Electronic Supplementary Material (ESI) for Chemical Communications

Remarkable photocytotoxicity of curcumin in HeLa cells in visible light and arresting its degradation on oxovanadium(IV) complex formation†

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Electronic Supplementary Information (ESI)

Experimental Section

Materials and measurements

All reagents and chemicals were procured from commercial sources (s.d. Fine Chemicals, India; Aldrich, USA) and used without further purification. Curcumin (95% curcuminoid content, 80% curcumin) was purchased from Sigma-Aldrich and purified into individual components by following a reported procedure.[S1] Solvents were purified by standard procedures.[S2] Supercoiled (SC) pUC19 DNA (cesium chloride purified) was purchased from Bangalore Genie (India). Tris-(hydroxymethyl)aminomethane-HCl (Tris-HCl) buffer solution was prepared using deionized and sonicated triple distilled water. Calf thymus (ct) DNA, agarose (molecular biology grade), distamycin, catalase, superoxide dismutase (SOD), ethidium bromide (EB) were procured from Sigma (USA). Dipyrido[3,2-a:2′,3′-c]phenazine (dppz) was prepared following a literature procedure using 1,10-phenanthroline-5,6-dione as a precursor.[S3,S4] Synthesis of the complexes was carried out under nitrogen atmosphere using Schlenk technique. Tetrabutylammonium perchlorate (TBAP) was prepared using tetrabutylammonium bromide and perchloric acid.

The elemental analysis was done using a Thermo Finnigan FLASH EA 1112 CHNS analyzer. The infrared, electronic spectra were recorded on Perkin Elmer Lambda 35 and Perkin Elmer spectrum one 55, respectively, at 25 °C. Molar conductivity measurements were done using a Control Dynamics (India) conductivity meter. Electrochemical measurements were made at 25 °C on an EG&G PAR model 253 VersaStat potentiostat/galvanostat with electrochemical analysis software 270 using a three electrode setup consisting of a glassy carbon working, platinum wire auxiliary and a saturated calomel reference electrode (SCE) in 20% DMF in Tris buffer. Tetrabutylammonium perchlorate (TBAP) (0.1 M) was used as a supporting electrolyte for the electrochemical measurements. Electrospray ionization (ESI) mass spectral measurements

were made using Bruker Daltonics make Esquire 300 Plus ESI model. The NMR spectra were recorded using Bruker Avance 400 (400 MHz) NMR spectrometer. Room temperature magnetic moment of the DMSO-d_6 solutions of the oxovanadium(IV) complexes containing 1% TMS (v/v) as the internal reference was obtained by a solution NMR method with a Bruker AMX-400 NMR spectrometer.[S5] Fluorescence microscopic investigations were carried out on Leica DM IL microscope with integrated Leica DFC400 camera and IL50 image software. Confocal microscopy was done using confocal scanning electron microscope (Leica, TCS SP5 DM6000). Flow cytometric analysis was performed using FACS Calibur (Becton Dickinson (BD) cell analyzer) at FL2 channel (595 nm).

Preparation of [VO(cur)(phen)Cl] (1) and [VO(cur)(dppz)Cl] (2):

Vanadyl sulfate (0.16 g, 1.0 mmol) and barium chloride (0.25 g, 1.0 mmol) together were dissolved in 15 ml of EtOH and 3 ml of water. The mixture was then stirred at room temperature for 1.5 h under inert atmosphere of nitrogen using Schlenk technique. The mixture was filtered using celite to remove white barium sulfate precipitate. The blue filtrate was deaerated and then saturated with nitrogen. An ethanolic solution (3 ml) of the corresponding phenanthroline base (0.19 g, phen; 0.28 g, dppz, (1.0 mmol)) was added to the filtrate. A deep greenish solution was formed after stirring the mixture for 20 min. To this mixture was then added the deaerated ethanol solution (25 ml) of curcumin (Hcur, 0.36 g, 1.0 mmol) previously neutralized with Et_3N (0.10 g, 1.0 mmol). The complex was precipitated out after stirring for 1 h. The precipitate was then filtered, isolated and washed with ethanol, THF and chloroform and finally dried in vacuum over P_4O_{10} [Yield: ~87% (**1**) and ~74% (**2**)].

Characterization data

Complex **1**: Anal. Calcd for $C_{33}H_{27}N_2ClO_7V$: C, 60.98; H, 4.19; N, 4.31. Found: C, 60.79; H, 4.26; N, 4.21. ESI-MS in CH_3CN: m/z 614.1257 $[M\text{-}Cl]^+$. IR data/ cm^{-1}: 3064 w, 1590 s, 1491 vs, 1420 s, 1381 m, 1276 s, 1152 m, 1122 m, 1035 w, 966 m, 840 m, 720 m, 555 w, 460 w (vs, very strong; s, strong; m, medium; w, weak). UV-visible in 10% DMF [λ_{max}/nm (ε/ dm^3 mol^{-1} cm^{-1})]: 721 (54), 453 sh (33000), 434 (35600), 265 (30400). Λ_M = 104 S m^2 M^{-1} in 10% aqueous DMF at 25 ºC. μ_{eff}, μ_B at 298 K: 1.67.

Complex **2**: Anal. Calcd for $C_{39}H_{29}N_4ClO_7V$: C, 62.28; H, 3.89; N, 7.45. Found: C, 62.41; H, 3.77; N, 7.42. ESI-MS in CH_3CN: m/z 716.49 $[M\text{-}Cl]^+$, IR data/ cm^{-1}: 3070 w, 1587 s, 1490 vs, 1422 s, 1377 m, 1279 s, 1154 m, 1117 m, 1031 w, 968 m, 810 m, 723 m, 558 w, 463 w, 435 m. UV-visible in 10% DMF [λ_{max}/nm (ε/ dm^3 mol^{-1} cm^{-1})]: 731 (64), 454 sh (38600), 434 (43900), 382 (25600), 361 (20300), 268 (46600). Λ_M = 96 S m^2 M^{-1} in 10% aqueous DMF at 25 ºC. μ_{eff}, μ_B at 298 K: 1.61.

Solubility and stability

The complexes showed good solubility in DMF and DMSO and moderate solubility in water, methanol, ethanol and acetonitrile. They had poor solubility in hydrocarbons. The complexes were found to be stable in the monocationic form due to dissociation of the chloride and the solution stability of the cationic species was ascertained from the ESI-MS and the molar conductivity data.

X-ray crystallographic procedures

The crystal structure of complex **1** was obtained by single crystal X-ray diffraction method. Crystals were obtained from slow evaporation of an acetonitrile solution of the complex.

Crystal mounting was done on glass fibre with epoxy cement. All geometric and intensity data were collected at low temperature using an automated Bruker SMART APEX CCD diffractometer equipped with a fine focus 1.75 kW sealed tube Mo-K_α X-ray source (λ = 0.71073 Å) with increasing ω (width of 0.3° per frame) at a scan speed of 5 sec per frame. Intensity data, collected using ω-2θ scan mode, were corrected for Lorentz–polarization effects and for absorption.[S6] The structure was solved and refined using SHELXL97[S7] present in the WinGx suit of programs (Version 1.63.04a).[S8] All non-hydrogen positions were initially located in the difference Fourier maps, and for the final refinement, the hydrogen atoms were placed in geometrically ideal positions and refined in the riding mode. Final refinement included atomic positions for all the atoms, anisotropic thermal parameters for all the non-hydrogen atoms and isotropic thermal parameters for all the hydrogen atoms. The electron density contributions from the highly disordered solvent molecules were removed using the SQUEEZE routine (PLATON).[S9] EADP restraint was used on the atoms C25 and C27 in order to avoid eccentric thermal ellipsoids. Perspective view of the molecule was obtained by ORTEP.[S10] Selected bond distances and angles in the complex are provided in Table S1.

Cell cytotoxicity assay

The photocytotoxicity of the complexes **1**, **2** and curcumin was studied using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which is based on the ability of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium rings of MTT forming dark purple membrane impermeable crystals of formazan that could be quantified from spectral measurement in DMSO.[S11] Approximately, 8000 cells of human cervical cancer HeLa cells were plated in 96 wells culture plate in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and after 24 h of incubation at 37 °C in CO_2 incubator. The stock solution

of the complexes **1**, **2** and curcumin that was prepared in DMSO was first diluted in culture medium to the desired concentration and then added to the 96 well plates. The quantity of DMSO was kept constant at 1% for all tests. Then cells were incubated for 4 h in dark. The medium that was subsequently replaced with PBS was photo-irradiated with visible light (400-700 nm) for 1 h using a Luzchem Photoreactor (Model LZC-1, Ontario, Canada) fitted with Sylvania make 8 fluorescent white tubes with a fluence rate of 2.4 mW cm^{-2} to provide a total dose of 10 J cm^{-2}. After photoexposure, PBS was removed and replaced with DMEM-FBS and incubation was continued for further 20 h in dark. After the incubation period, 20 μL of 5 mg mL^{-1} of MTT was added to each well and incubated for an additional 3 h. The culture medium was finally discarded and 200 μL of DMSO was added to dissolve the formazan crystals and its absorbance at 595 nm was measured using a BIORAD ELISA plate reader. Cytotoxicity of **1, 2** and curcumin was measured as the percentage ratio of the absorbance of the treated cells to the untreated controls. The IC_{50} values were determined by nonlinear regression analysis (GraphPad Prism).

DNA fragmentation analysis by agarose gel electrophoresis

DNA fragmentation analysis was conducted to confirm the apoptotic mechanism induced by curcumin, **1** and **2** for cell death. Briefly $0.3x10^6$ cells were taken in each 60 mm dish. It was grown for 24 h and later treated with **1** (15 μM), **2** (5 μM) and curcumin (10 μM) incubated for 4 h in dark. One dish containing the complex or curcumin was exposed to light for 1 h and again the cells were left to grow for 4 h along with its dark control in another dish. After 4 h, cells were trypsinized, washed with DPBS and re-suspended in 0.4 ml of lysis buffer (10 mM Tris-HCl; pH, 8.0, 20 mM EDTA, 0.2% triton-X 100) with an incubation of 20 min on ice. Lysed cells were

centrifuged for 20 min at 13000 rpm and their supernatant (which had soluble chromosomal DNAs including both high molecular weight DNA and nucleosomal DNA fragments) was collected. Phenol chloroform was performed to remove the protein present. Later supernatant was precipitated with 1/10 volume of 3M sodium acetate (pH, 5.8) and 2 volume of ethanol at –20 °C for overnight. DNA pellet was washed with 70% alcohol and re-suspended in Tris-EDTA (pH 8) containing RNAse (100 µg/ml RNAse) followed by incubation at 37 °C for 2 hrs. DNA samples were resolved on 1.5% agarose gel at 80 V for approximately 2 h and photographed under UV light.

Confocal studies: uptake and localization

Uptake of fluorescent complexes **1**, **2** and curcumin into the cell was visualized using a confocal scanning electron microscope (Zeiss, LSM510 apocromat). HeLa cells were grown on glass cover slips in each 12 well plates at a seeding density of 50,000 cells in 1.5 ml of culture medium for 24 h. Cells were then treated with the complex for 2 h, and 4 h in dark. Cells were fixed and permeabilized with chilled methanol for 5 min at -20 °C. Methanol was subsequently removed followed by washing with 1X PBS. It was later incubated with propidium iodide (1 mg/ml) to stain the nucleus for 2 min and visualized under a confocal scanning electron microscope.

DNA binding methods

Absorption titration

DNA binding experiments were done in Tris-HCl/NaCl buffer (5 mM Tris-HCl, 5 mM NaCl, pH 7.2) using DMF solution of the complexes. Calf thymus (ct) DNA (ca. 350 µM NP) in this buffer medium gave a ratio of UV absorbance at 260 and 280 nm of ca. 1.9:1 indicating that the DNA is apparently free from protein. The concentration of ct-DNA was estimated from its absorption intensity at 260 nm with a known molar extinction coefficient value (ε) of 6600 M^{-1}

cm^{-1}.[S12] Absorption titration experiments were made by varying the concentration of the ct-DNA while keeping the metal complex concentration constant. Due correction was made for the absorbance of ct-DNA itself. Each spectrum was recorded after equilibration of the sample for 5 min. The intrinsic equilibrium binding constant (K_b) and the binding site size (s) of the complexes to ct-DNA were obtained by McGhee-von Hippel (MvH) method using the expression of Bard et al. by monitoring the change of the absorption intensity of the spectral bands with increasing concentration of ct-DNA by regression analysis using equation

$$(\varepsilon_a - \varepsilon_f)/(\varepsilon_b - \varepsilon_f) = (b - (b^2 - 2K_b^2 C_t[\mathrm{DNA}]_t/s)^{1/2})/2K_bC_t$$

where $b = 1 + K_bC_t + K_b[\mathrm{DNA}]_t/2s$ and ε_a is the extinction coefficient observed for the absorption band at a given DNA concentration, ε_f is the extinction coefficient of the complex free in solution, ε_b is the extinction coefficient of the complex when fully bound to DNA, K_b is the equilibrium binding constant, C_t is the total metal complex concentration, $[\mathrm{DNA}]_t$ is the DNA concentration in nucleotides and s is the binding site size in base pairs.[S13,14] The non-linear least-squares analyses were done using Origin Lab, version 8.1.

DNA thermal denaturation studies were carried out by monitoring the absorption intensity of ct-DNA (190 μM) at 260 nm on varying the temperature from 40 to 90 °C in absence and presence of the complexes or curcumin (20 μM). Measurements were carried out using a Cary 300 bio UV-visible spectrometer with a Cary temperature controller at an increase rate of 0.5 °C per min of the solution.

DNA cleavage experiments

The cleavage of supercoiled (SC) pUC19 DNA (0.2 μg, 50 μM, 2686 base-pairs) was studied by agarose gel electrophoresis using curcumin and the complexes in 50 mM Tris–HCl

buffer (pH 7.2) and 50 mM NaCl containing 10% DMF. The photo-induced DNA cleavage reactions were carried out under illuminated conditions using a diode laser of 785 nm wavelength (Model: LQC785-100C from Newport Corporation with LD module, continuous-wave (CW) circular beam). The laser power was 100 mW, measured using Spectra Physics CW Laser Power Meter (Model 407A). Before light exposure, each sample was incubated for 1.0 h at 37 °C and analyzed for the photo-cleaved products by gel electrophoresis method as described earlier by us.[S15] The mechanistic studies were carried out using different additives (NaN_3, 0.5 mM; DMSO, 4 μL; KI, 0.5 mM; catalase, 4 units; SOD, 4 units). For the D_2O experiment, this solvent was used for dilution of the sample up to 20 μL final volume. The samples after incubation in a dark chamber were added to the loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol (3 μL) and the solution was finally loaded on 1% agarose gel containing 1.0 μg/mL ethidium bromide (EB). Electrophoresis was carried out in a dark chamber for 2.0 h at 60 V in TAE (Tris-acetate EDTA) buffer. Bands were visualized by UV light and photographed. The extent of DNA cleavage was measured from the intensities of the bands using UVITEC Gel Documentation System. Due corrections were made for the low level of nicked circular (NC) form present in the original supercoiled (SC) DNA sample and for the low affinity of EB binding to SC compared to NC and linear forms of DNA.[S16] The concentrations of the complexes and additives corresponded to that in the 20 μL final volume of the sample using Tris buffer. The observed error in measuring the band intensities was ~5%.

References

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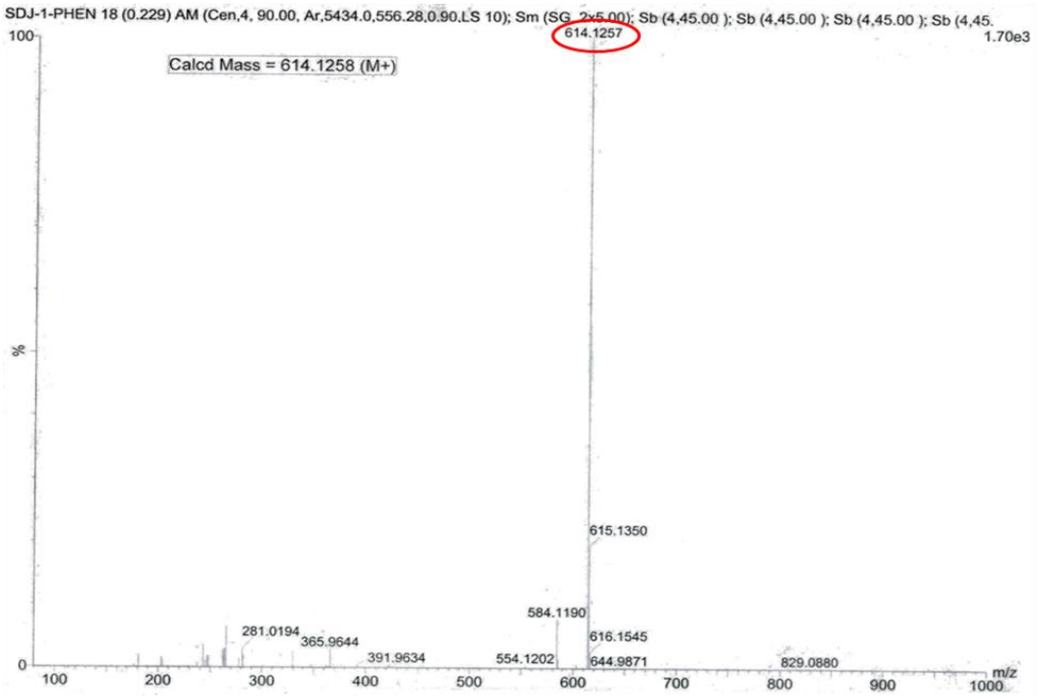
Table S1. Selected bond distances (Å) and angles (°) for [VO(cur)(phen)Cl] (**1**) with e.s.d.s in the parentheses

V(1)-N(1)	2.128(5)	N(2)-V(1)-O(3)	163.4(2)
V(1)-N(2)	2.116(5)	N(2)-V(1)-O(4)	92.28(17)
V(1)-O(3)	1.973(5)	N(2)-V(1)-O(7)	95.0(2)
V(1)-O(4)	1.959(4)	N(2)-V(1)-Cl(1)	80.71(14)
V(1)-O(7)	1.536(5)	O(3)-V(1)-O(4)	90.46(17)
V(1)-Cl(1)	2.783(2)	O(3)-V(1)-O(7)	100.4(2)
N(1)-V(1)-N(2)	78.12(18)	O(3)-V(1)-Cl(1)	83.53(15)
N(1)-V(1)-O(3)	94.10(18)	O(4)-V(1)-O(7)	101.96(19)
N(1)-V(1)-O(4)	160.3(2)	O(4)-V(1)-Cl(1)	81.47(14)
N(1)-V(1)-O(7)	96.1(2)	O(7)-V(1)-Cl(1)	174.65(14)
N(1)-V(1)-Cl(1)	79.98(15)		

(a)



(b)

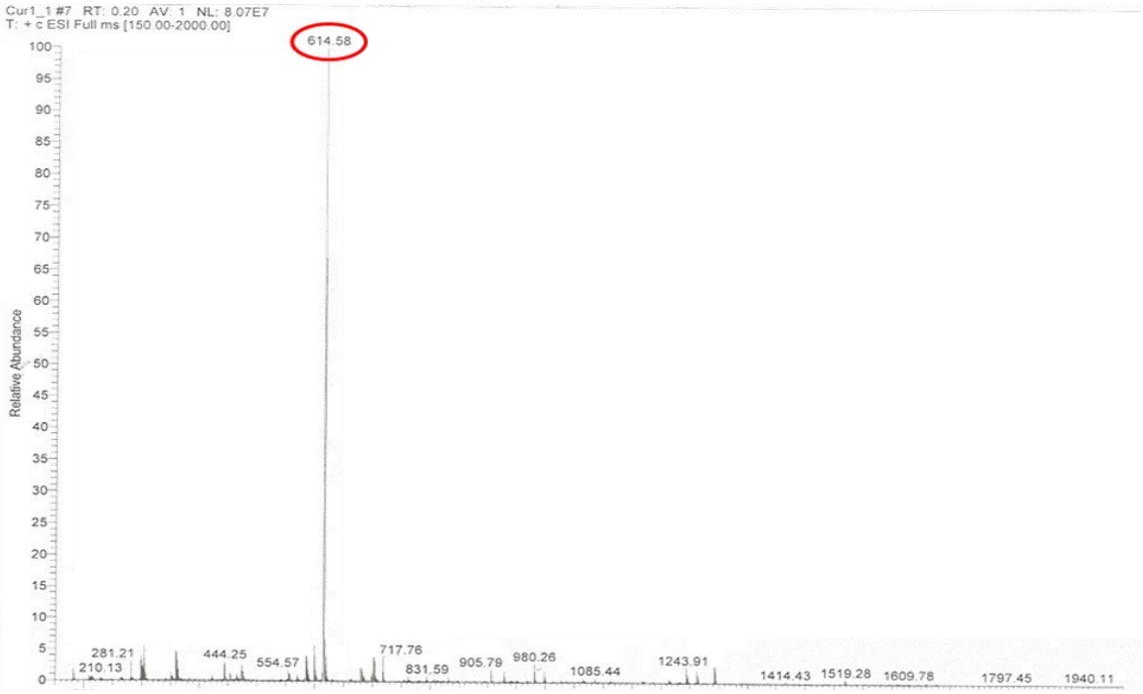
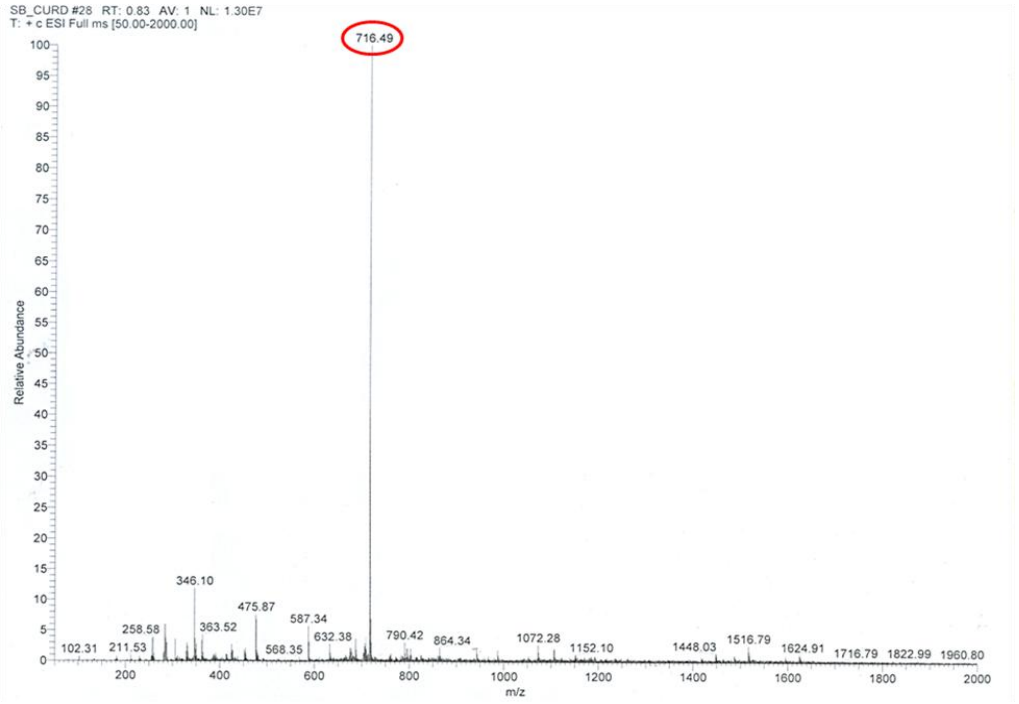


Fig. S1. The ESI-MS spectrum of complex **1** showing the prominent $[M\text{-}Cl]^+$ peak in CH_3CN (a) and aqueous CH_3CN (1:1 v/v) (b) after incubation for 4 h at 37 °C**.**

(a)



(b)

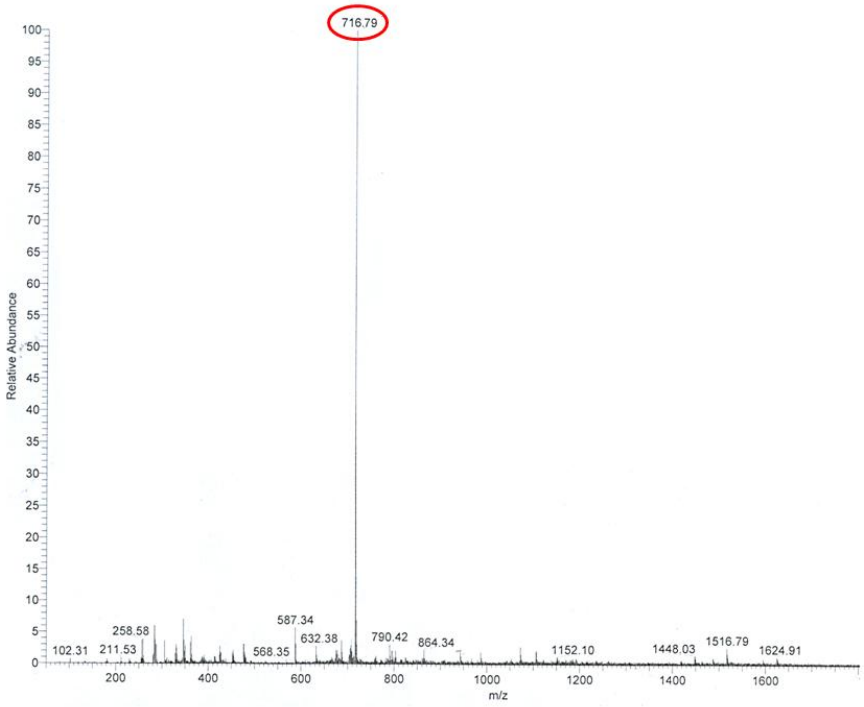


Fig. S2. The ESI-MS spectrum of complex **2** showing the prominent $[M\text{-}Cl]^+$ peak in CH_3CN (a) and 50% aqueous CH_3CN (b) after incubation for 4 h at 37°C**.**

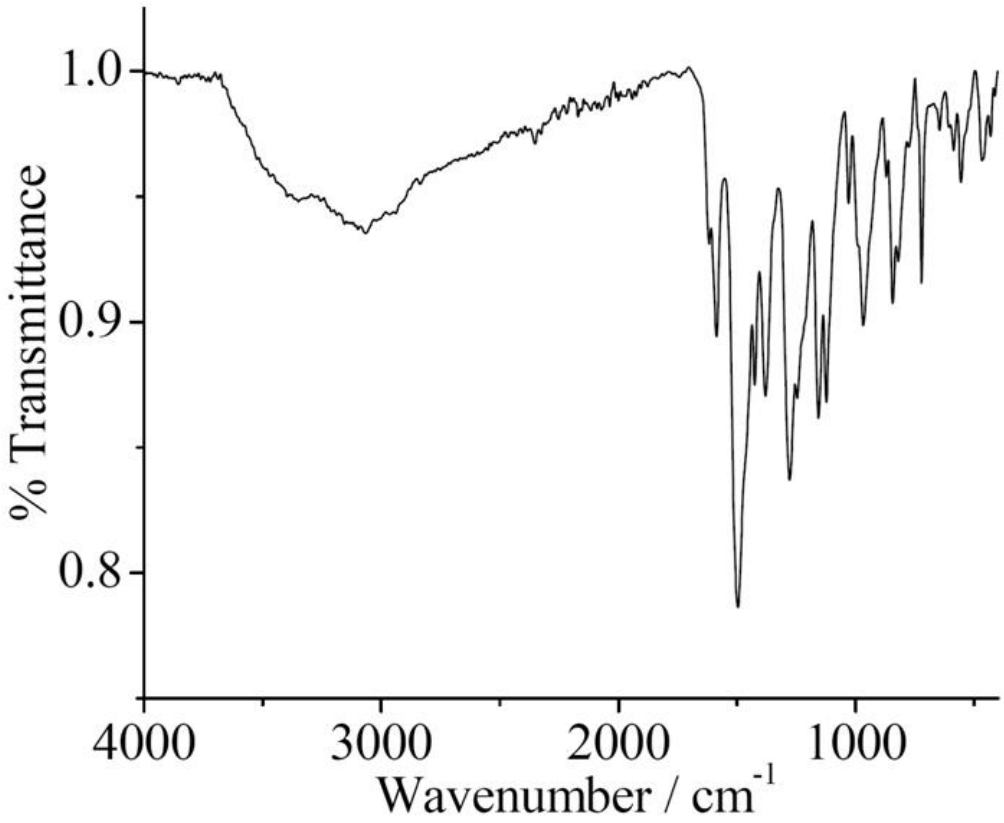


Fig. S3. IR spectrum of complex [VO(cur)(phen)Cl] (**1**).

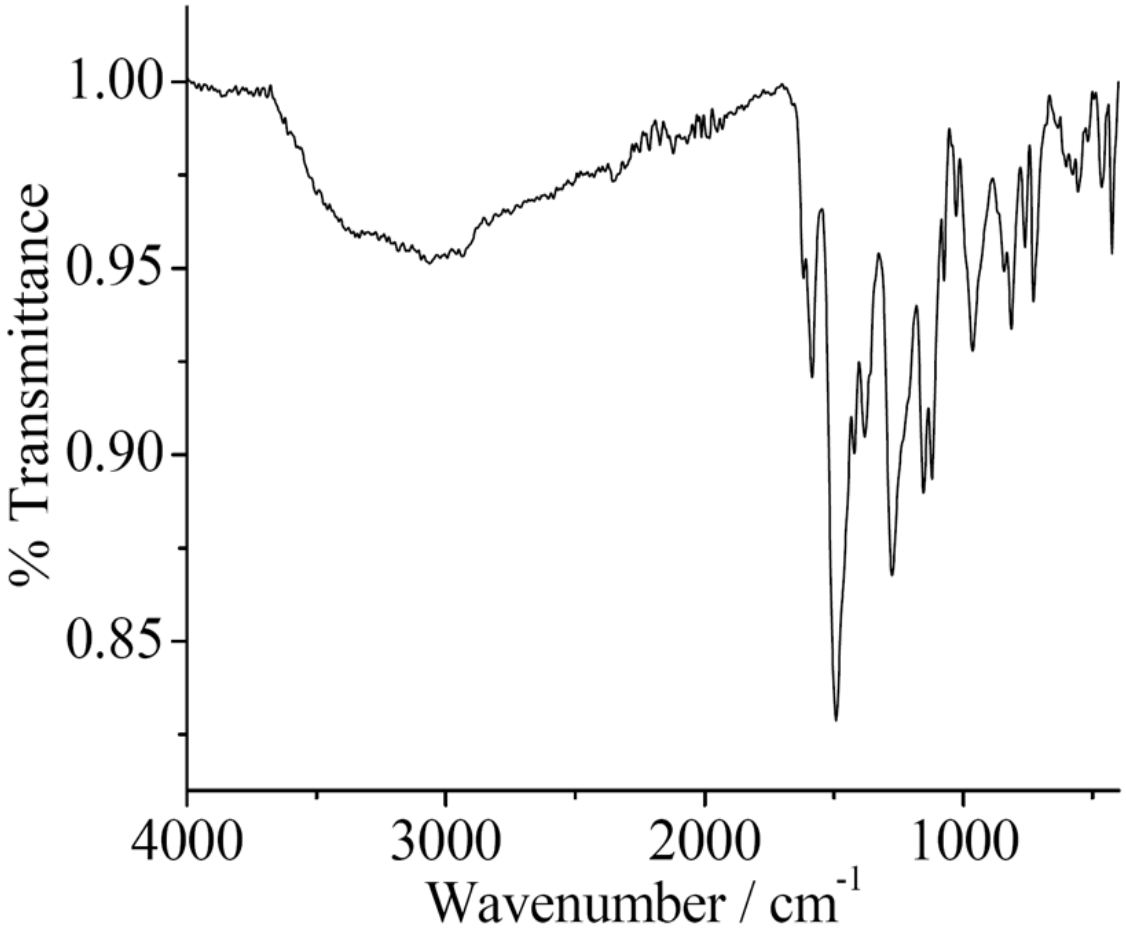


Fig. S4. IR spectrum of complex [VO)(cur)(dppz)Cl] (**2**).

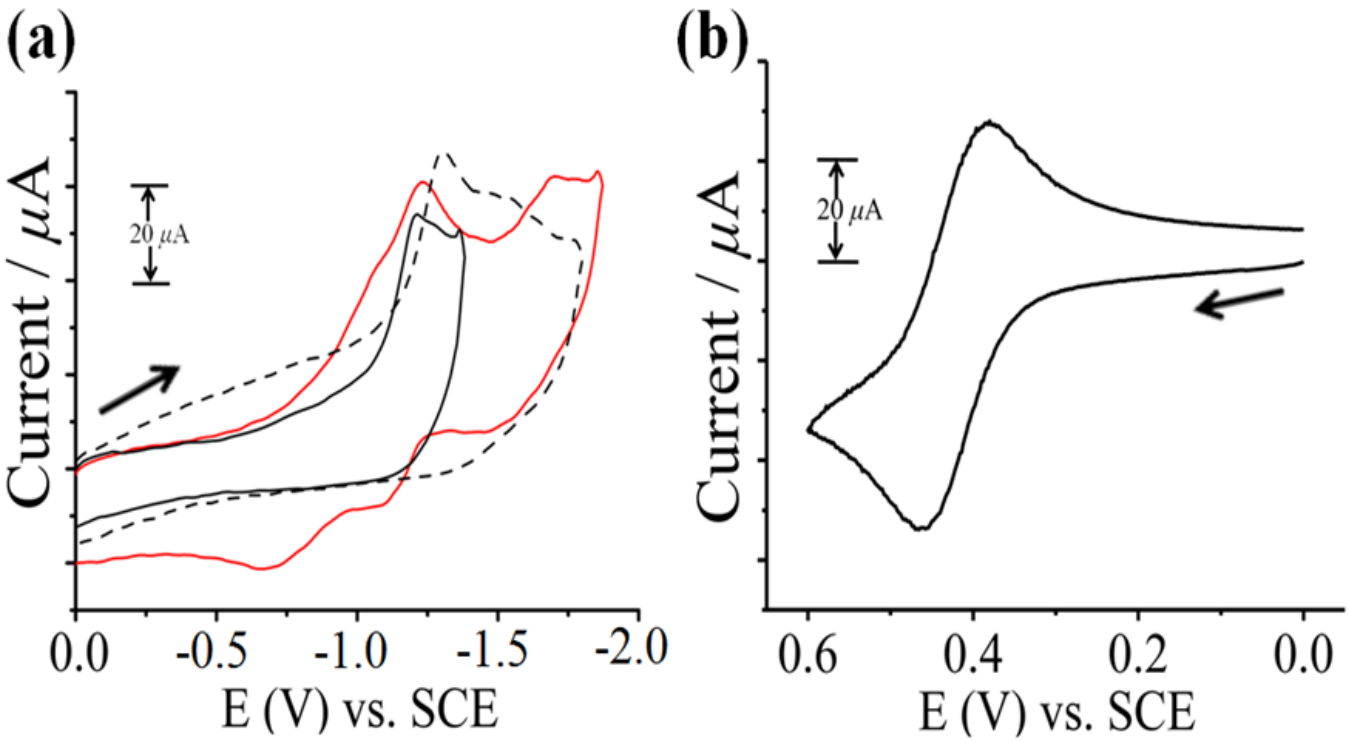


Fig. S5. (a) Cyclic voltammograms of the complexes **1** (—, ---) and **2** (—) and (b) of ferrocene in 20% aqueous DMF at a scan speed of 50 mV s^{-1} and 0.1 M TBAP as a supporting electrolyte. E_f (ΔE_p) of ferrocene is 0.42 V (80 mV) vs. SCE.

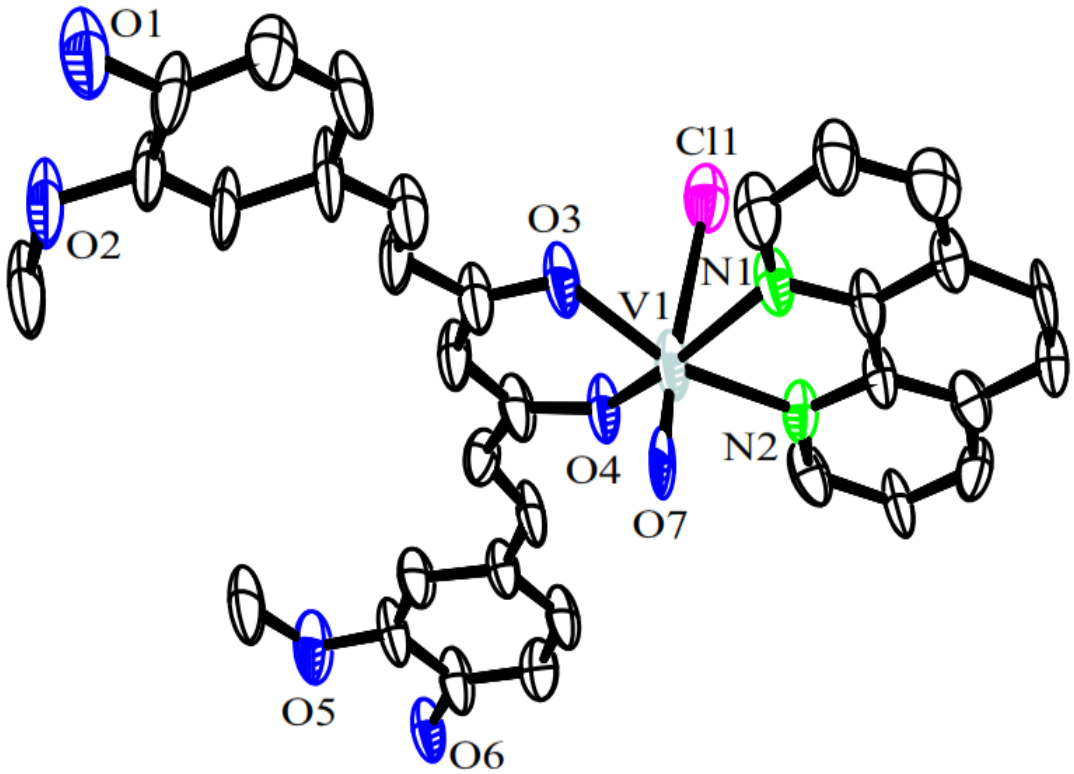


Fig. S6. An ORTEP view of complex **1** showing atom labeling scheme for the metal and hetero atoms and 50% probability thermal ellipsoids. The hydrogen atoms are not shown for clarity.

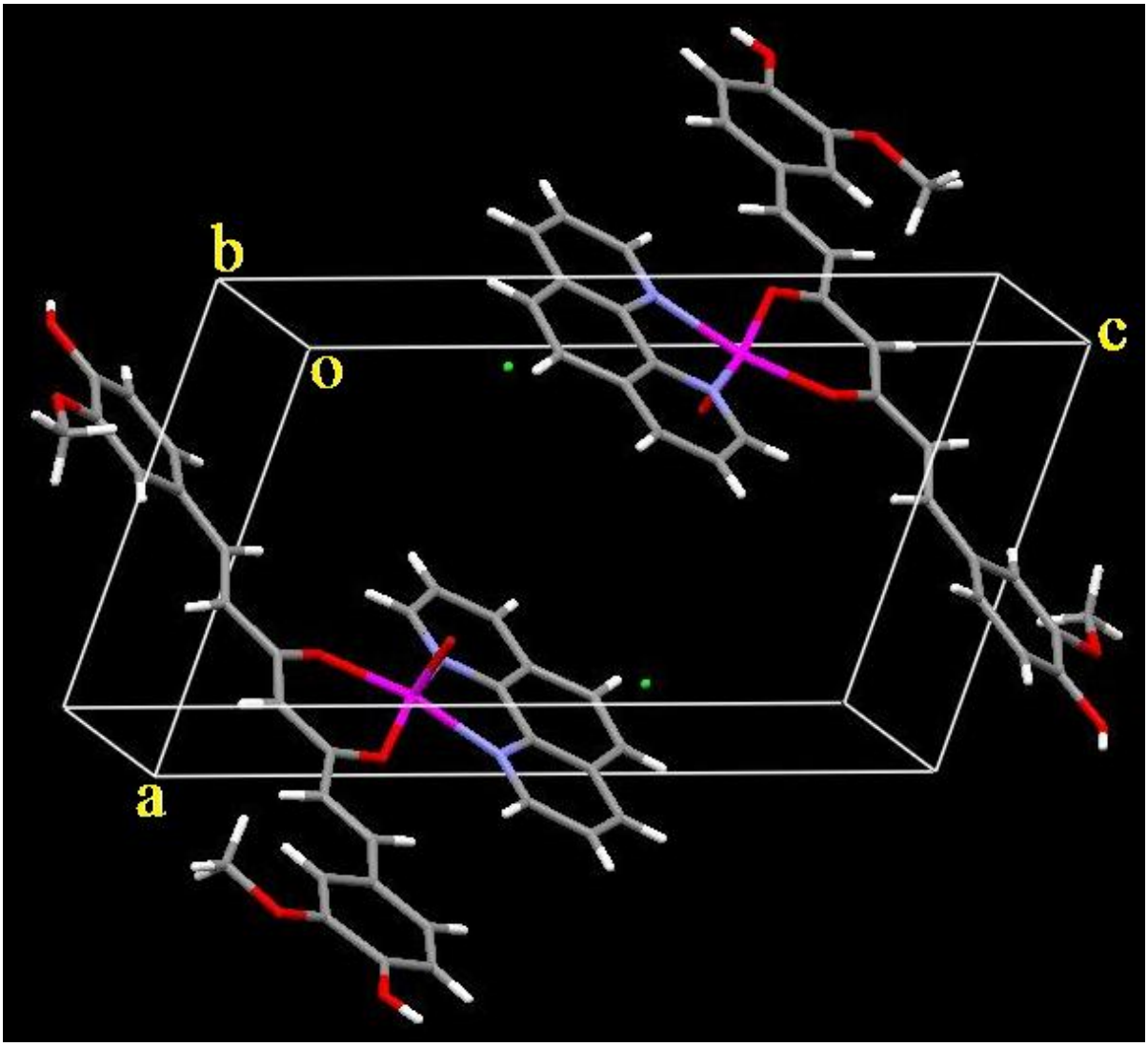


Fig. S7. Unit cell packing diagram of **1**.

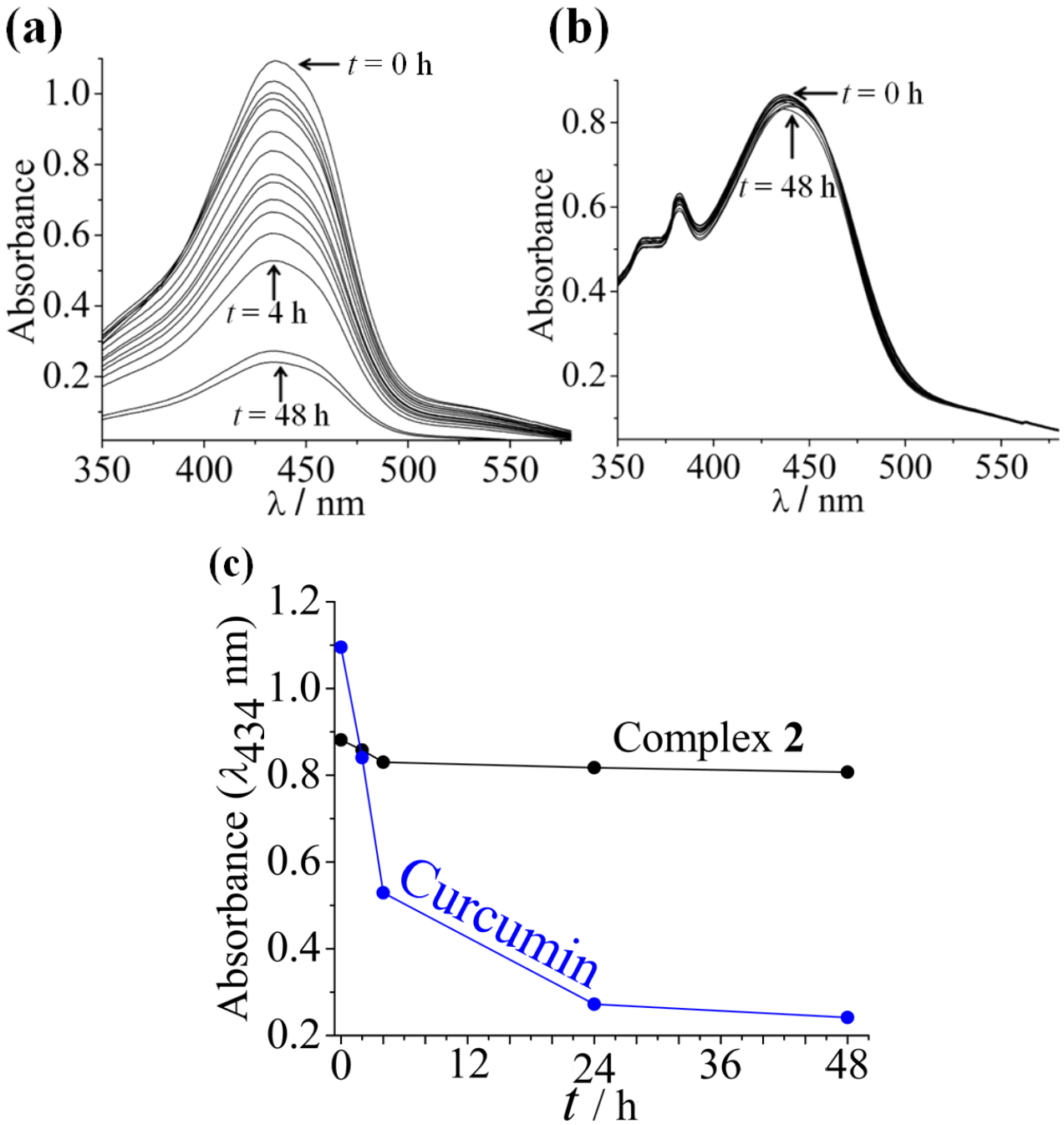


Fig. S8. Absorption spectral traces of (a) curcumin and (b) complex **2** in DMSO-Tris buffer (1:1 v/v, pH = 7.2, 37 °C). The spectra were recorded at an interval of 20 min for initial 4 h after incubation of the sample at 37 °C. The spectral data show significant degradation of curcumin over 4 h (a). The degradation is arrested on complex formation (b). (c) Plots showing the time dependence absorbance of curcumin (blue) and complex **2** (black) in 1:1 DMSO-Tris buffer (pH = 7.2, 37 ºC) over a period of 48 h.

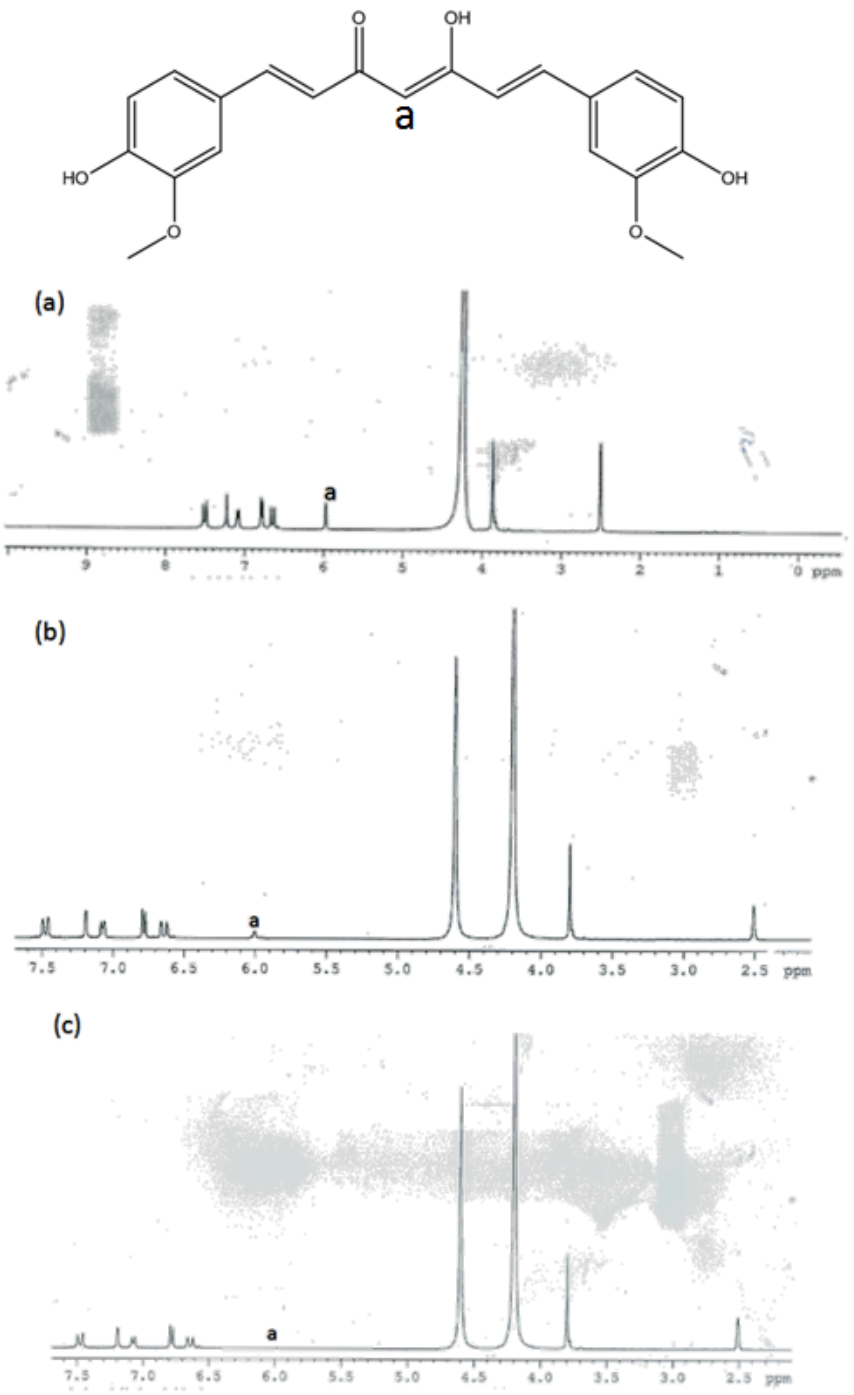


Fig. S9. ^{1}H NMR spectrum of curcumin in (a) DMSO-d_6 (0.75 ml), (b) DMSO-d_6 (0.75 ml + D_2O (0.1 ml) recorded after 4 h and (c) DMSO-d_6 (0.75 ml + D_2O (0.1 ml) recorded after 24 h. The spectra in (b) and (c) show gradual disappearance of the peak (marked as **a**) at 5.9 ppm corresponding the C-H proton of the β-diketone moiety.

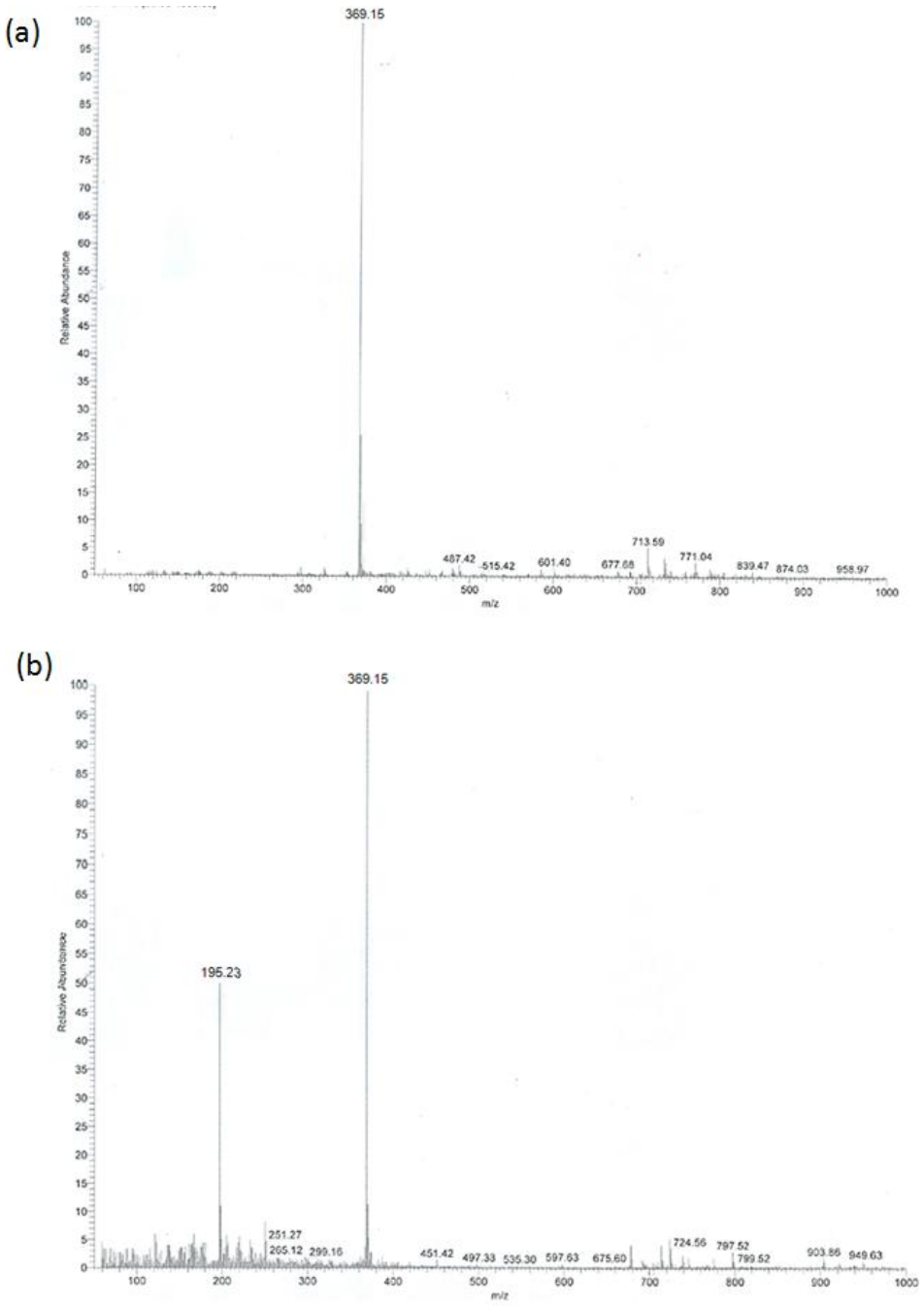


Fig. S10. ESI-MS spectrum of (a) curcumin in aqueous acetonitrile showing $[M+H]^+$ peak and (b) curcumin in aqueous acetonitrile after incubation for 4 h at 37 °C showing a peak at 195.23 that corresponds to ferulic acid generated from the degradation of curcumin.

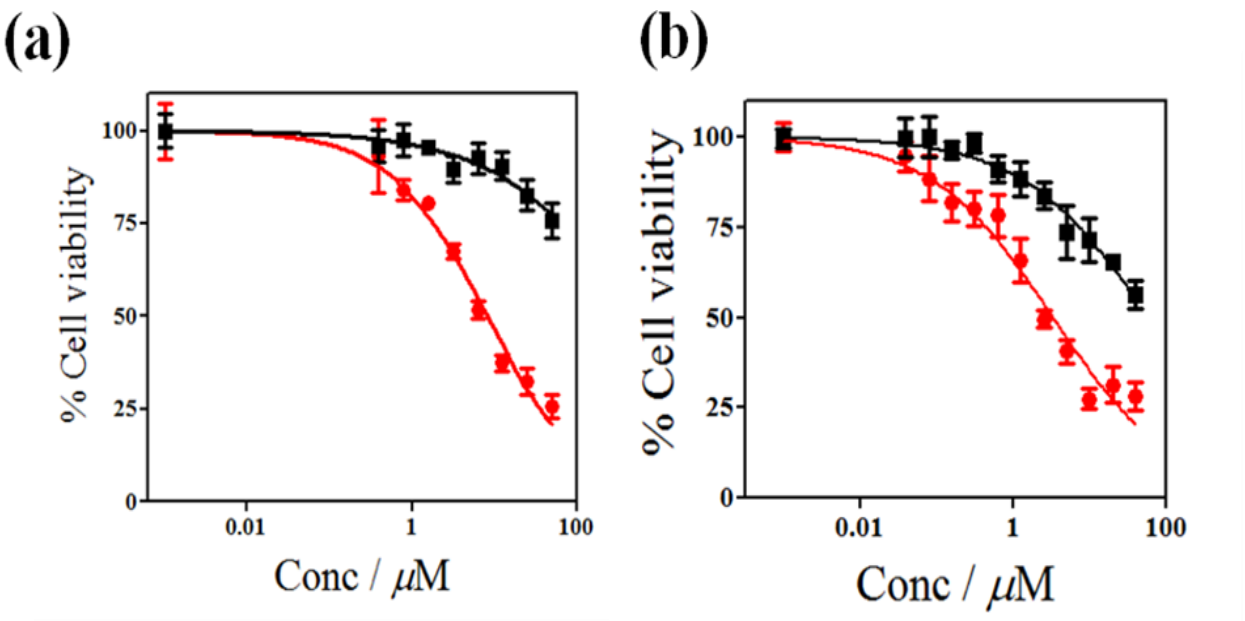


Fig. S11. Photocytotoxicity of complex **1** (a) and **2** (b) in HeLa cancer cells on 4 h incubation in dark followed upon photo-irradiation in visible light (400 to 700 nm) for 1 h as determined by MTT assay. The photo-exposed and dark-treated cells are shown in red and black color symbols, respectively.

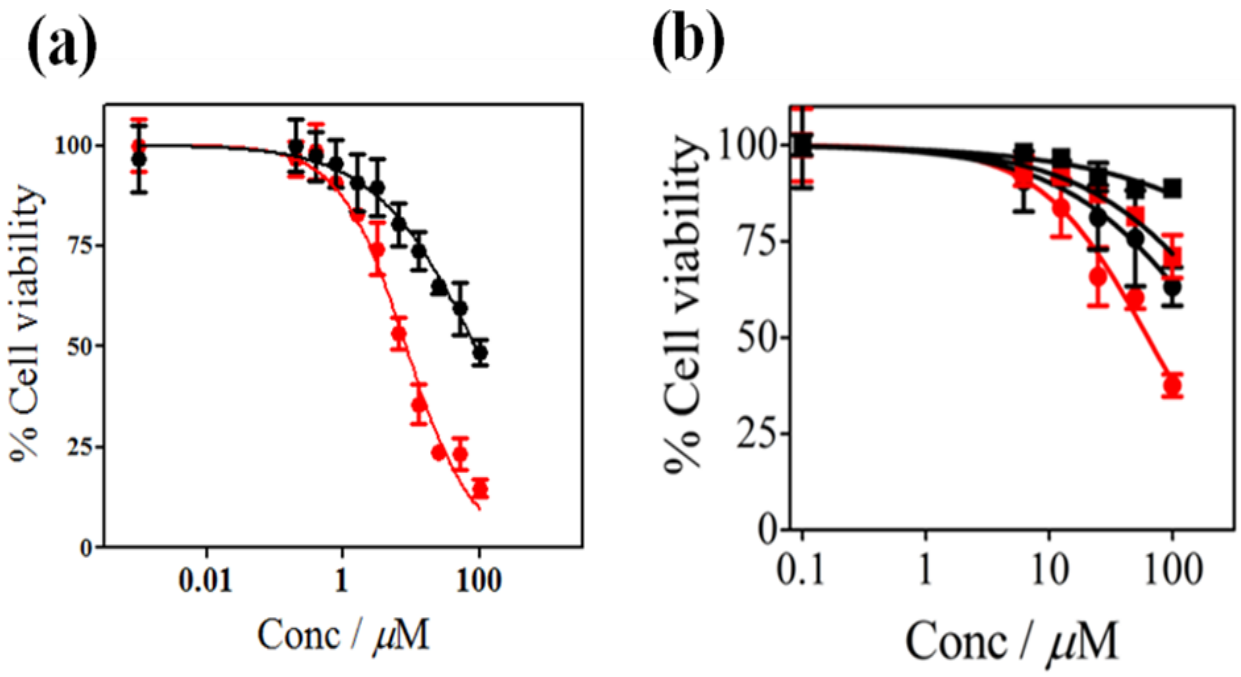


Fig. S12. Cell viability plot showing the photocytotoxicity of (a) curcumin (●) and (b) $VOSO_4$ (■) and dppz (●) in HeLa cells on 4 h incubation in dark followed by exposure to visible light (400-700 nm, 10 J cm^{-2}) for 1 h, as determined from the MTT assay. Red and black color symbols indicate data in visible light and in dark, respectively.

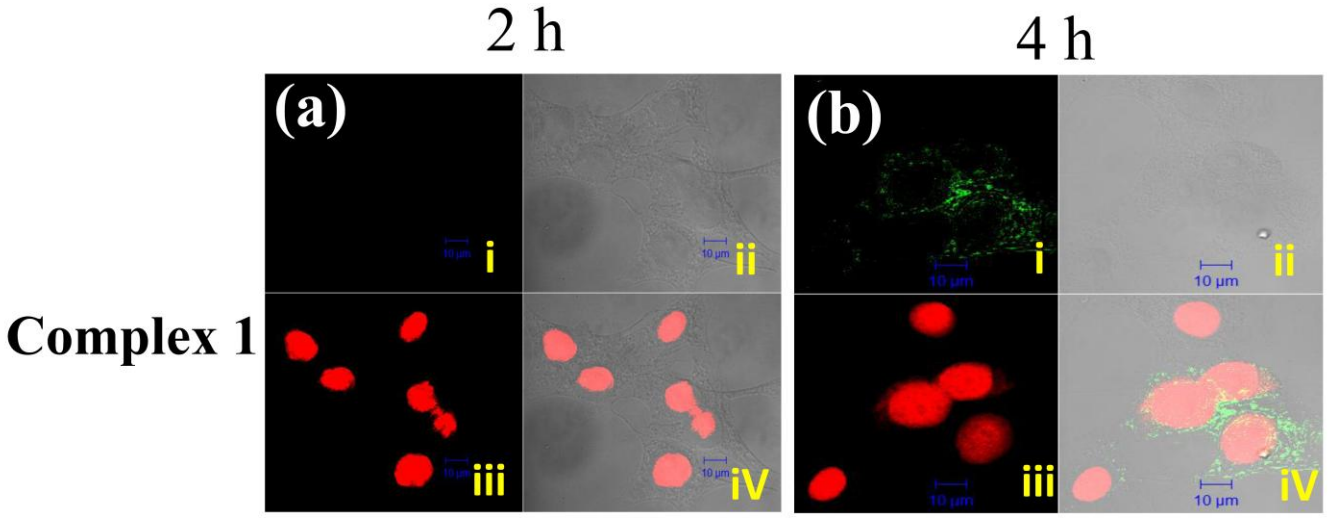


Fig. S13. A time-course collection of confocal microscopic images of the HeLa cells treated with complex **1** (10 μM) and propidium iodide (PI, 10 mg/ml). Panels (a-b, i) show the green emission of complex **1**. Panels (a-b, ii) show the bright field images. Panels (a-b, iii) correspond to the red emission of PI. Panels (a-b, iv) show the merged images.

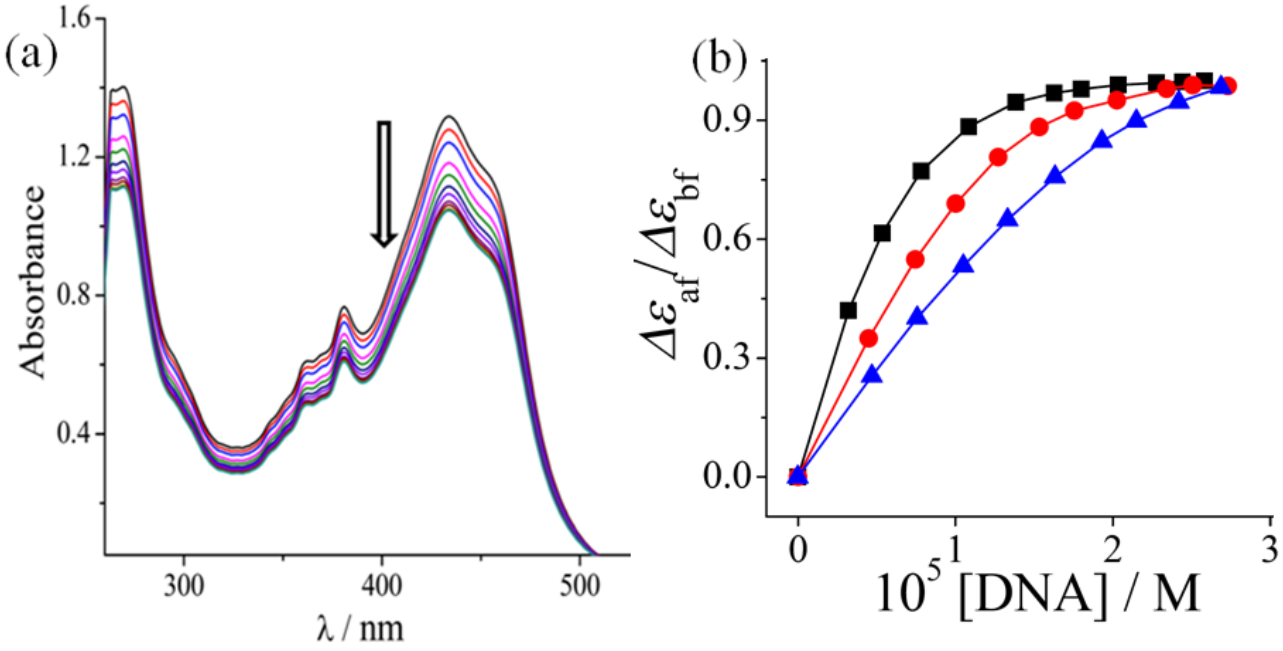


Fig. S14. (a) Absorption spectral traces of complex **2** in 5 mM Tris-HCl buffer (pH 7.2) on increasing the quantity of calf thymus DNA. (b) The least-squares fit of $\Delta\varepsilon_{af}/\Delta\varepsilon_{bf}$ *vs.* [DNA] for curcumin (▲) and for complexes **1** (●) and **2** (■).

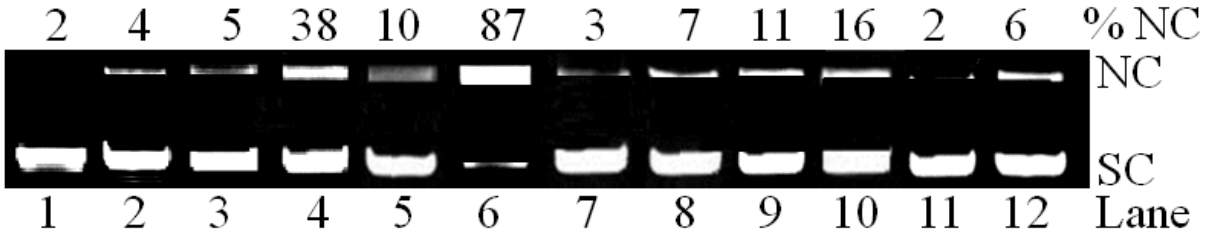


Fig. S15. Cleavage of SC pUC19 DNA (0.2 μg, 50 μM) by [VO(cur)(L)Cl] (L = phen, **1**; dppz, **2**) (40 μM), $VOSO_4$ and ligands in 50 mM Tris-HCl/NaCl buffer (pH, 7.2) containing 10% DMF on photo-irradiation with near-IR light of 785 nm (1 h exposure): lane 1, DNA control (in dark); lane 2, DNA control (in light); lane 3, DNA +**1** (in dark); lane 4, DNA + **1** (in light); lane 5, DNA + **2** (in dark); lane 6, DNA + **2** (in light); lane 7, DNA + $VOSO_4$ (in dark) ; lane 8, DNA + $VOSO_4$ (in light); lane 9, DNA + curcumin (in dark); lane 10, DNA + curcumin (in light); lane 11, DNA + dppz (in dark); lane 12, DNA + dppz (in light).