Electronic Supplementary Material (ESI) for New Journal of Chemistry.

A new *7*-diethylamino-*4*-hydroxycoumarin based reversible colorimetric/fluorometric probe for sequential detection of Al^{3+}/PPi and its potential use in biodetection and bioimaging applications

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UV-vis absorption titration

1 mM stock solution of the probe was prepared in DMF solvent. 15 μL (1 mM) ligand solution was diluted to 3 mL of DMF-Tris-HCl (6:4, v/v, pH 7.2) to make the final concentration of 5 μM. UV-Vis titration experiment was carried out between the probe and Al^{3+} by adding an incremental amount of (0-2 equivalent) 1 mM Al^{3+} ions to the probe solution, and the absorbance peak was monitored at room temperature.

Fluorescence titration

1 mM stock solution of the probe was prepared by dissolving 3.80 mg of the compound in 10 mL of DMF. For titration experiments, 5 μM solutions were prepared by addition of 15 μL of 1 mM solution to 3 mL of DMF-Tris-HCl (6:4, v/v, pH 7.2) in emission cuvette. Incremental amount of metal salt solution (1 mM) (Al^{3+}) was added to the above solution from 0 to 1 equivalent and corresponding emission spectra were recorded at room temperature.

Quantum yield Calculation

The fluorescence quantum yield experiment was performed by using following formula. Quinine sulfate was used as a standard at 365 nm with a known ϕ value of 0.546 in 0.5 M H_2SO_4.[1]

$$\Phi_s = \left(\frac{A_s}{A_r}\right)\left(\frac{f_s}{f_r}\right)\left(\frac{\eta_r^2}{\eta_r^2}\right)\Phi_r$$

Where, Φ_s and Φ_r are fluorescence quantum yield of sample and reference respectively. A_s and A_r are the area under the curve of sample and reference emission spectrum. The terms f_s and f_r represent the absorption optical density value for sample and reference while η is the refractive index of the solvent

Calculation of the binding stoichiometry and association constant

The binding stoichiometry of the metal ion to the probe **L** was determined by using the mole ratio method of Job plot experiments.[2]

The association constant (K_I) of the metal ion (Al^{3+}) binding to the probe **L** determined by a non-linear least squares fit of the data with the following equation as referenced elsewhere.[3]

$$F(x) = \frac{a + b \times cx^n}{1 + cx^n}$$

Where, **x** is the concentration of aluminium, **F(x)** is the intensity, **a** is the intensity of probe without aluminium, **b** is the intensity at the saturation, **n** is the binding stoichiometry and **c** is the association constant.

The association constant of the Al^{3+} ensemble binding to the PPi was determined from the fluorescence titration data based on the reported Benesi-Hildebrand equation.[4]

$$1/\Delta A \textit{ vs } 1/[M^+]$$

Calculation of Limit of Detection

To calculate the Limit of detection (LOD) 5 μM concentration of probe **L** and 1 mM concentration of metal ions were prepared. Fluorescence titration was carried out by gradual addition of metal ions (Al^{3+}) to the solution of **L**. Fluorescence titration showed a linear response up to saturation point. Then, LOD was calculated by the following equation

$$LOD = 3s/m$$

Where, s is the standard deviation of blank measurements and m is the slope value obtained from the calibration curve.

Extraction of aluminium content from the pharmaceutical tablet

The finely powdered tablet sample (Digene) was dissolved in 20 mL of 2N nitric acid and stirred for 8 hours, after which the solutions were filtered, and the filtrate was diluted with double distilled water. The volume of the final solution was made up to 100 mL and the pH of the extract was adjusted to 7.2 and used for the emission titration at ambient temperature.

Polymerase Chain Reaction (PCR)

PCR is a well-established technique to detect or amplify target DNA. Here we used commercial PCR-kit GoTaq® G2 Colorless Master Mix (Promega) which is a premixed ready-to-use solution containing GoTaq® G2 DNA Polymerase, dNTPs, $MgCl_2$ in reaction buffers. The PCR reaction mixture was prepared by the addition of 0.2 picomoles forward and reverses primers in the 1X PCR master mix along with 10 nanograms of template plasmid DNA. The

PCR reaction was carried out in Mastercycler® nexus (Eppendorf) at 10, 20, 30 and 40 cycles of denaturation (95°C/30 s), annealing (50°C/30 s), and extension (72°C/60 s). Forward Primer 5'-CCTGTCTGGTCCCGAAAGAAC-3' and Reverse primer 5'-= GACGTGATCCGATCCACAATG-3' were used and the amplified product size is was 131 base pairs.

Measurement of PPi in biological extracts and bio-imaging

The mammalian C2C12 cell line obtained from National Centre for Cell Science, India and Escherichia coli DH5α cells were used for this experiment. The experimental details were mentioned elsewhere in our previous publication[5]. Briefly, the mammalian cells were grown in 20 % FBS in DMEM with appropriate antibiotics and antimycotics at 37 °C in a humidified incubator with 5 % CO_2. The cells were collected at 70 % confluence by trypsinization. Total cell extracts were prepared by suspending cells in RIPA lysis buffer followed by sonication. Similarly, freshly inoculated DH5α cells cultured at 37 °C in the LB media overnight were collected and washed with PBS. The cells were lysed by sonication in bacterial lysis buffer. Cell debris from mammalian and bacterial cell lysates was removed by centrifugation. Protein concentrations of the supernatants were determined by the Bio-Rad protein assay system. Total cell extracts at various concentrations were used to measure the PPi.

For bio-imaging applications, 50-60 % confluent cells were treated with a different combination of probe **L** (5 μM), Al^{3+} (25 μM) and PPi (25 μM and 50 μM) for 5 minutes followed by fluorescence imaging with Nuclear counterstain DAPI (4′,6-diamidino-2-phenylindole) in an IX73 Inverted Fluorescence Microscope (Olympus). The fluorescence signal in the cells was quantified using ImageJ Ver1.52a software (NIH, USA) and represented as the corrected total cell fluorescence (CTCF) using the below-mentioned formula. CTCF = Integrated Density – (Area of selected cell × Mean fluorescence of background readings)

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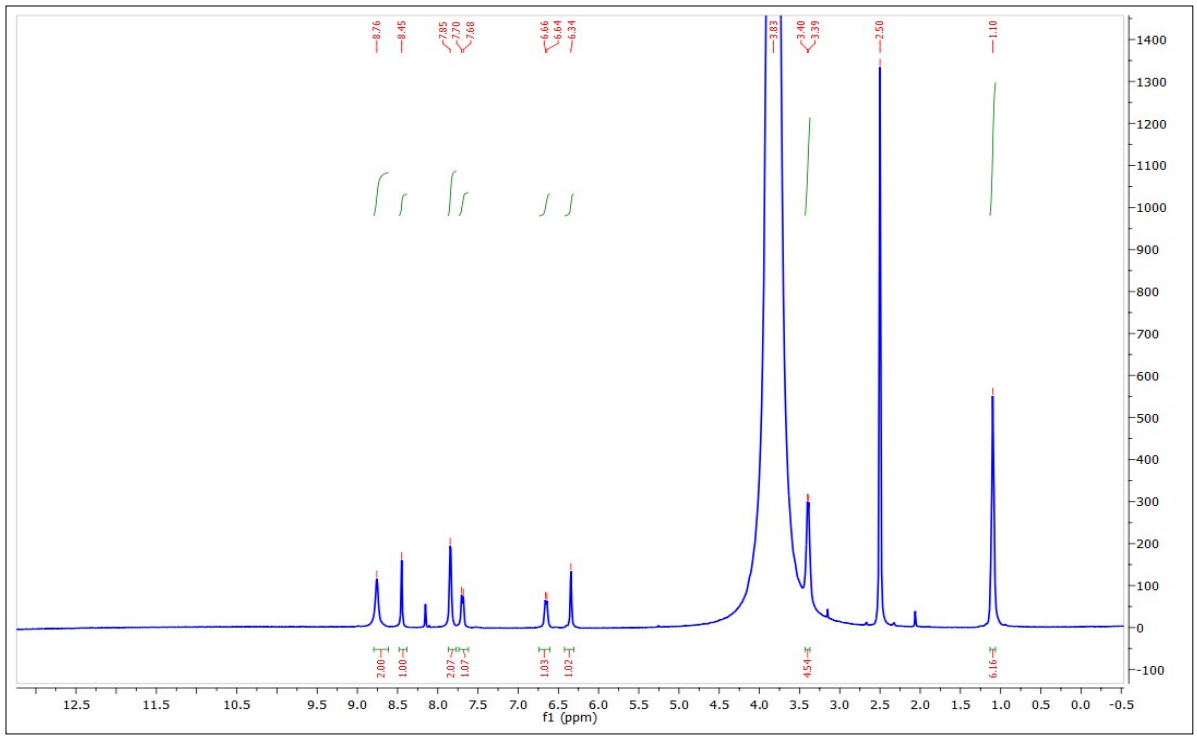


Figure. S1. ^{1}H NMR spectrum of **L** in DMSOd_6.

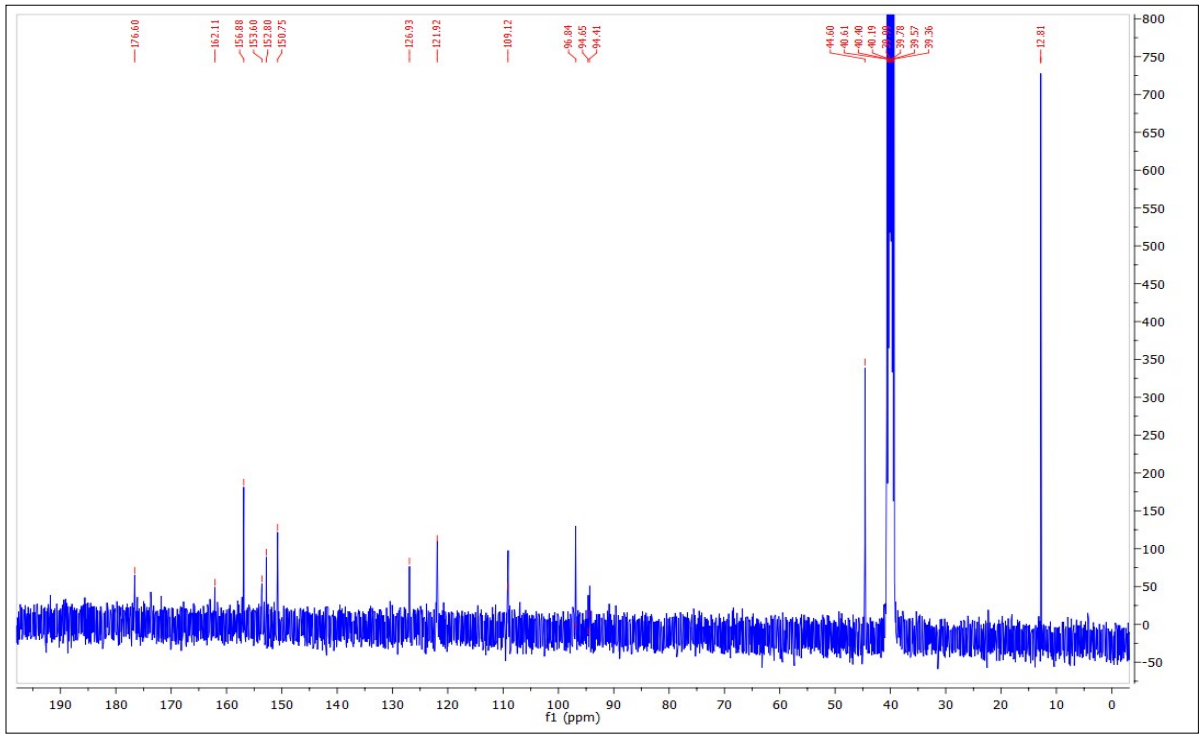


Figure. S2. ^{13}C NMR spectrum of **L** in DMSOd_6.

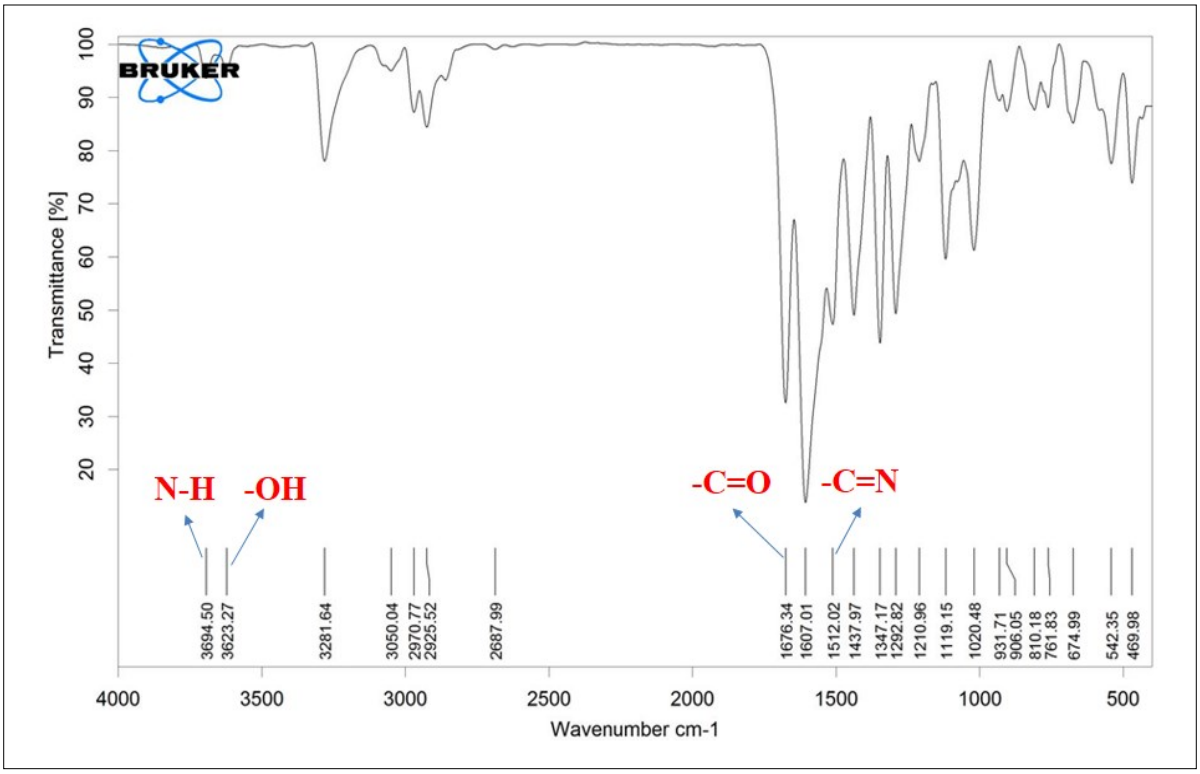


Figure. S3. FT-IR spectrum of **L**.

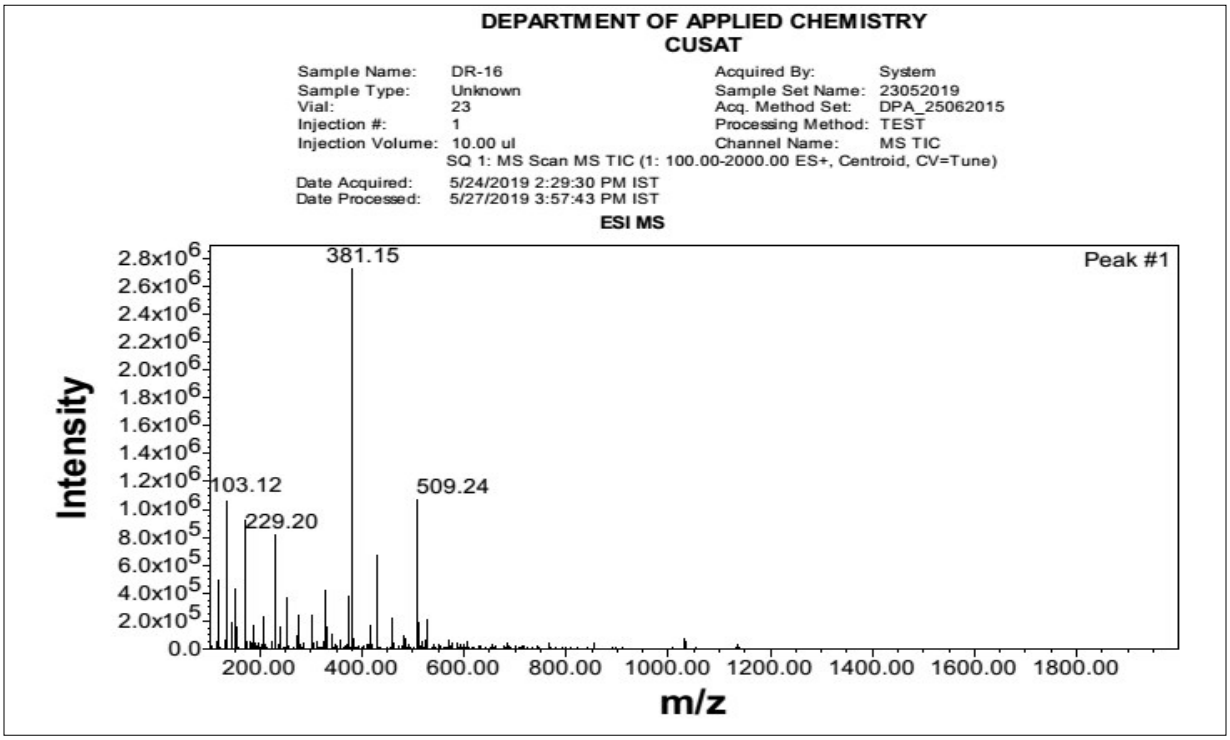


Figure. S4. ESI-Mass spectrum of **L** in DMSO.

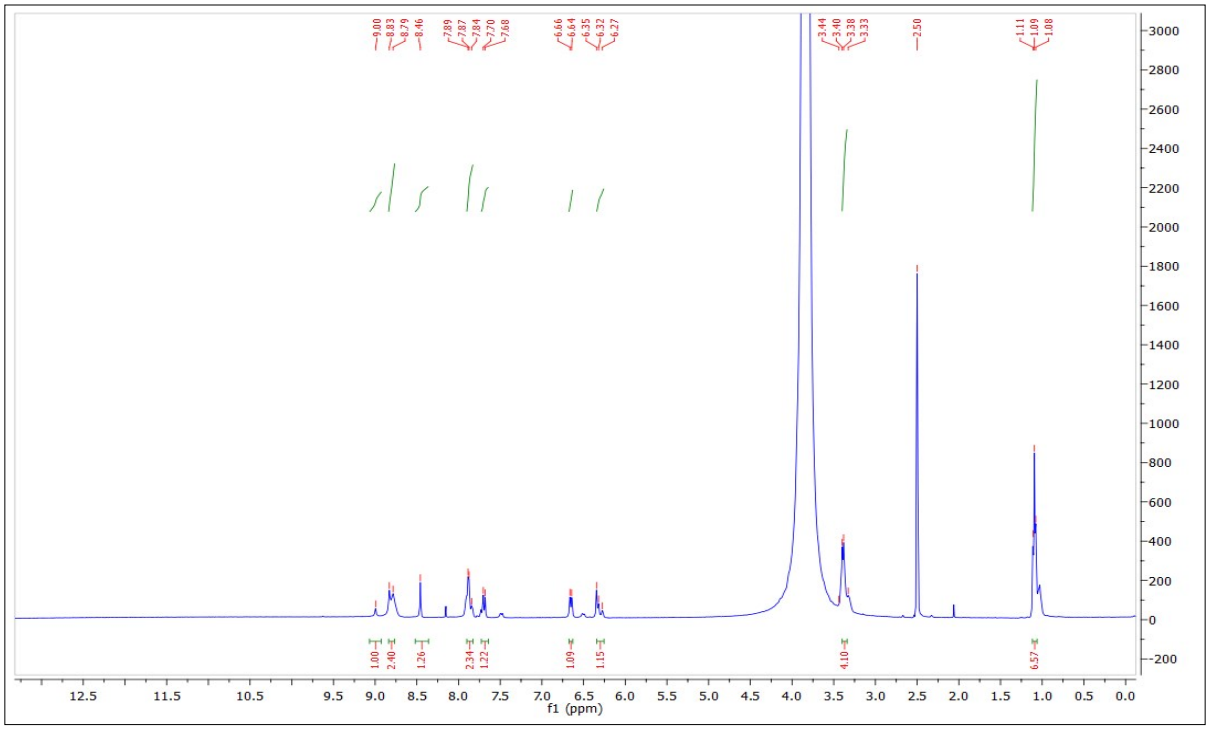


Figure. S5. ^{1}H NMR spectrum of **L-Al^{3+}** complex in DMSOd_6.

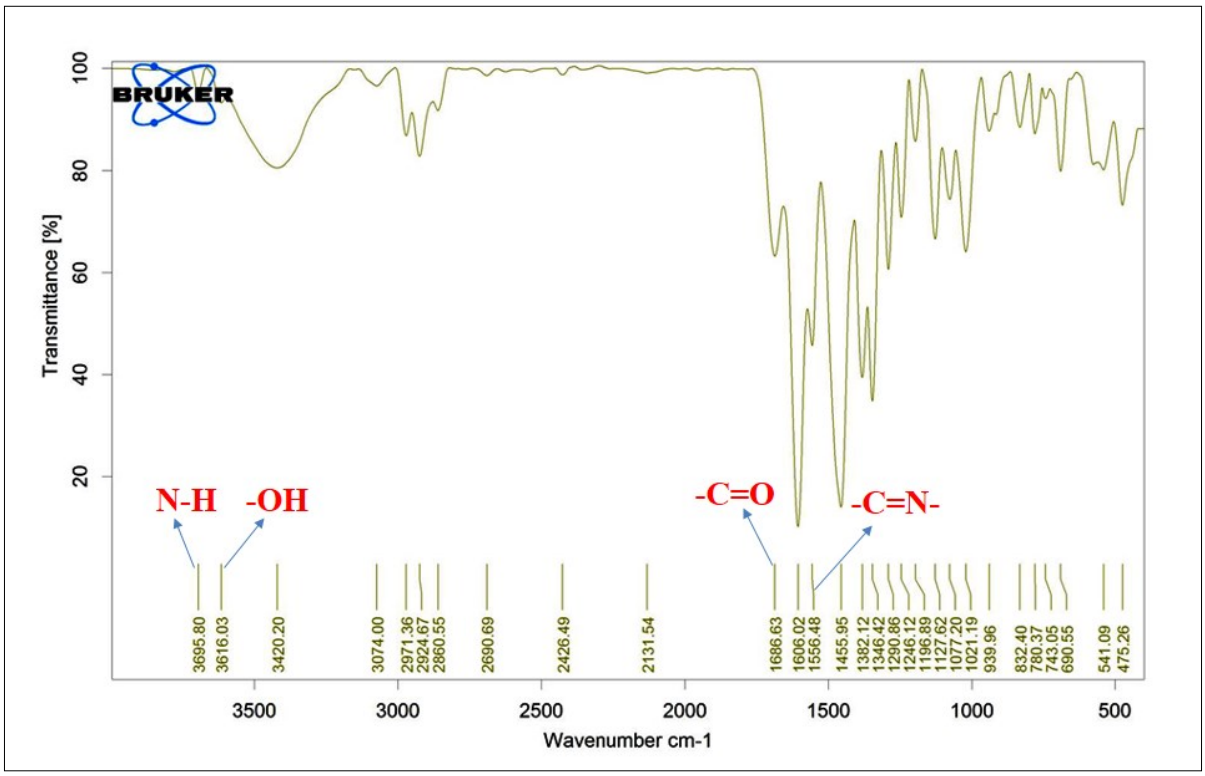


Figure. S6. FT-IR spectrum of **L-Al^{3+}** complex.

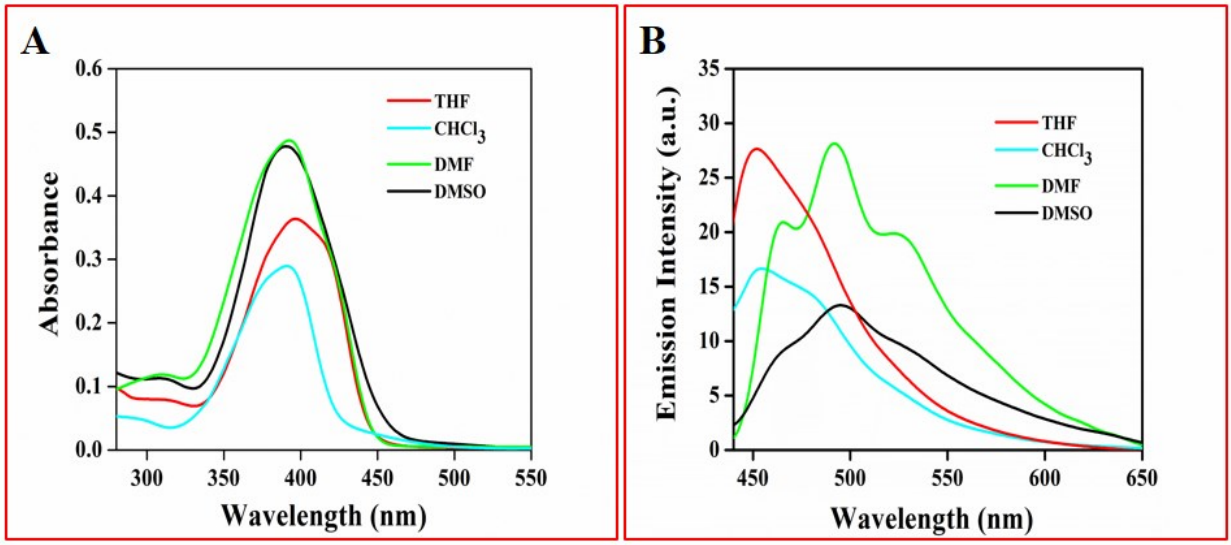


Figure. S7. A) Absorption spectra of the compound **L** in different solvents. B) Emission spectra of the compound **L** in different solvents.

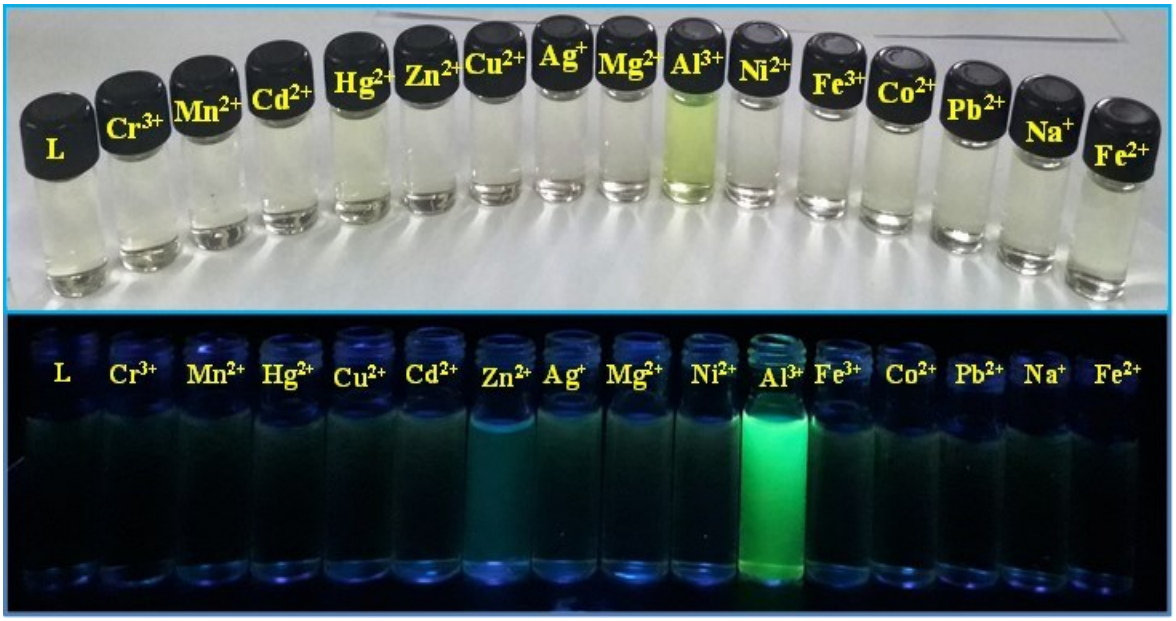


Figure. S8. Naked eye visible color changes of **L** (5 μM**)** upon addition of different metal ions under daylight (upper) and UV light (lower) (10 equiv.).

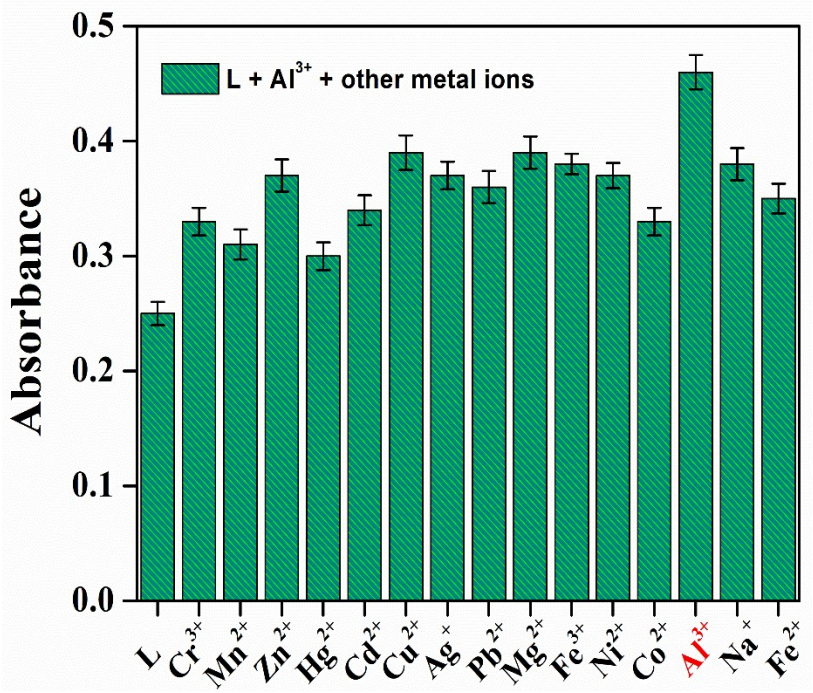


Figure S9. Absorption competitive selectivity of **L** with 10 equiv. of different metal ions in the presence of Al^{3+} (2 equiv.).

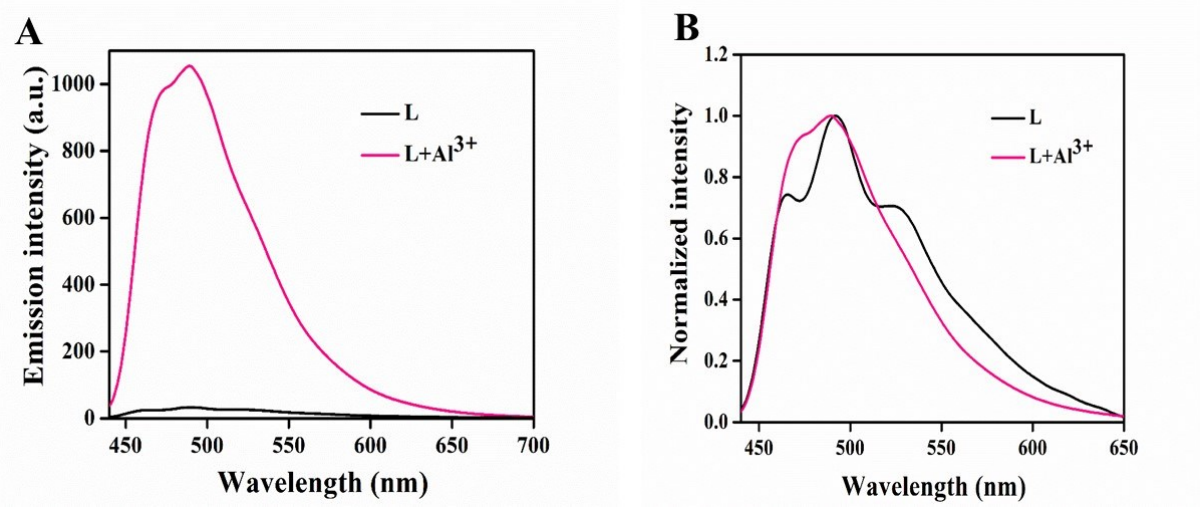


Figure S10. A) Un-normalized emission spectra of **L** and **L-Al^{3+}** ions (1 equiv.). B) Normalized emission spectra of **L** and **L-Al^{3+}** ions.

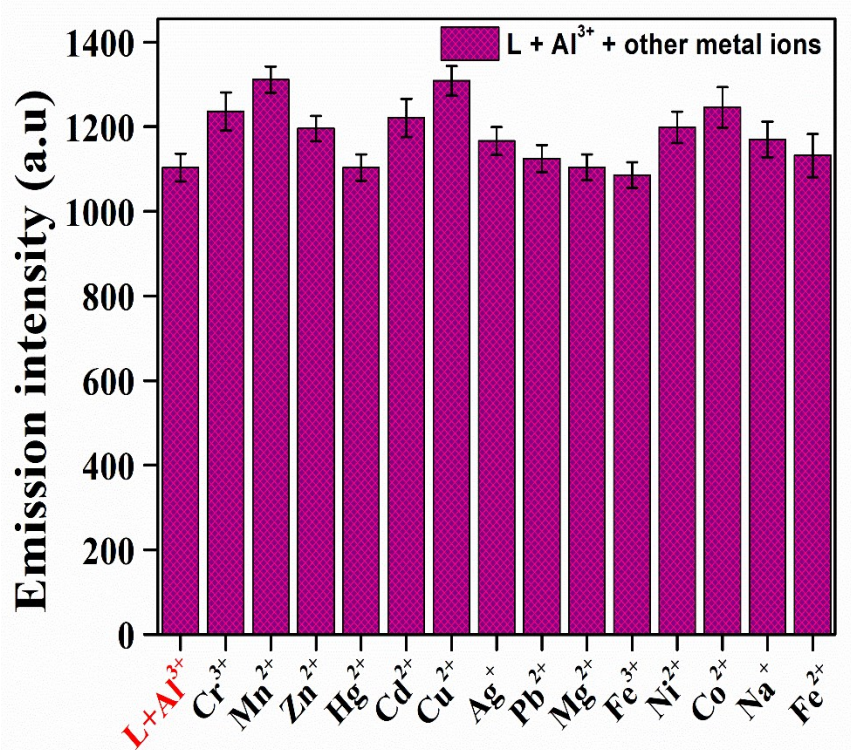


Figure S11. Emission competitive selectivity of **L**+Al^{3+} with 10 equivalents of different metal ions.

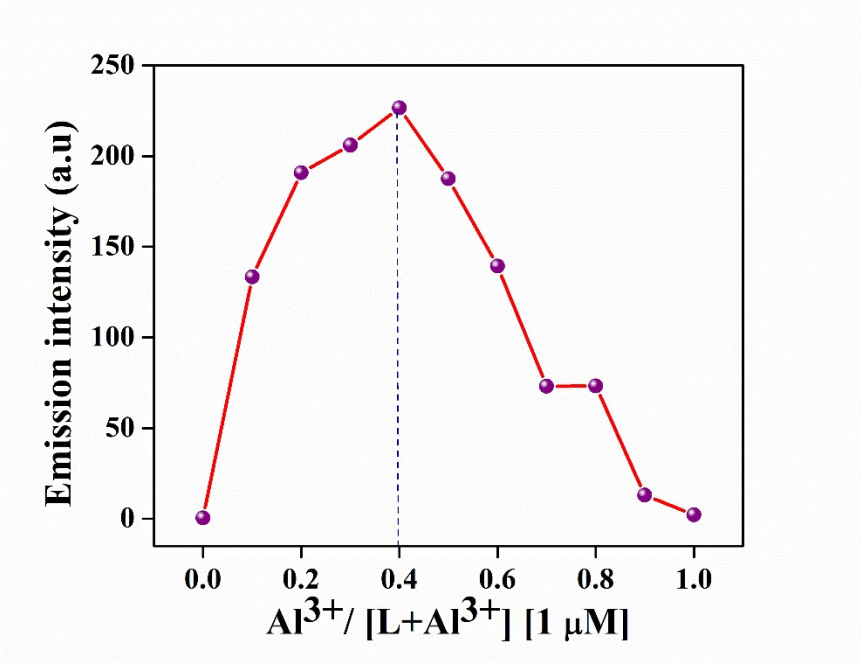


Figure S12. Job's plot of **L** with Al^{3+} showing 2:1 binding stoichiometry.

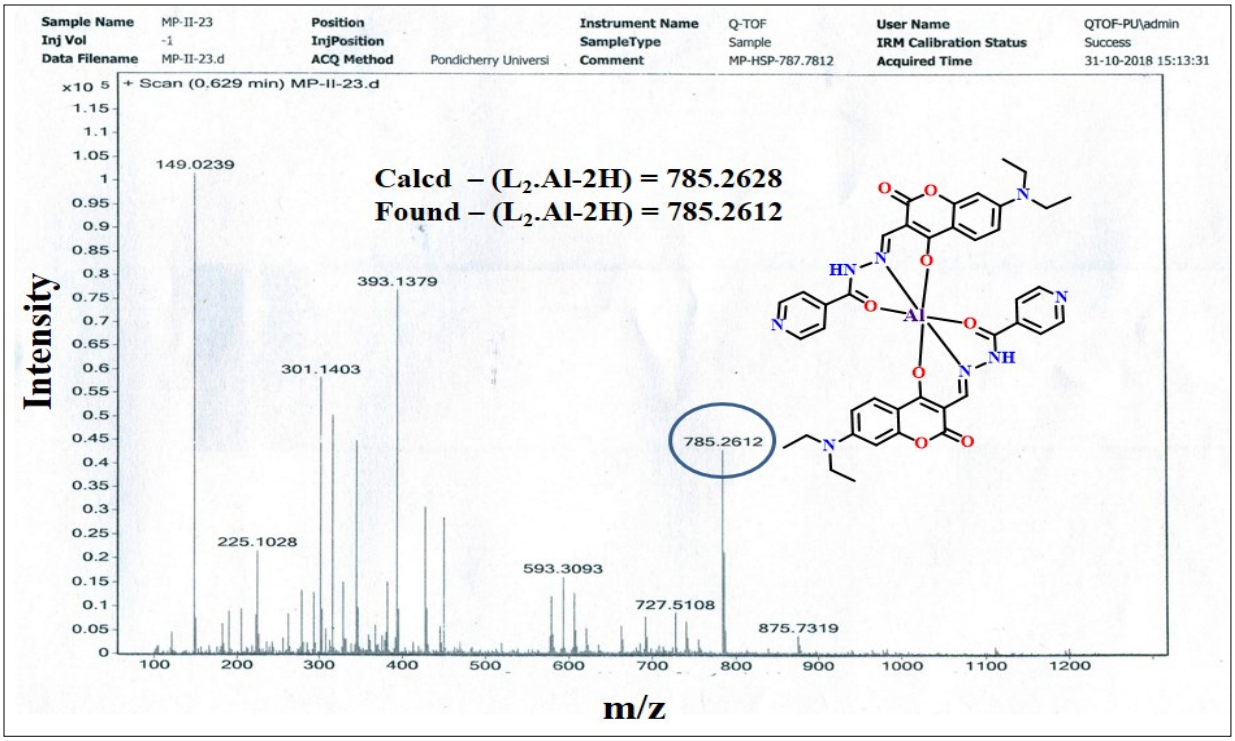


Figure. S13. ESI-Mass spectrum of **L-Al^{3+}** complex in DMSO.

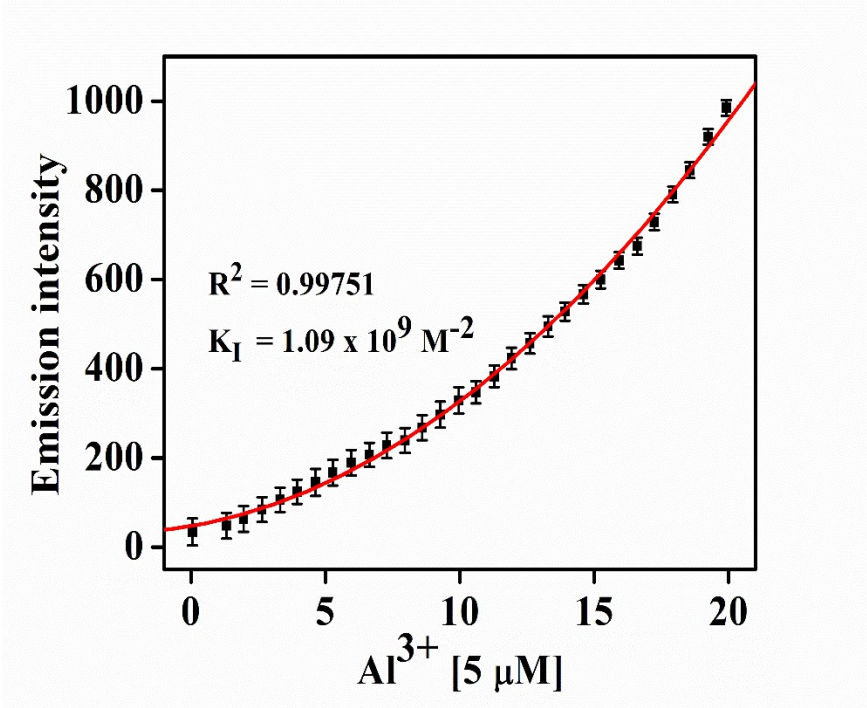


Figure S14. Non-linear curve fitting plot of emission titration data for determination of association constant of **L**.

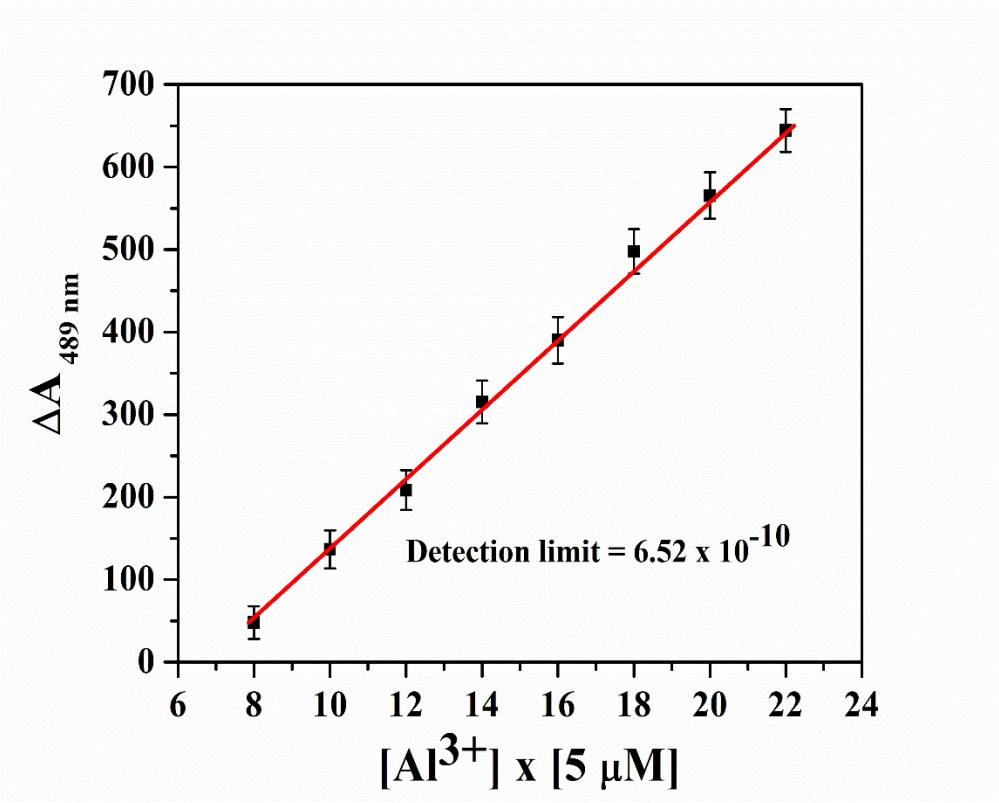
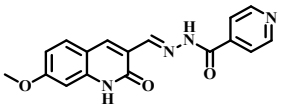
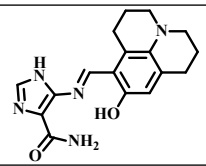
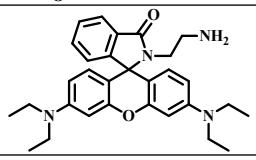
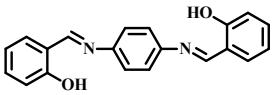
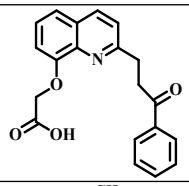
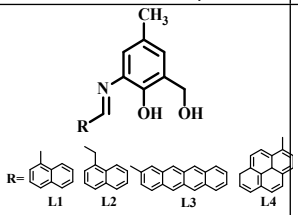
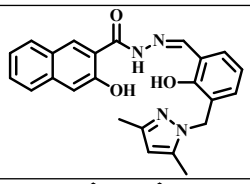
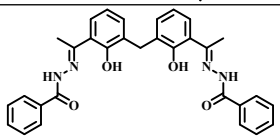
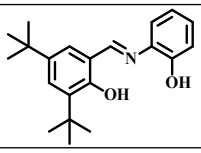
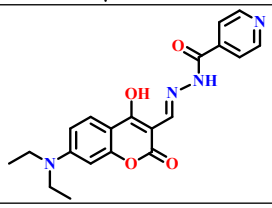


Figure S15. Limit of detection plot of emission titration data for **L** with Al^{3+}.

Table S1. Comparison of Al^{3+} sensing by sensor **L** with some of the previously reported Al^{3+} and PPi sensors.

Previous literature	Ligand Structure	Solvent system	Detection limit for L-Al^{3+}	Detection limit for L-Al^{3+}PPi	Association constant for L-Al^{3+}	Association constant for L-Al^{3+}PPi	PCR study	Al^{3+} tablet study	Cell imaging study
Inorg. Chem. 2017, 56, 3315−3323		Aqueous solution	5.60×10^{-10} M	1.40×10^{-8} M	695 M^{-2}	-	No	No	Yes
Dyes and Pigments 139 (2017): 136-147.		10 mM bis-tris buffer pH 7.0	9.24 μM	20.5 μM	2.3×10^{2} M^{-1}	3.70×10^{3} M^{-1}	No	No	Yes
Analyst, 2010, 135, 2079–2084		1 mM HEPES buffer solution pH 7.4	-	7.3 μM	2.77×10^{8} M^{-2}	-	No	No	No

Journal of Photochemistry and Photobiology A: Chemistry 358 (2018): 92-99.		H_2O/DMF (1:4)	3.9×10^{-7} M	7.5×10^{-7} M	Correlation coefficient 0.9908	Correlation coefficient 0.9904	No	No	No
ChemistrySelect 2019, 4, 10643–10648		DMF/HEPES (5:1,v/v,10 mM, pH=7.2)	4.96×10^{-7} M	5.70×10^{-7} M	5.27×10^{3} M^{-1}	1.23×10^{3} M^{-1}	No	No	Yes
Inorg. Chem. 2019, 58, 15, 10364–10376		EtOH (containing 0.5% THF)	5.86 µM 4.64 µM 1.46 µM 0.38 µM	-	1.4×10^{3} M^{-1} 7.6×10^{3} M^{-1} 6.6×10^{3} M^{-1} 16×10^{3} M^{-1}	-	No	No	No
Photochem. Photobiol. Sci., 2018,17, 200-212		HEPES buffer pH 7.2.	114.54 nM	0.56 µM	3.4×10^{4} M^{-1}	-	No	No	Yes
Spectrochimica Acta, Part A (2020), 118600.		H_2O/DMF (1:1)	0.65 µM	-	3.7×10^{5} M^{-1}	-	No	No	No
Dalton Trans., 2015,44, 11352-11359		HEPES buffer (20 mM, 1% EtOH, pH = 7.4).	2.94×10^{-8} M	2.74×10^{-7} M	$5.96 \times 10^{3}M^{-1}$	-	No	No	Yes
Present work		DMF-Tris-HCl (6:4, v/v, PH 7.2)	6.52×10^{-10} M	2.36×10^{-8} M	1.09×10^{9} M^{-2}	2.10×10^{3} M^{-1}	Yes	Yes	Yes

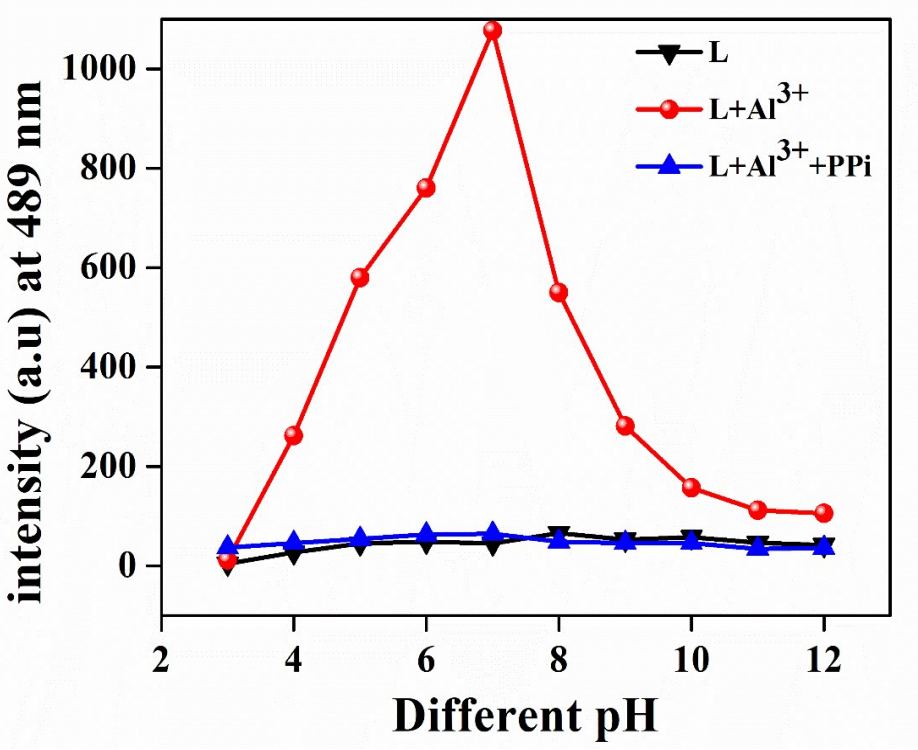


Figure S16. Fluorescence intensity changes at 489 nm of **L** (5 μM), **L-Al^{3+}** (1 equiv.) and **L-Al^{3+}-PPi** (45 equiv.) in DMF-Tris-HCl (6:4, v/v, PH 7.2) solution at different pH scale (3-12).

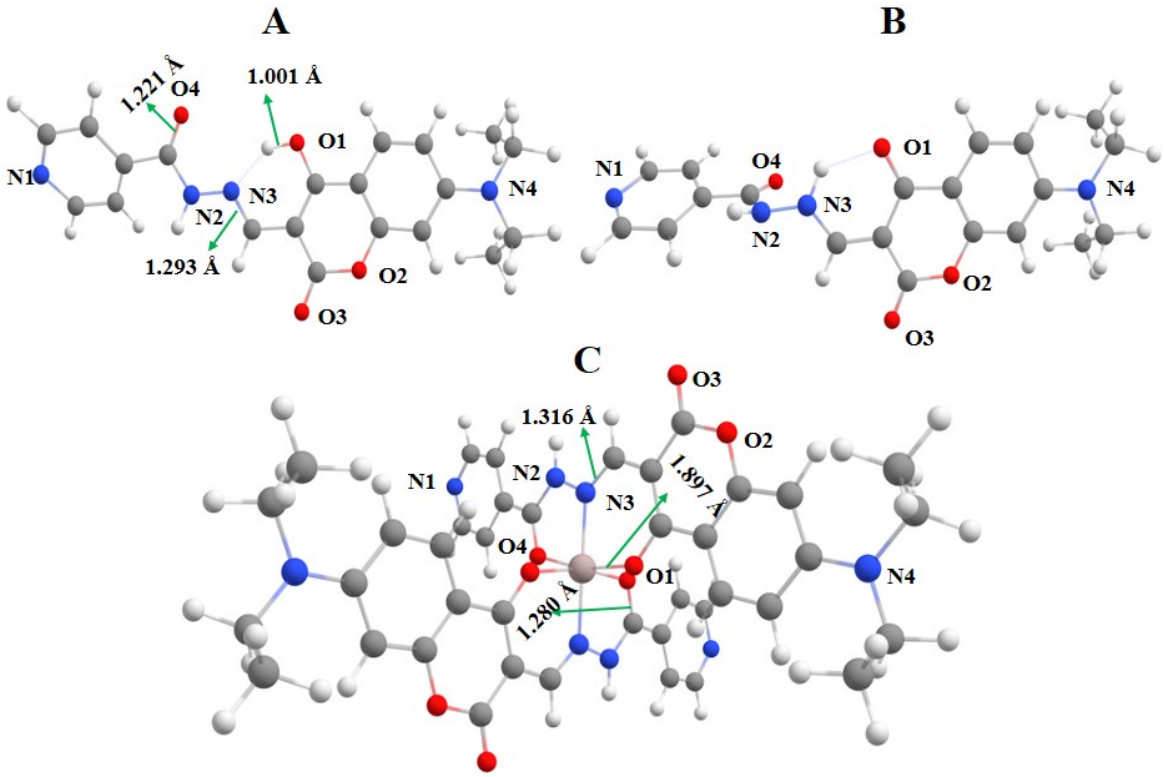


Figure S17. A) Optimized structures of **L** enol form. B) **L** keto form. C) **L-Al^{3+}** complex.

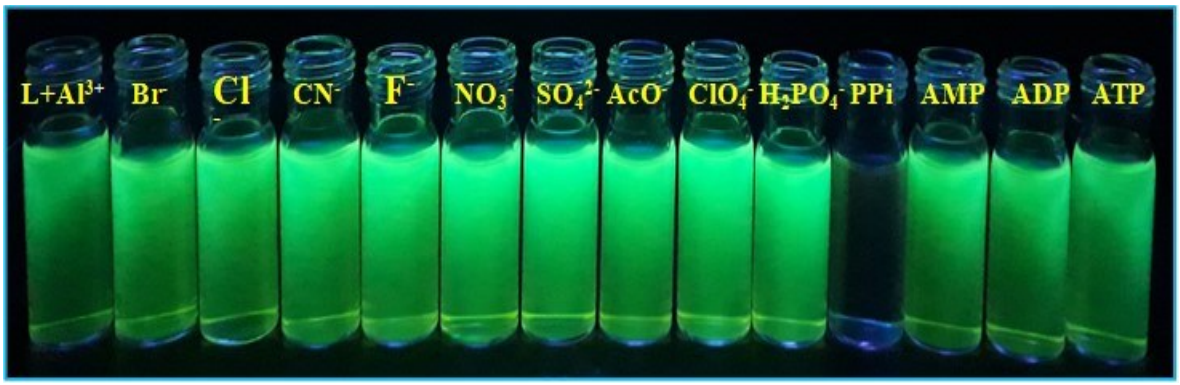


Figure S18. Photographic image of **L-Al^{3+}** ensemble with 50 equiv. of different anions under UV light 365 nm.

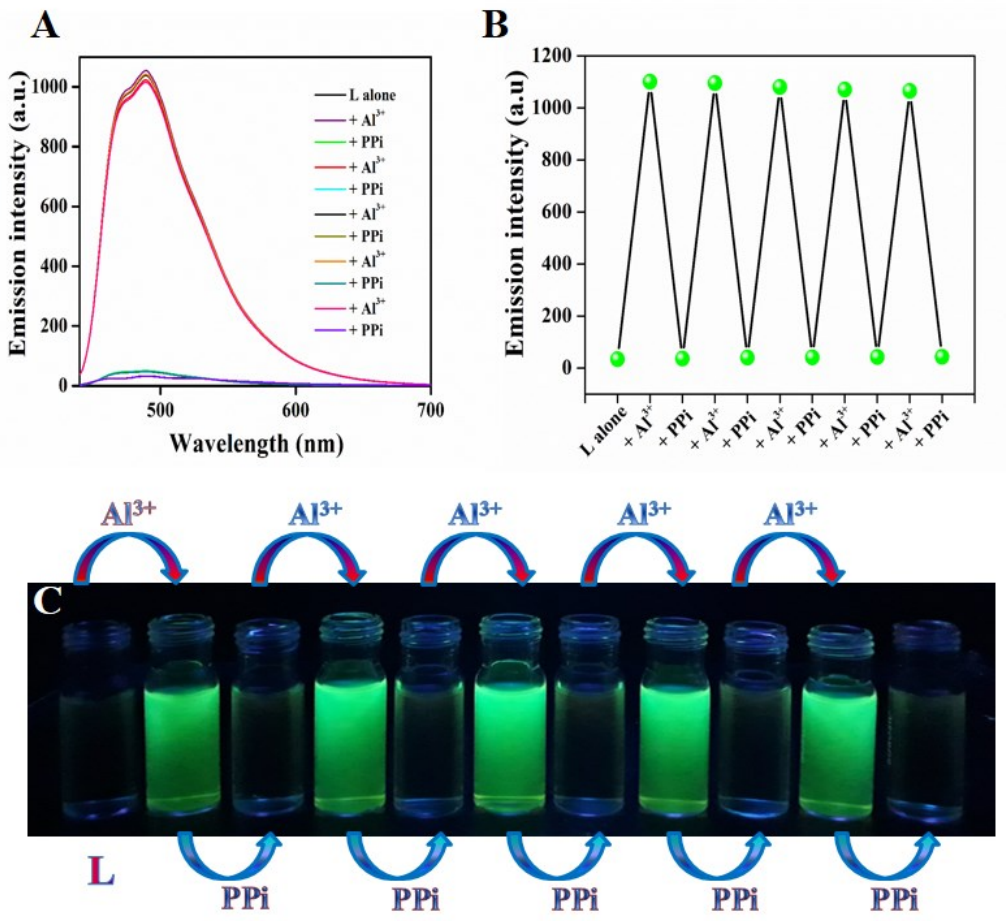


Figure S19. A) Emission spectral data of **L** with continues addition of Al^{3+} followed by PPi. B) Reversible fluorescence switching cycle of **L** with Al^{3+} and PPi. C) Photographic image of **L** shows reversible on-off emissive behavior with Al^{3+} and PPi under UV light 365 nm.

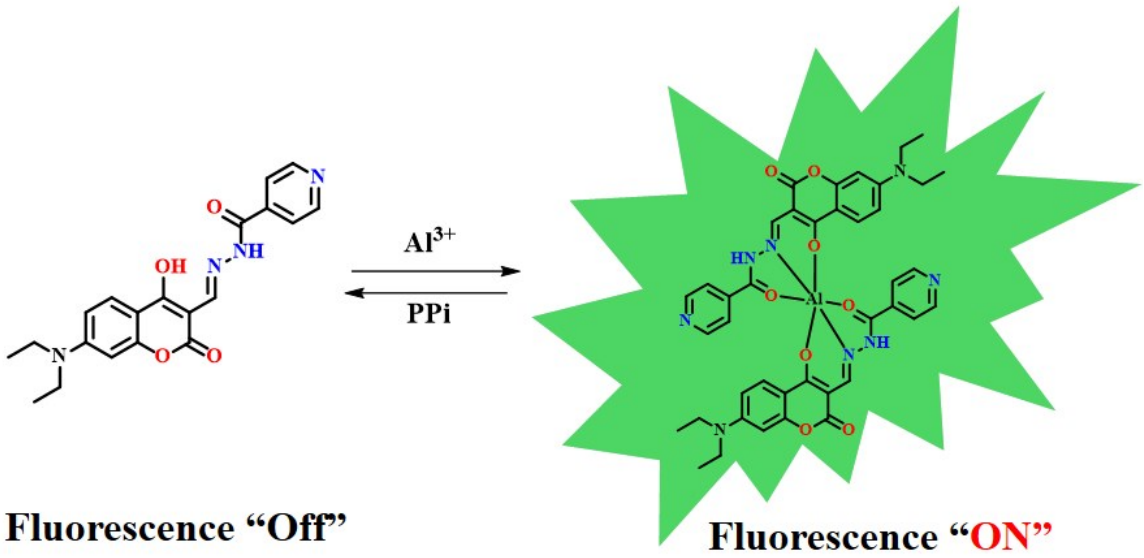


Figure S20. Detection mechanisms for the probe with Al^{3+} ion and probe-Al^{3+} ensemble with PPi.

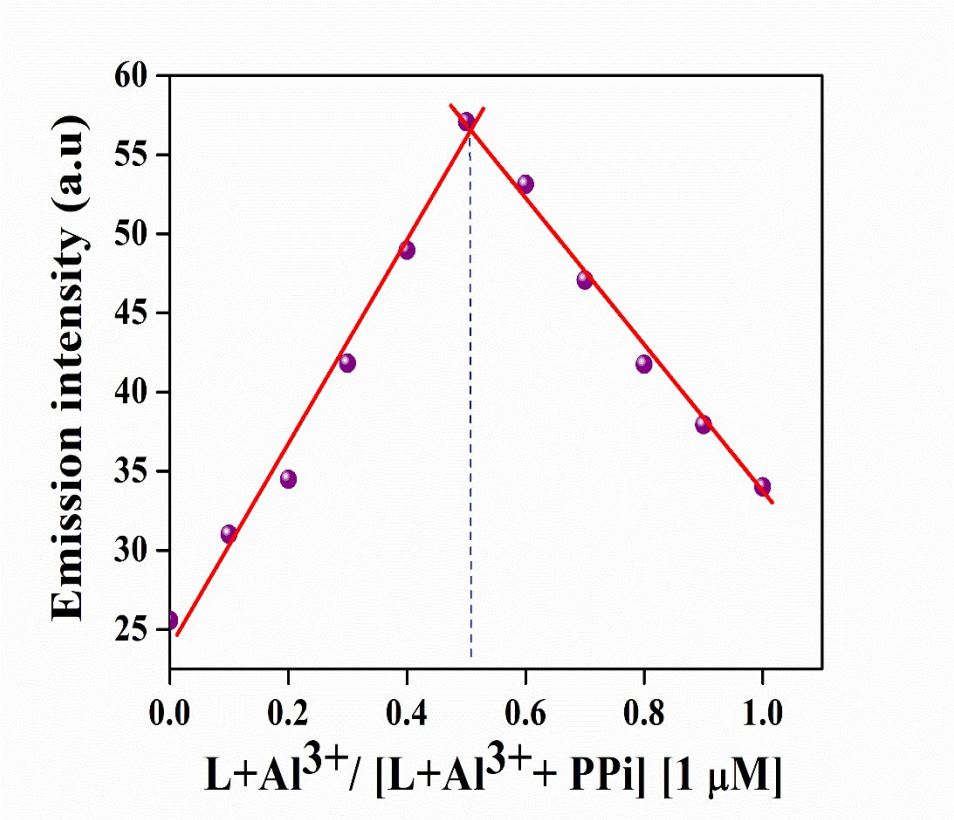


Figure S21. Job's plot of **L.Al**$^{3+}$ with PPi showing 1:1 binding stoichiometry.

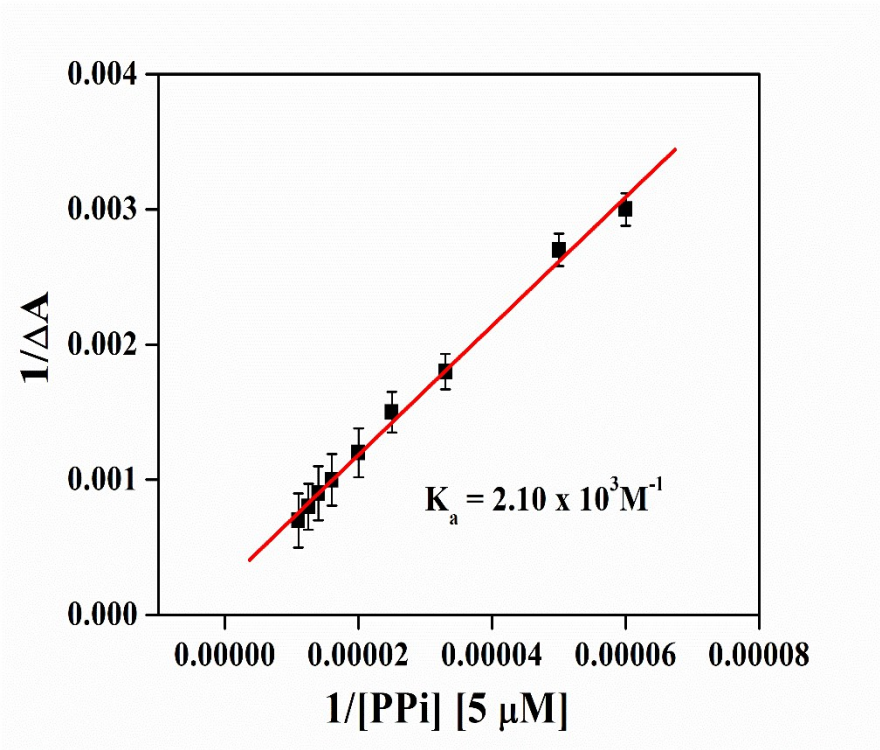


Figure S22. Benesi-Hildebrand plot (emission 489 nm) of **L-Al^{3+}** assuming 1:1 stoichiometry for association between **L-Al^{3+}** ensemble and PPi.

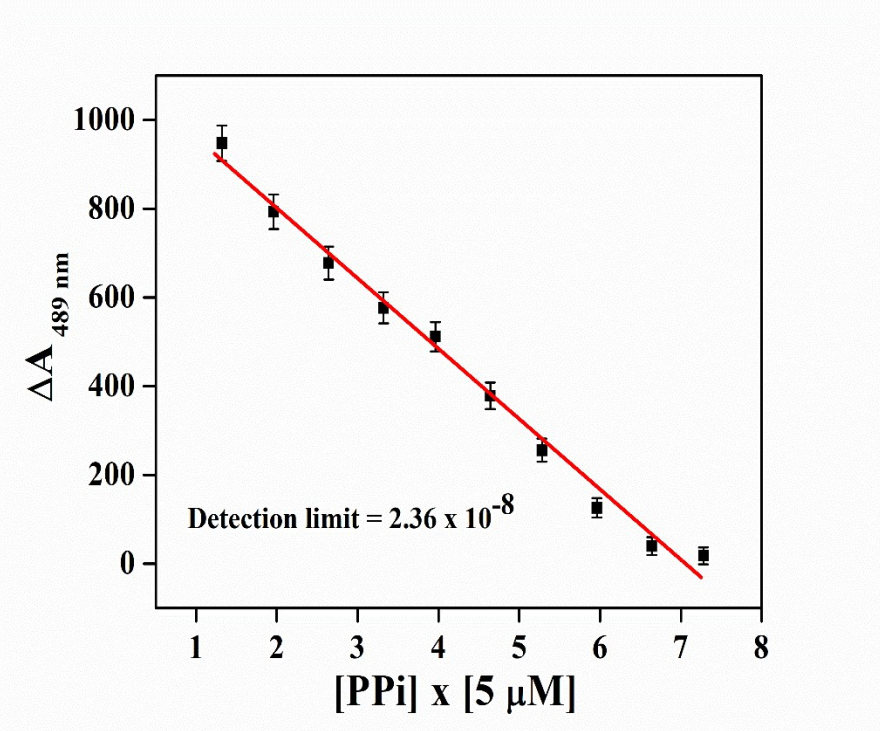


Figure S23. Limit of detection plot of emission titration data for **L-Al^{3+}** with PPi.

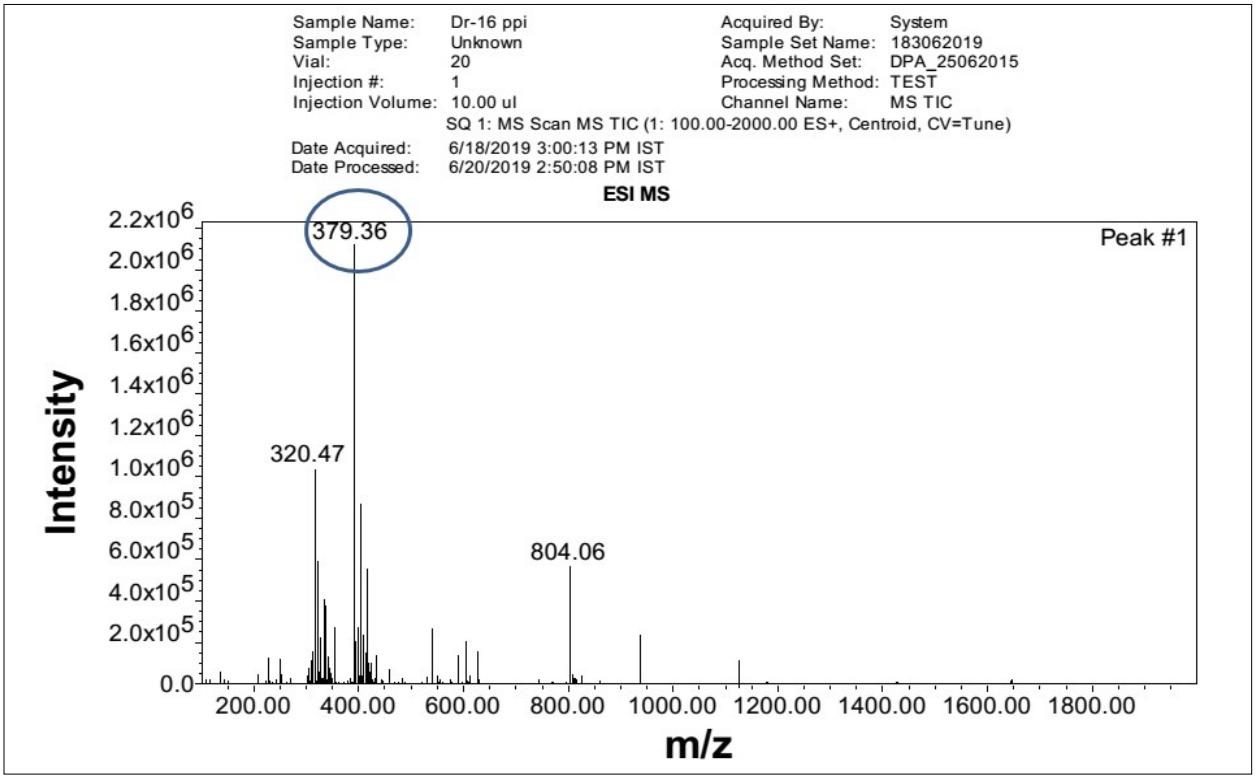


Figure. S24. ESI-Mass spectrum of **L-Al^{3+}-PPi** complex in DMSO.

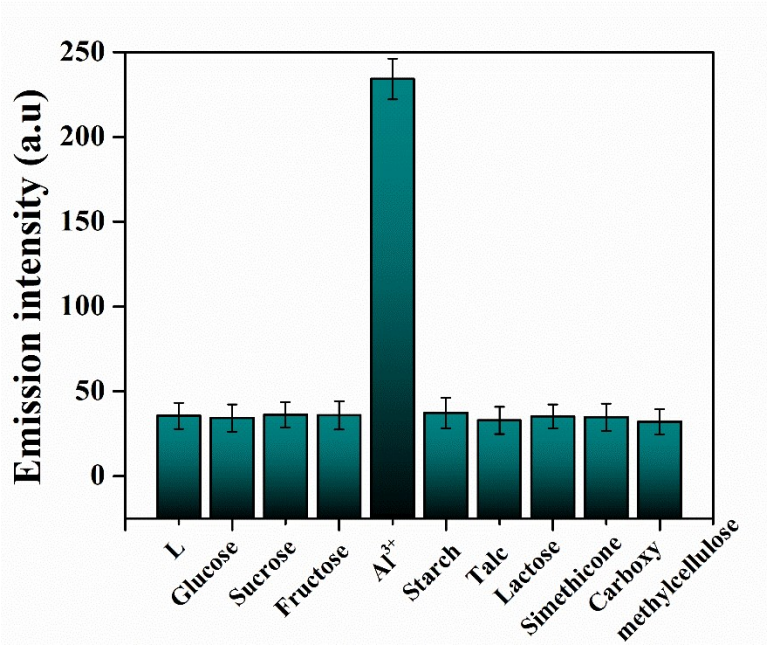


Figure S25. Emission intensity changes of **L** (5 μM), with different analytes (10 equivalents) present in pharmaceutical tablets.

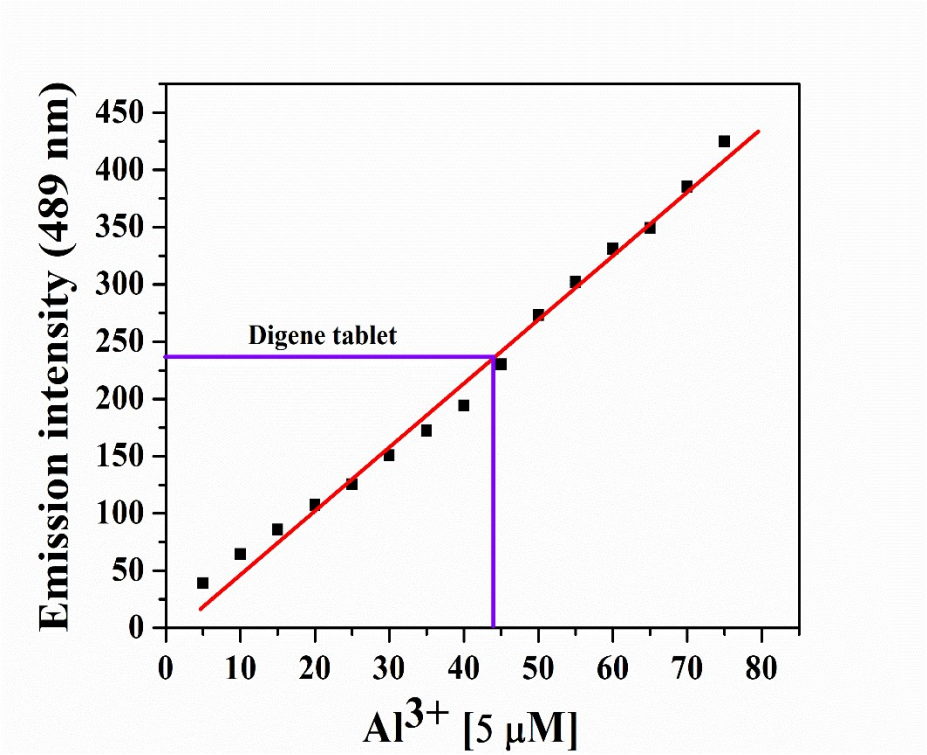


Figure S26. Emission titration curve for **L** (5 μM) *vs* Al^{3+} ions (λ_{ex} = 390 nm) for the determination of unknown concentration of Al^{3+} ions from Digene tablet extracts.

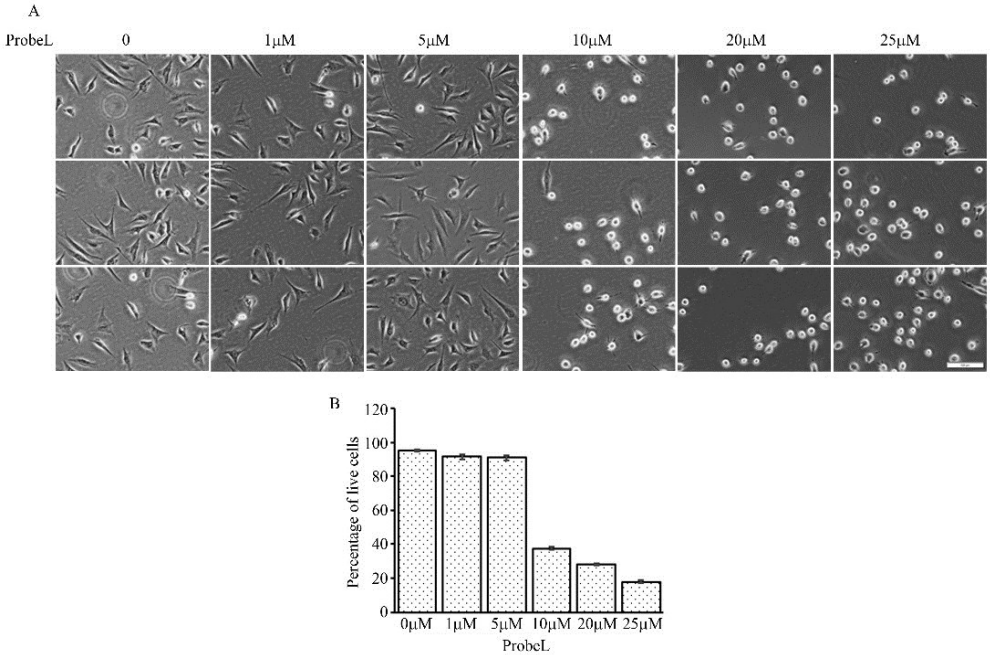


Figure S27. Toxicity study for the probe **L** in live cells.

Table S2. Crystallographic data for compound **L**.

Empirical formula	$C_{20}H_{20}N_4O_4$
Formula weight	380.40
Temperature	296(2) K
Wavelength	0.71073 Å
Crystal system	Triclinic
Space group	P-1
Unit cell dimensions	a = 4.9758(2) Å α = 63.6610(10)° b = 13.6254(5) Å β = 84.3720(10)° c = 15.0972(6) Å γ = 82.3240(10)°
Volume	908.20(6) $Å^3$
Z	2
Density (calculated)	1.391 Mg/m^3
Absorption coefficient	0.099 mm^{-1}
F(000)	400
Crystal size	0.339 x 0.137 x 0.042 mm^3
Theta range for data collection	2.700 to 28.321°
Index ranges	-6<=h<=6, -18<=k<=18, -20<=l<=20
Reflections collected	52560
Independent reflections	4515 [R(int) = 0.0478]
Completeness to theta = 25.242°	99.8 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7457 and 0.7129
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	4515 / 0 / 253
Goodness-of-fit on F^2	1.090
Final R indices [I>2sigma(I)]	R1 = 0.0868, wR2 = 0.2355
R indices (all data)	R1 = 0.1088, wR2 = 0.2509
Extinction coefficient	n/a
Largest diff. peak and hole	0.643 and -0.326 $e.Å^{-3}$

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