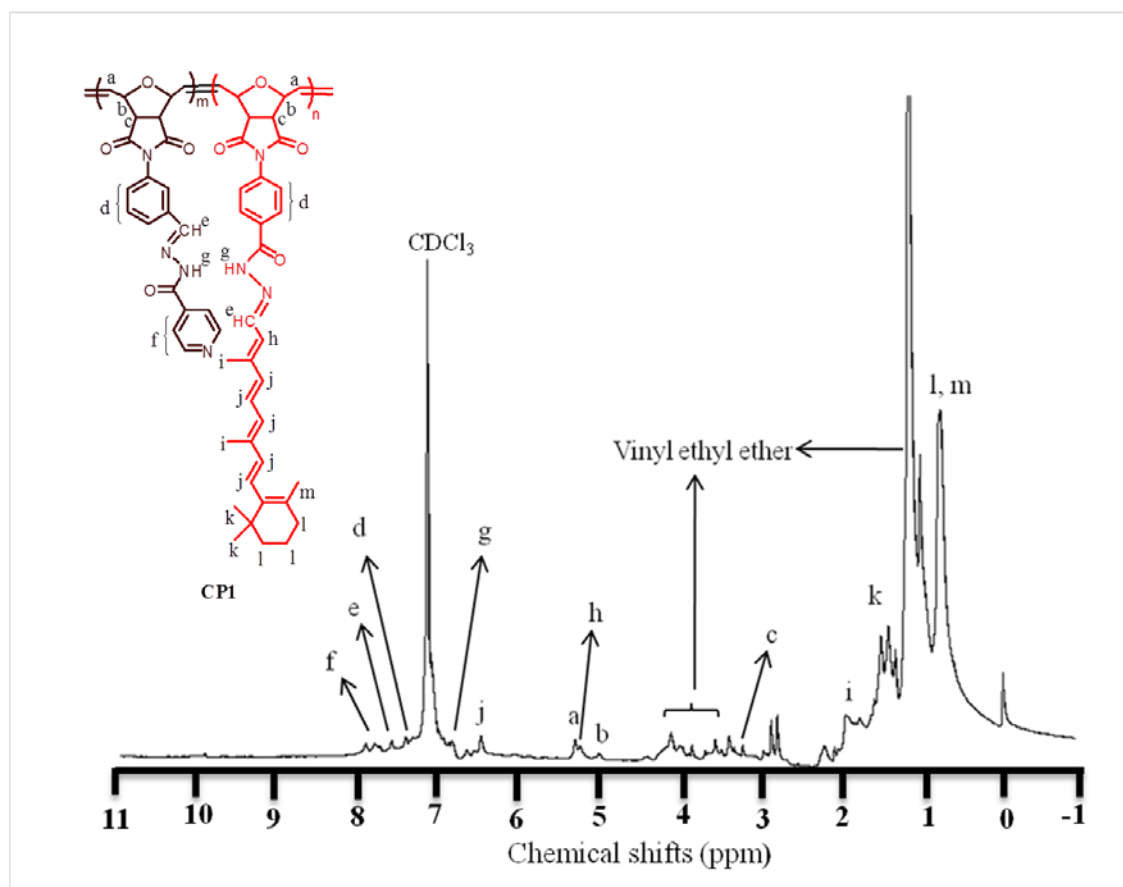


Fig S14: ¹H NMR spectrum of CP 1 in CDCl₃.

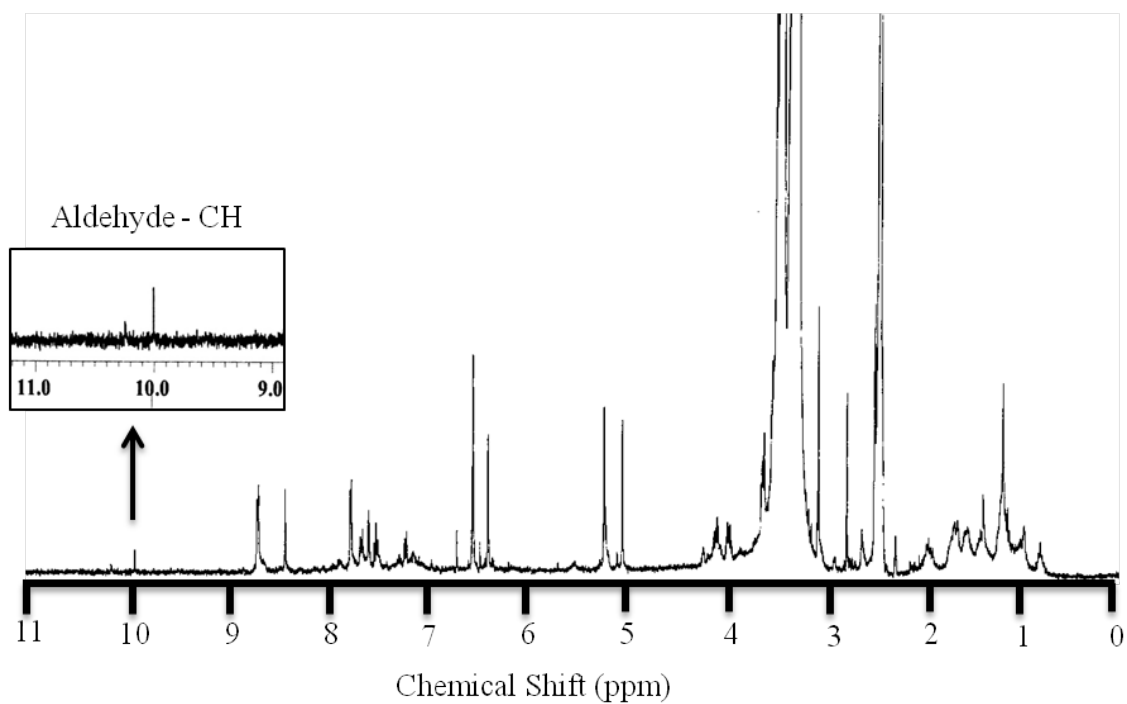


Fig S15: ^1H NMR spectrum of CP 2 in DMSO-d_6 after cleavage in acidic condition.

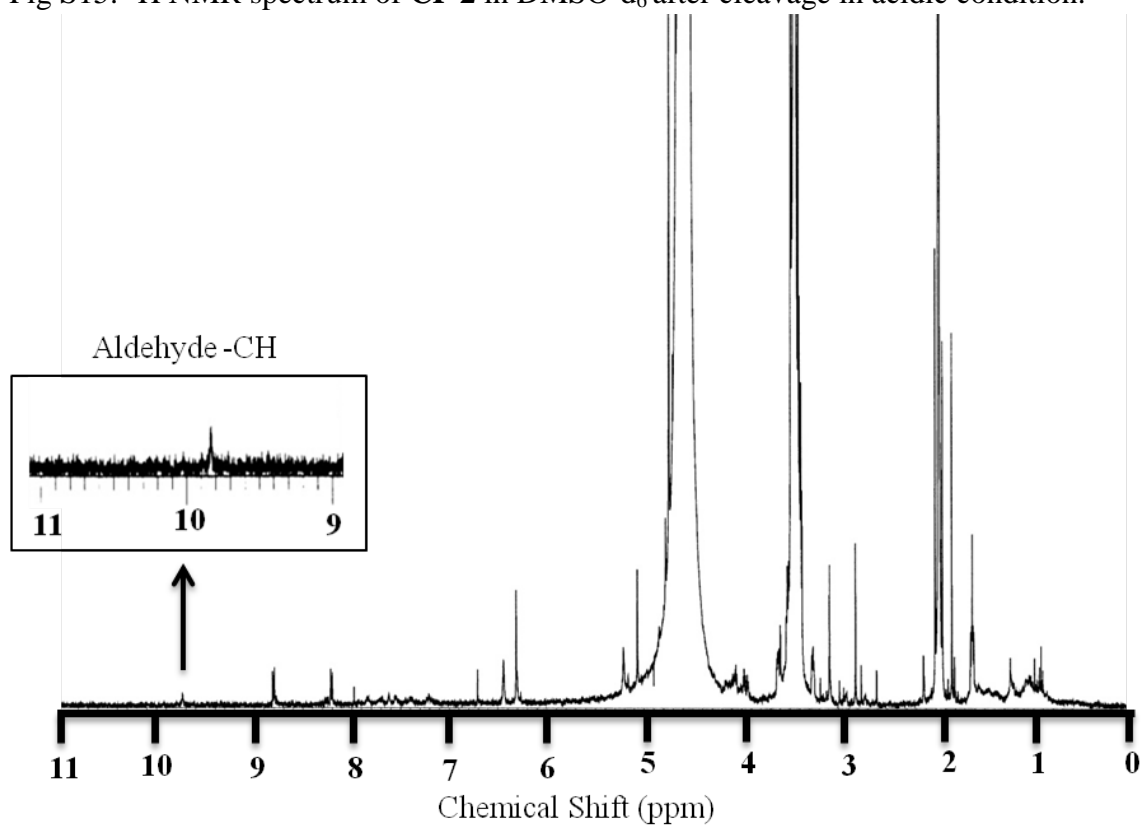


Fig S16: ^1H NMR spectrum of CP 2 in D_2O after cleavage in acidic condition.

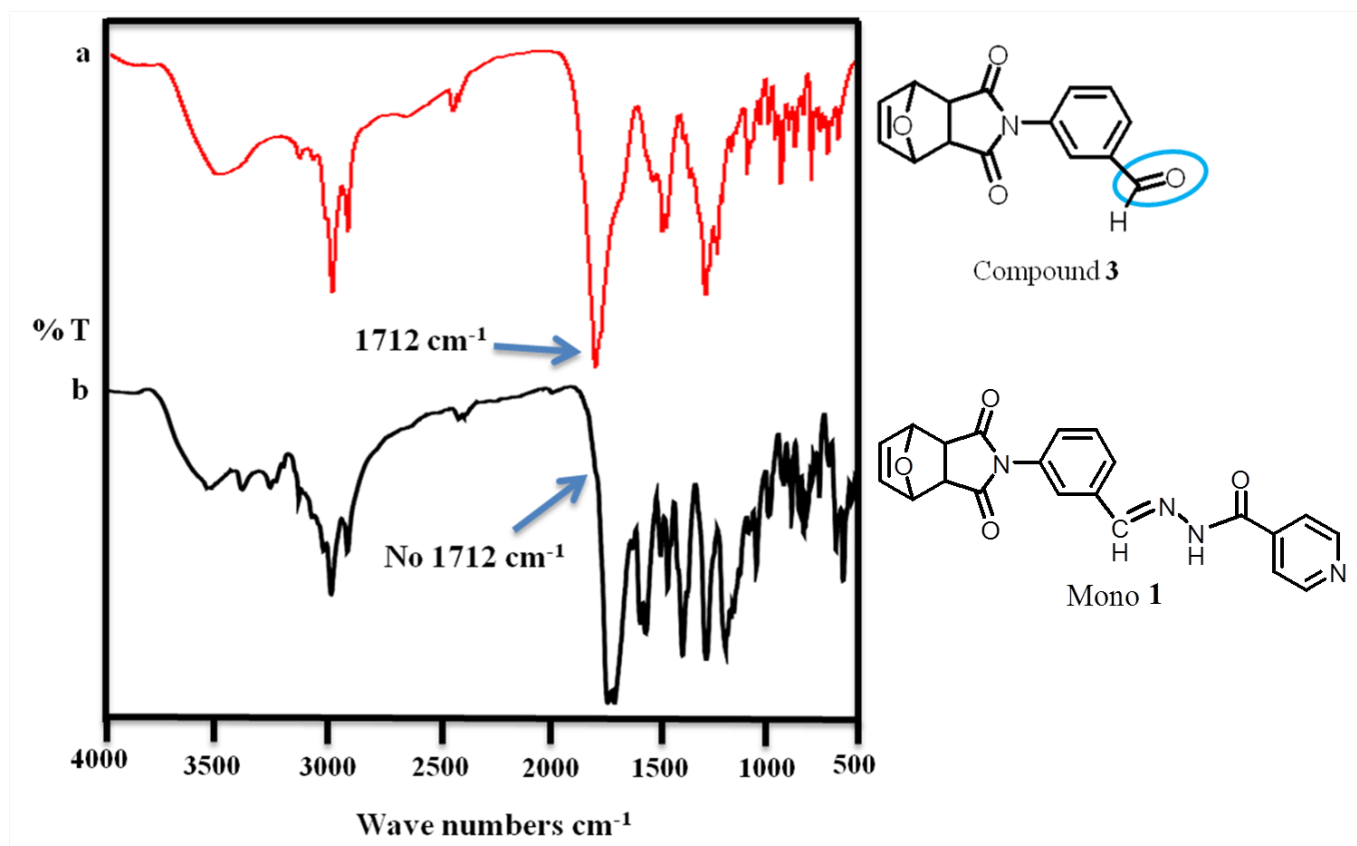


Fig S17. FT-IR spectrum of (a) compound 3 and (b) Mono 1.

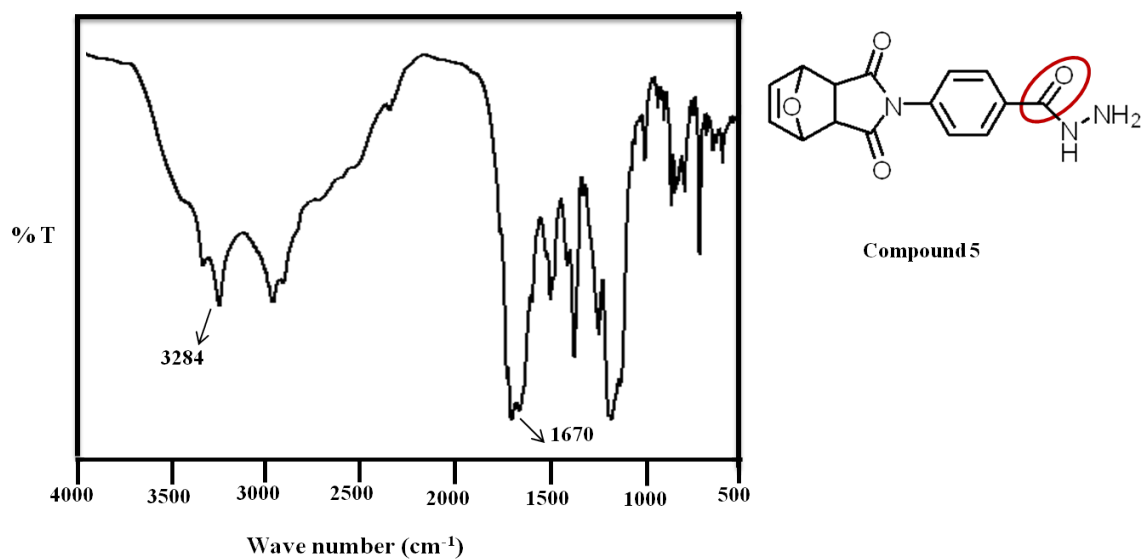


Fig S18: FT-IR spectrum of compound 5.

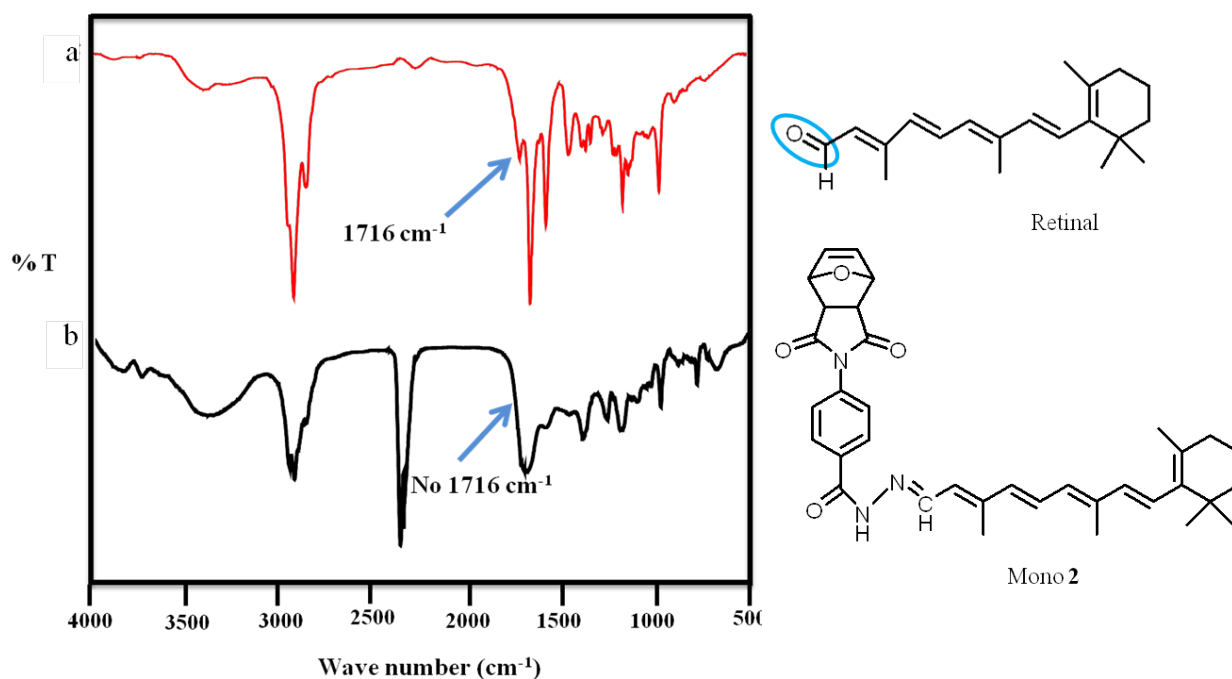


Fig S19: FT-IR spectrum of (a) retinal and (b) Mono 2.

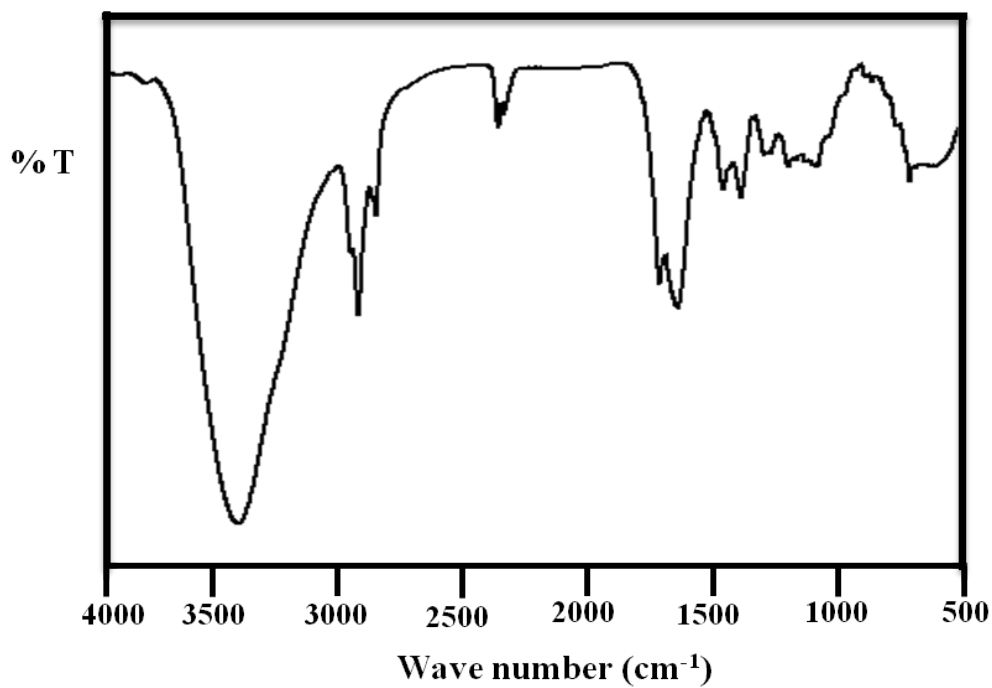


Fig S20: FT-IR spectrum of CP 1.

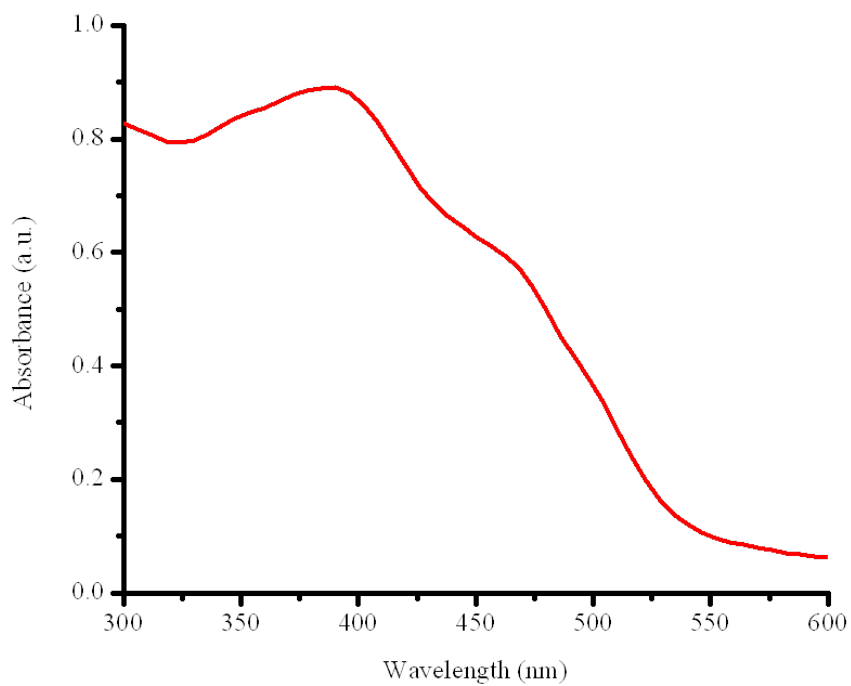


Fig S21: UV spectra of **CP 2**.

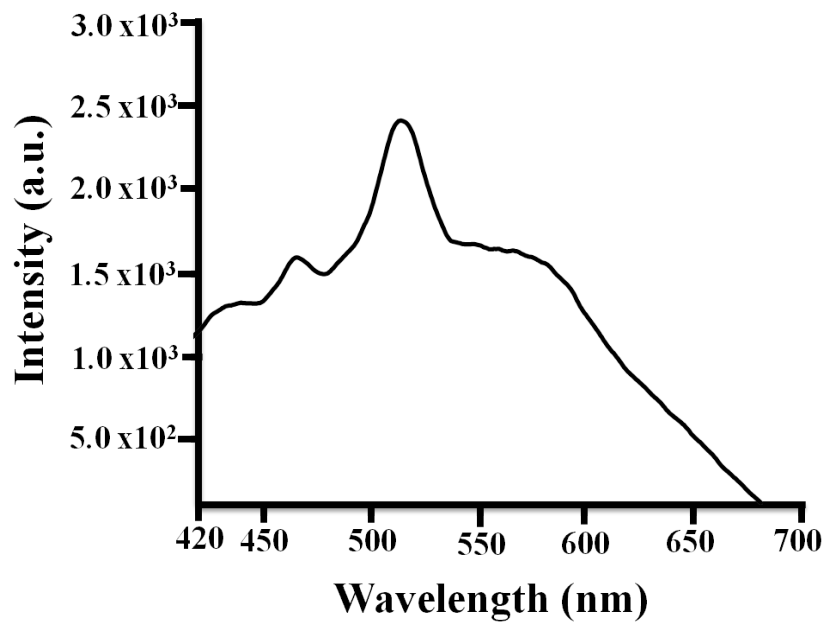


Fig S22: Fluorescence spectra of **CP 2** excited at 390 nm.

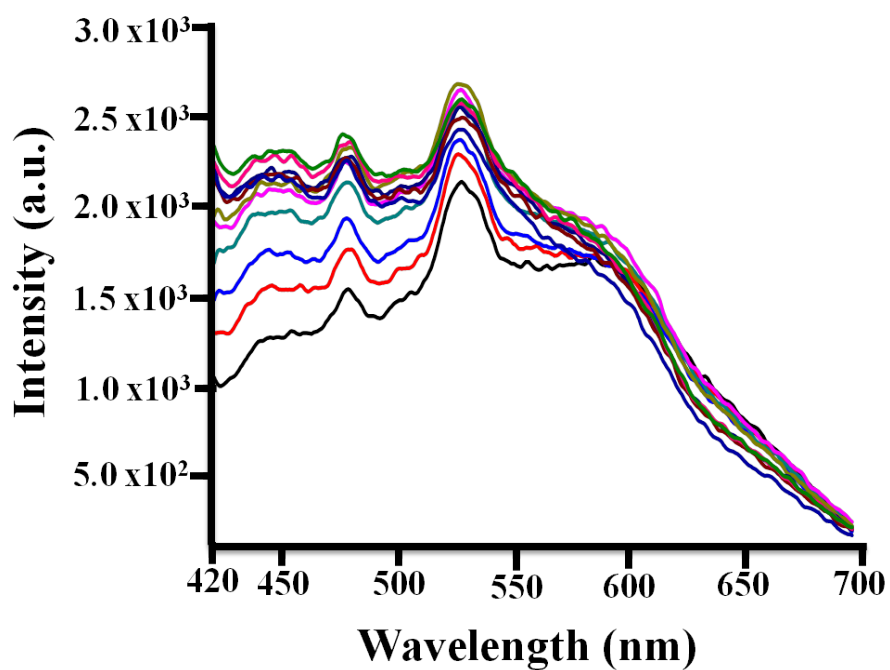


Fig S23: Fluorescence spectra for drug release from **CP 2** at pH 4 by Dialysis method.

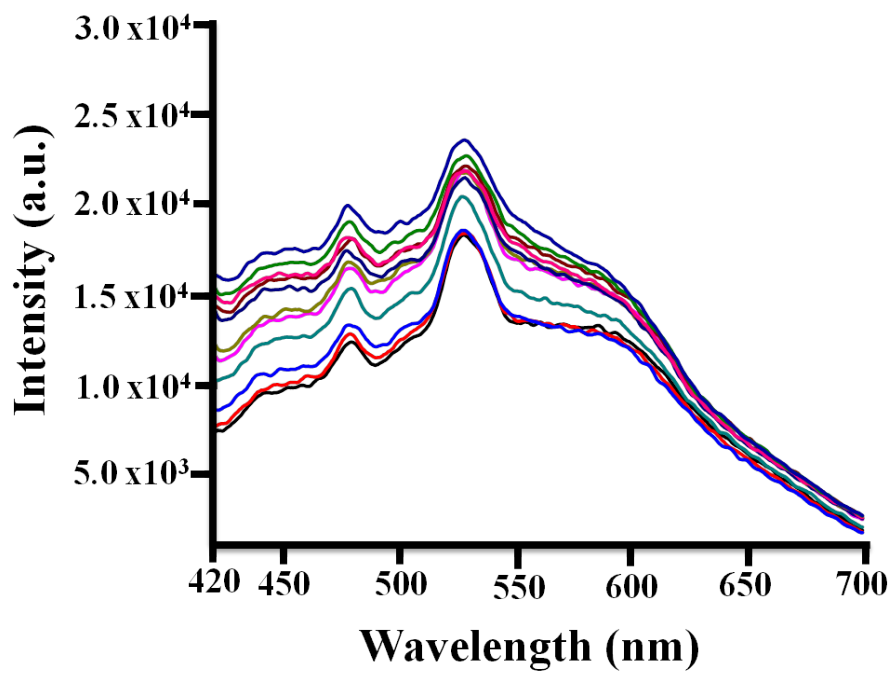


Fig S24: Fluorescence spectra for drug release from **CP 2** at pH 5.5 by Dialysis method.

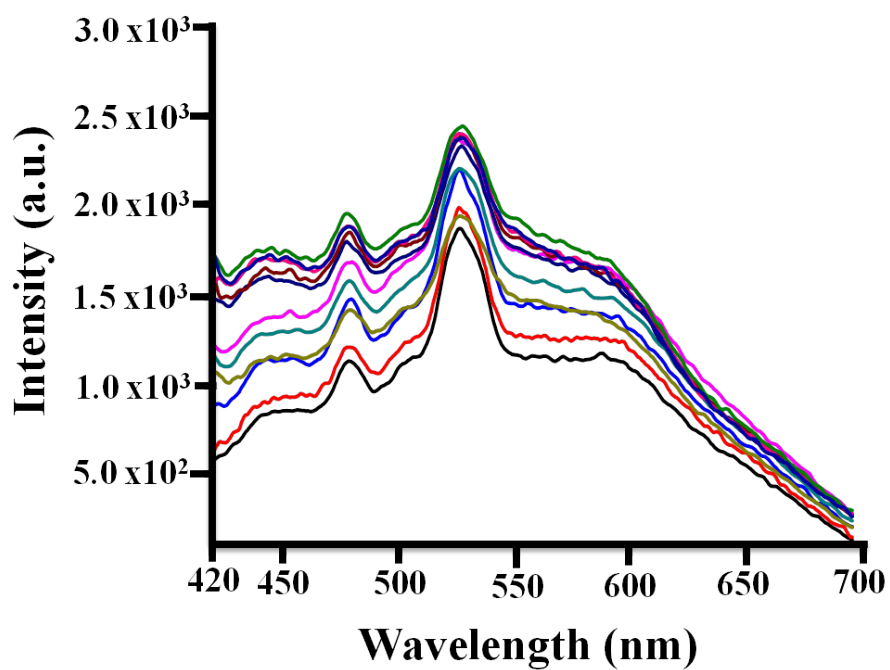


Fig S25: Fluorescence spectra for drug release from **CP 2** at pH 6.5 by Dialysis method.

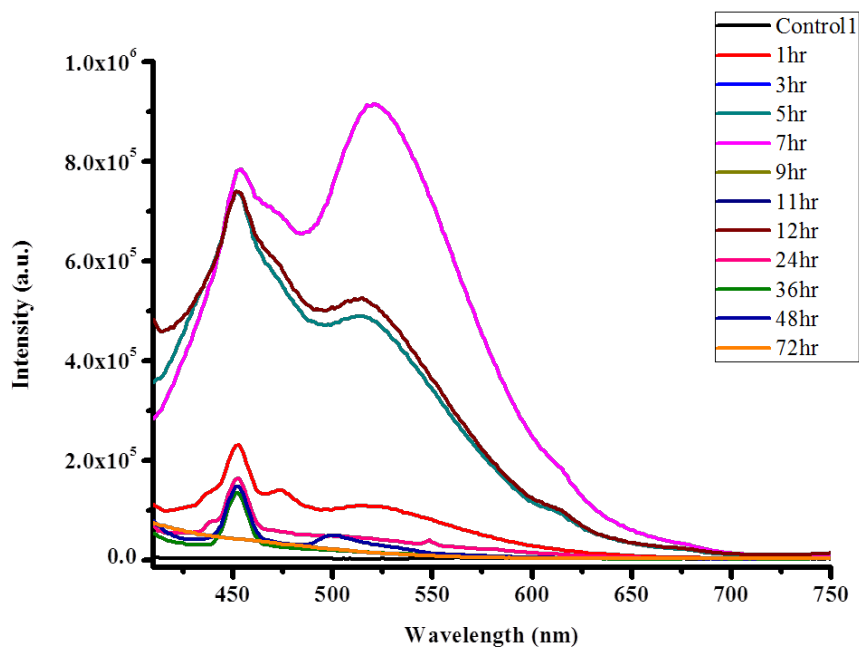


Fig S26: Fluorescence spectra of **CP 2** in urine collected as renal clearance from mouse **1**. The excitation wavelength is 390 nm.

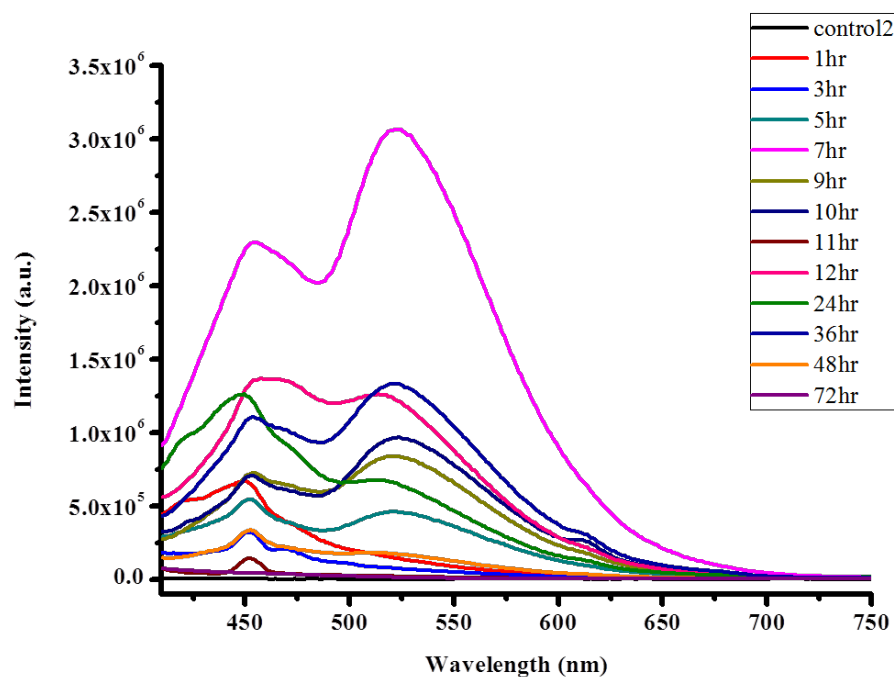


Fig S27: Fluorescence spectra of **CP 2** in urine collected as renal clearance from mouse **2**. The excitation wavelength is 390 nm.

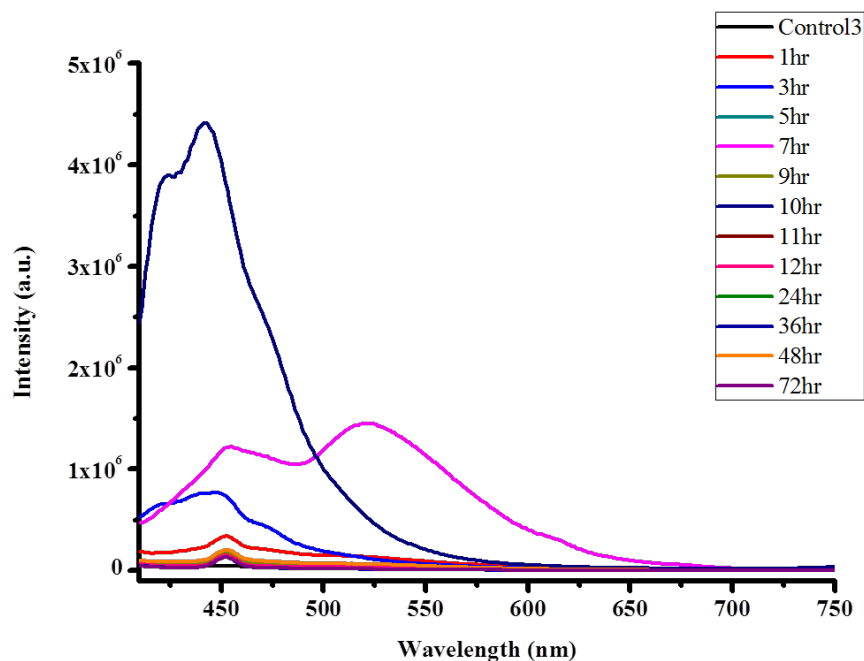


Fig S28: : Fluorescence spectra of **CP 2** in urine collected as renal clearance from mouse **3**.The excitation wavelength is 390 nm.

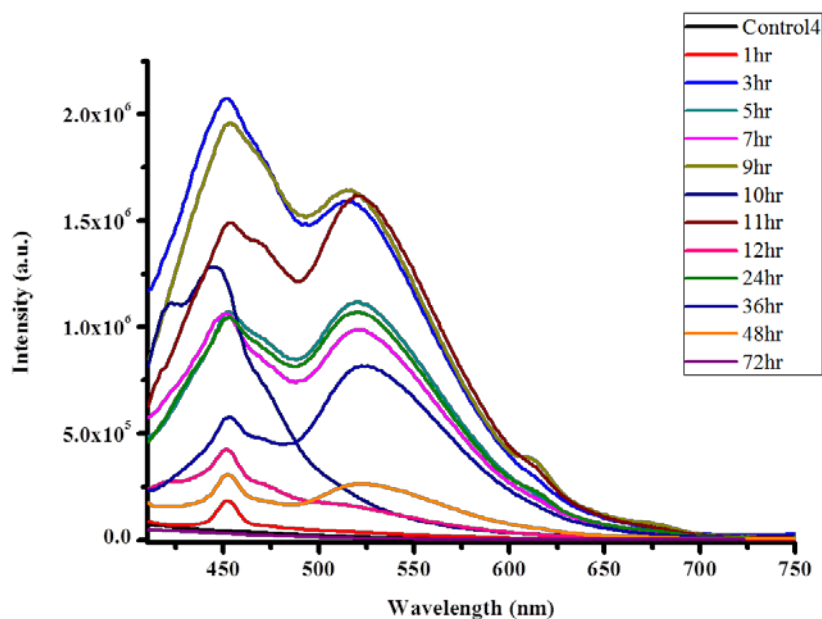


Fig S29: : Fluorescence spectra of **CP 2** in urine collected as renal clearance from mouse **4**.The excitation wavelength is 390 nm.

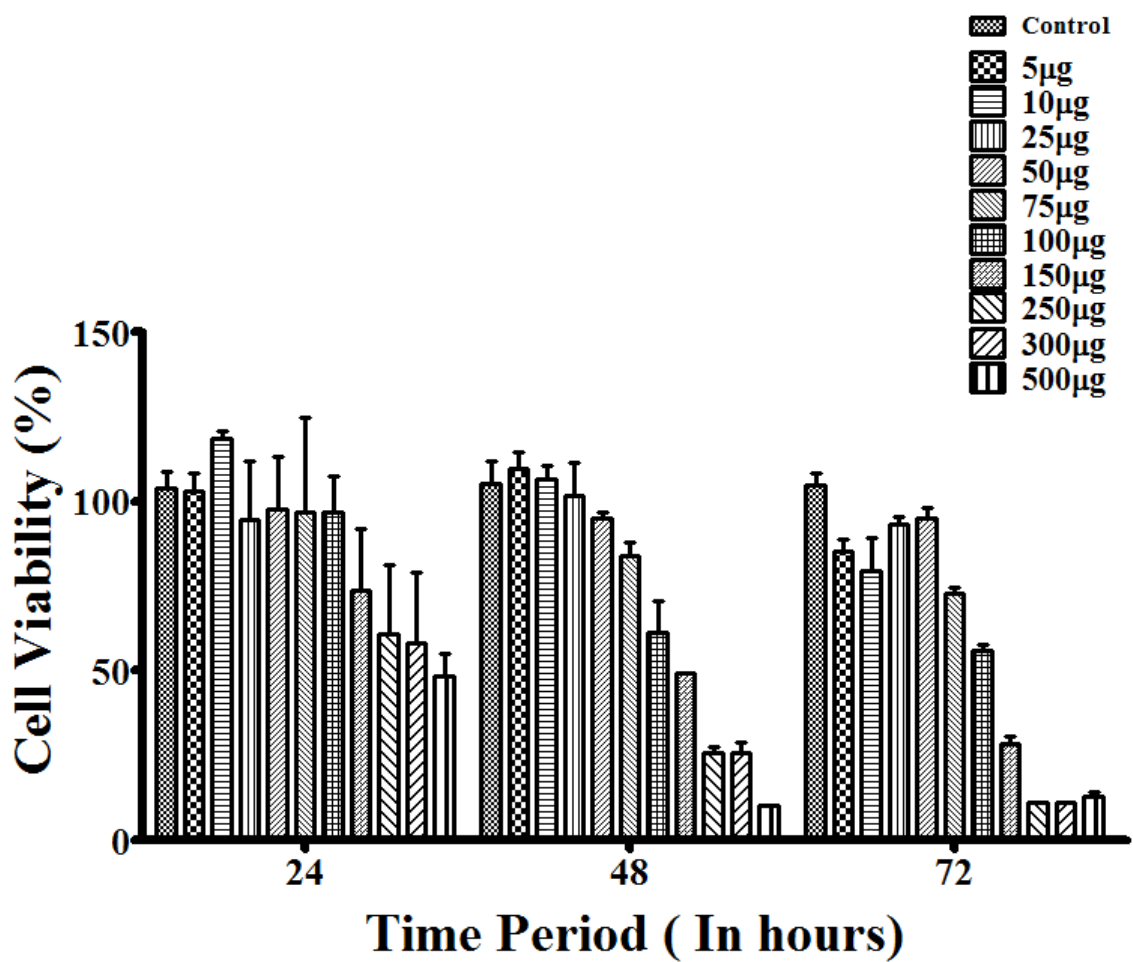


Fig. S30 : Cytotoxicity assay of Isoniazid.

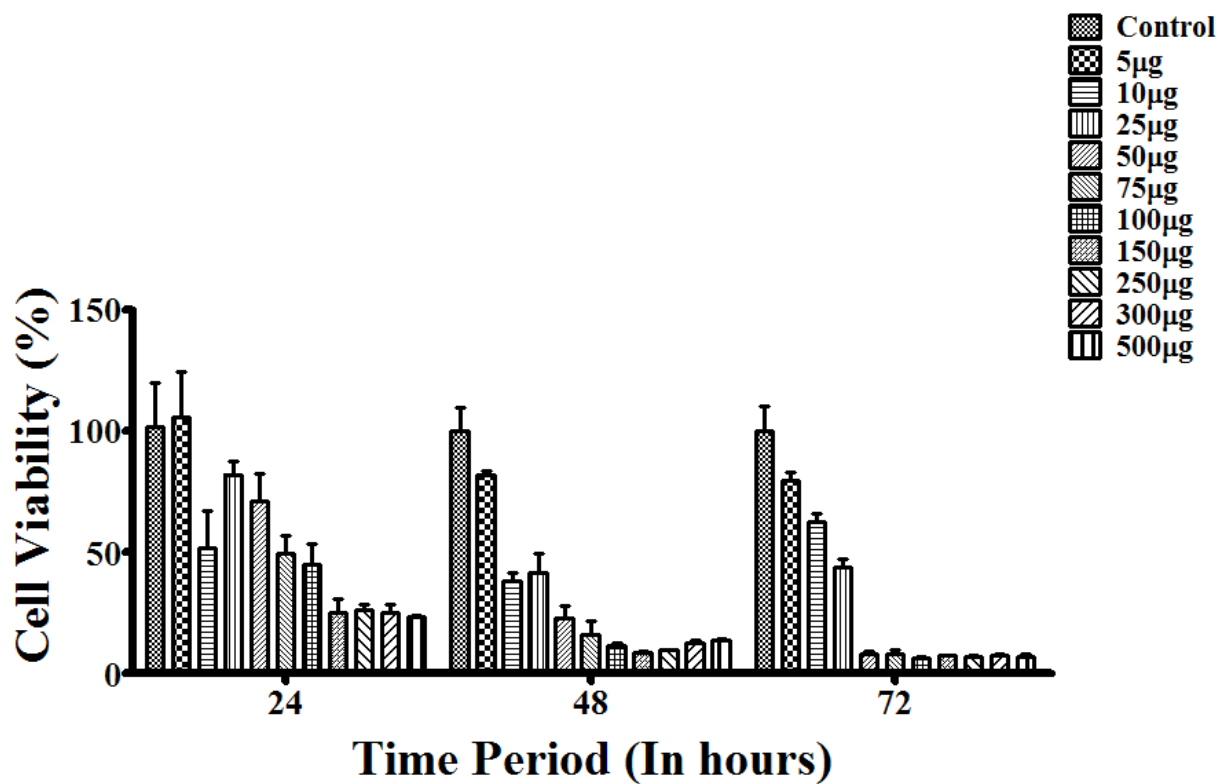


Fig. S31 : Cytotoxicity assay of Retinal.