Electronic Supplementary Information

A discrete $Cu_2(Pd\text{-}bpy)_2L_2$ heterometallic compound with superoxide dismutase enzyme like activity

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Material

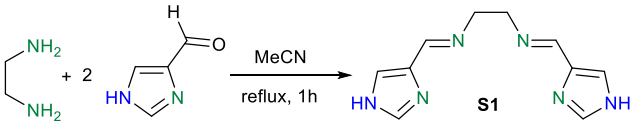
Palladium chloride, copper nitrate trihydrate, sodium borohydride, 2,2'-bipyridine, 4-imidazolecarboxyaldehyde, tris-Cl, bromophenol blue, ethanol, chloroform and glycine were purchased from Sigma-Aldrich. Dithiothreitol was supplied by Biogene, USA. Ethylenediamine, silver nitrate, acetonitrile and methanol were purchased from CDH, India. Sucrose, EDTA, riboflavin, phenylmethanesulphonyl fluoride (PMSF), sodium dodecyl sulphate (SDS), acrylamide, bis-acrylamide, TEMED, ammonium persulphate (APS), nitrobluetetrazolium (NBT), KCl and KCN were purchased from SISCO Research Laboratory, Mumbai, India. H_2O_2 was obtained from SD Fine chemicals, Mumbai, India. All other chemicals used were of the analytical grade. *cis*-$[Pd(bpy)(NO_3)_2]$, was prepared as reported in the literature.[1]

Physical measurements

NMR spectra were obtained at room temperature with a Jeol 400 MHz spectrometer. Mass spectra were recorded on a MICROMASS Q-TOF mass spectrometer or Thermo TSQ quantum access max equipped with standard electrospray source. Melting points were determined using a Deep vision apparatus and were uncorrected. The single crystal X-ray analysis was carried out using a Rigaku Oxford diffractometer with model no. XTALAB supernova equipped with PILATUS 200 K detector, the X-ray source being Cu Kα. UV visible was measured by ThermoFisher genesys 10S bio. IR was analysised in Thermo scientific Nicolet iS5 mid infrared instrument. Conductance measurements were performed in Labard Lim-CON01 equipment. Elico LI 120 was used to measure the pH.

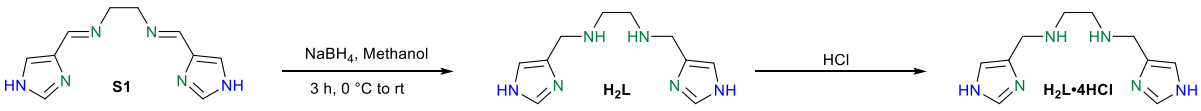
Experimental procedure

Synthesis of **S1**:



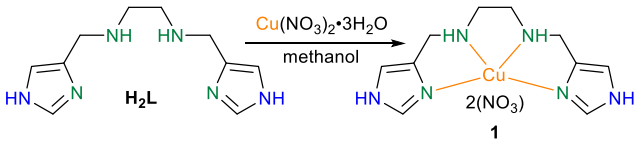
To a clear colourless solution of ethylenediamine (0.3005 gm, 5 mmol) in 60 ml of acetonitrile, 4-imidazolecarboxyaldehyde (0.9609 gm, 10 mmol) was added. It was refluxed for 1 h, filtered and washed two times with acetonitrile. Dried in vacuum to get an off white compound (yield =1.0666 gm, 99%), mp 232 °C (decomposed). ^{1}H NMR (δ, 400 MHz, DMSO-d_6, 298 K): 8.17 (2H, s, H_a), 7.66 (2H, s, H_b), 7.3-7.5 (2H, br, H_c), 3.73 (4H, s, H_d) ppm. ESI-HRMS: observed m/z 217.1193 $[M+H]^+$, 239.1007 $[M+Na]^+$; calculated m/z 217.1196 $[M+H]^+$, 239.1016 $[M+Na]^+$. Found: C, 55.44; H, 5.61; N, 38.95. $C_{10}H_{12}N_6$ requires C, 55.54; H, 5.59; N, 38.86%.

Synthesis of **H_2L·4HCl**:



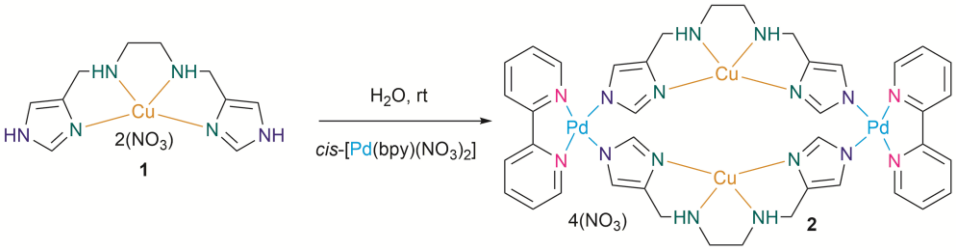
To a white suspension of **S1** (0.0526 gm, 0.243 mmol) in 3 ml MeOH, sodium borohydride (0.023 g, 0.608 mmol) was added, while keeping the bath temperature below 0 °C. The solution was stirred at room temperature for 3 h. Colourless solution was observed. To this solution gradually conc. HCl was added, while keeping the bath temperature below 0 °C. White precipitate was observed after addition of ~ 1.5 ml of conc. HCl. Washed with MeOH, centrifuged and dried in vacuum (yield = 0.0454 gm, 51%,), mp 250 °C (decomposed). ^{1}H NMR (δ, 400 MHz, D_2O, 298 K): 8.64 (2H, s, H_a), 7.57 (2H, s, H_b), 4.36 (4H, s, H_c), 3.38 (4H, s, H_d) ppm. ^{13}C NMR (δ, 100 MHz, D_2O, 298 K): 135.5, 122.7, 121.8, 42.7 and 40.7 ppm. ESI-HRMS: observed m/z 221.1505 $[M+H]^+$, 243.1314 $[M+Na]^+$; calculated m/z 221.1509 $[M+H]^+$, 243.1329 $[M+Na]^+$. Found: C, 32.90; H, 5.52; N, 22.91. $C_{10}H_{20}N_6Cl_4$ requires C, 32.81; H, 5.51; N, 22.96%.

Synthesis of **1**:



To a white suspension of **S1** (0.0432 gm, 0.2 mmol) in 5 ml MeOH, sodium borohydride (0.0189 g, 0.5 mmol) was added, while keeping the bath temperature below 0 °C. The solution was stirred at room temperature for 3 h to produce the ligand **H_2L**. Copper nitrate trihydrate (0.0483 gm, 0.2 mmol) was added to the above solution. Deep blue colour solution was observed. To this diethyl ether was added to get a blue precipitate and it was filtered, washed with diethyl ether and vacuum dried to get a blue colour solid (yield = 0.0693 gm, 85 %), mp 223 °C (decomposed). ESI-MS: observed m/z 327.99 $[M-\{2(NO_3)\}+HCOO^-]^+$, 282.01 $[M-\{2(NO_3)+H\}]^+$, 141.55 $[M-\{2(NO_3)\}]^{2+}$; calculated m/z 328.07 $[M-\{2(NO_3)\}+HCOO^-]^+$, 282.06 $[M-\{2(NO_3)+H\}]^+$, 141.54 $[M-\{2(NO_3)\}]^{2+}$. Found: C, 30.85; H, 5.57; N, 22.32. $C_{13}H_{28}N_8O_9Cu$ requires C, 30.98; H, 5.60; N, 22.24%.

Synthesis of **2**:



To an aqueous solution of compound **1** (0.0901 gm, 0.221 mmol), *cis*-$[Pd(bpy)(NO_3)_2]$ (0.0854 gm, 0.221 mmol) was added to give a clear deep blue solution which on evaporation at room temperature gave a blue solid (yield = 0.1078 gm, 73 %), mp 190 °C (decomposed). Found: C, 34.25; H, 3.7; N, 19.95. $C_{20}H_{26}N_{10}O_8CuPd$ requires C, 34.1; H, 3.72; N, 19.88%.

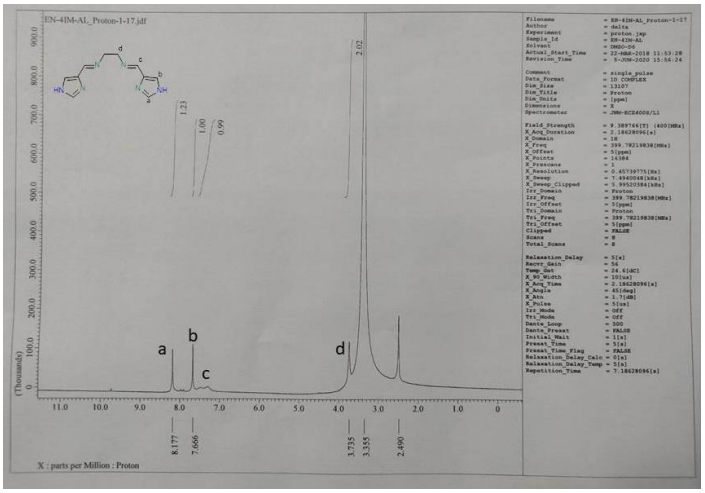


Figure S1. 400 MHz ^{1}H NMR of **S1** in DMSO-d_6.

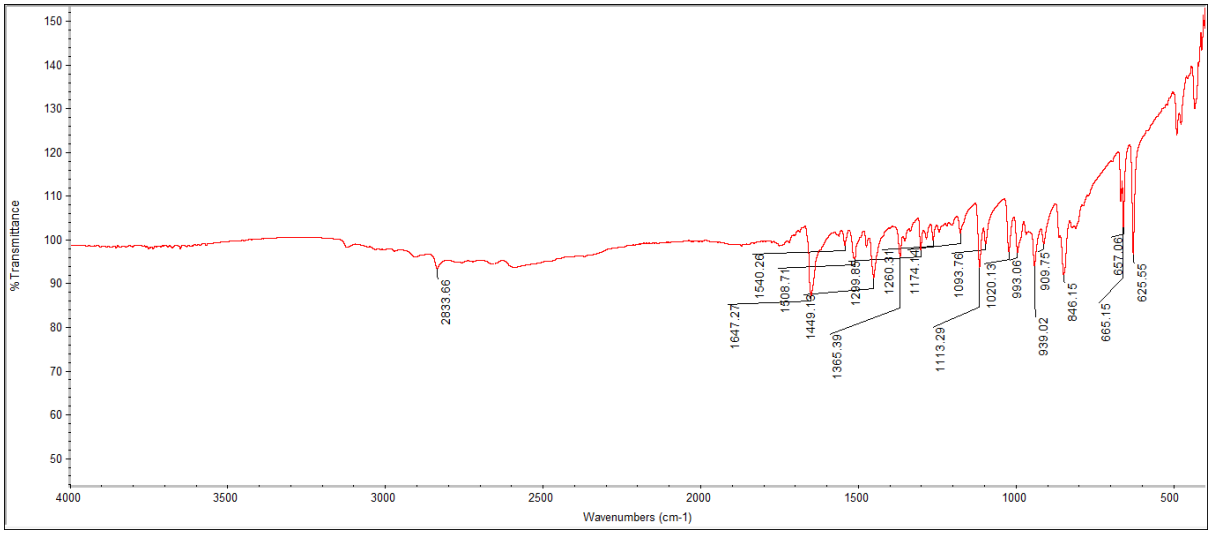
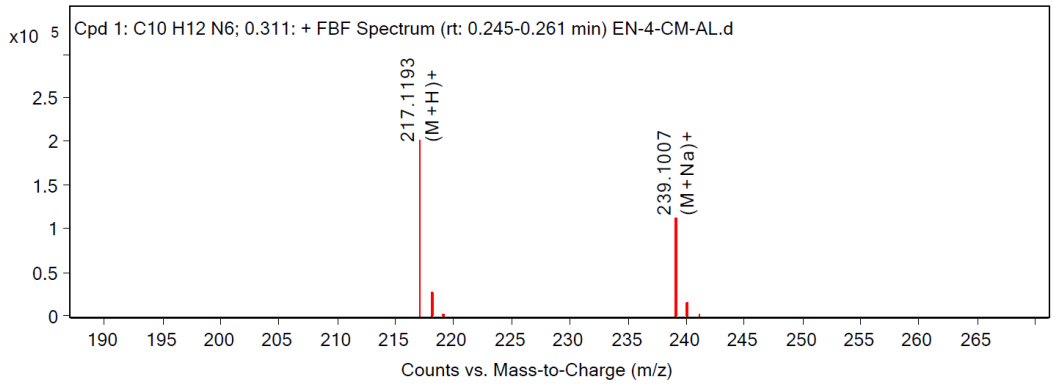


Figure S2. IR spectrum of **S1**.



MS Spectrum Peak List

m/z	z	Abund	Ion
217.1193	1	200471.73	(M+H)+
218.1215	1	22093.6	(M+H)+
219.1233	1	1215.78	(M+H)+
239.1007	1	111073.2	(M+Na)+
240.1029	1	12504.33	(M+Na)+
241.106	1	450.72	(M+Na)+

Figure S3. HRMS of **S1**.

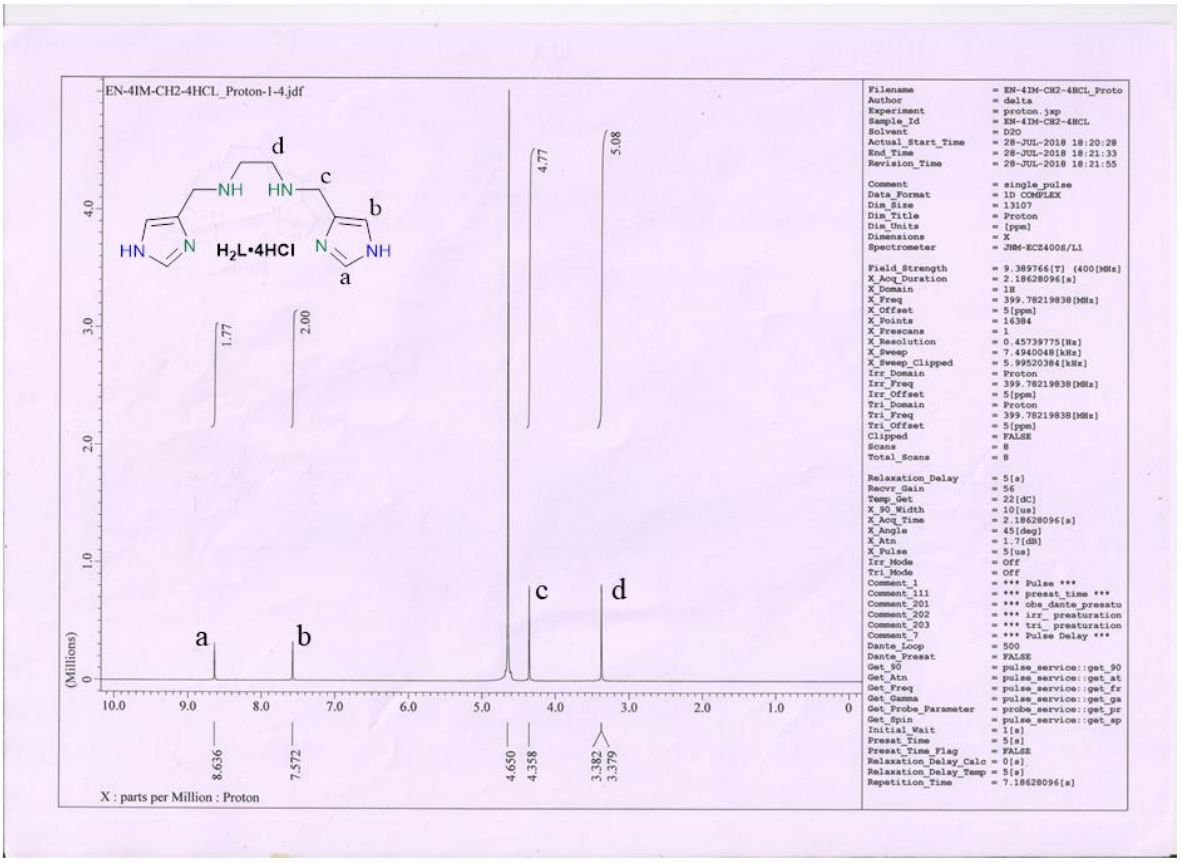


Figure S4. 400 MHz ^{1}H NMR of **H_2L·4HCl** in D_2O.

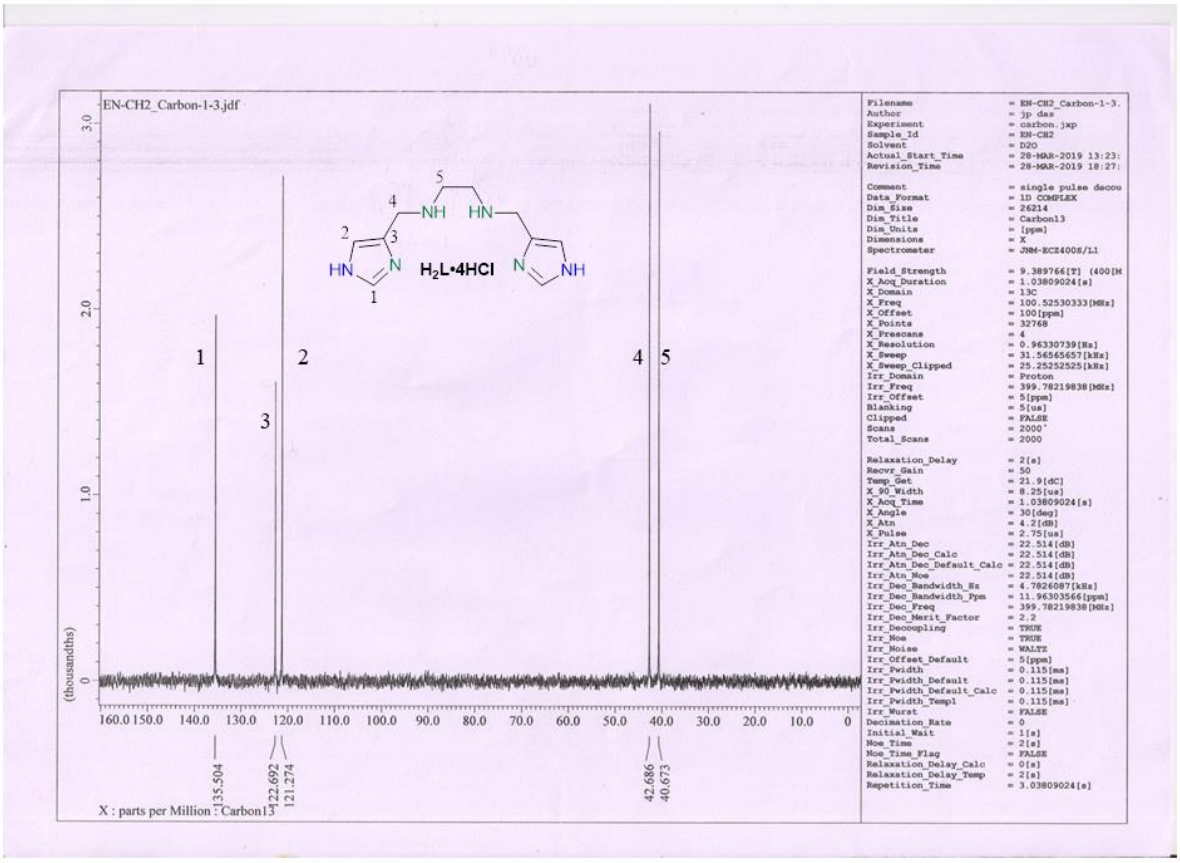


Figure S5. 100 MHz ^{13}C NMR of **H_2L·4HCl** in D_2O.

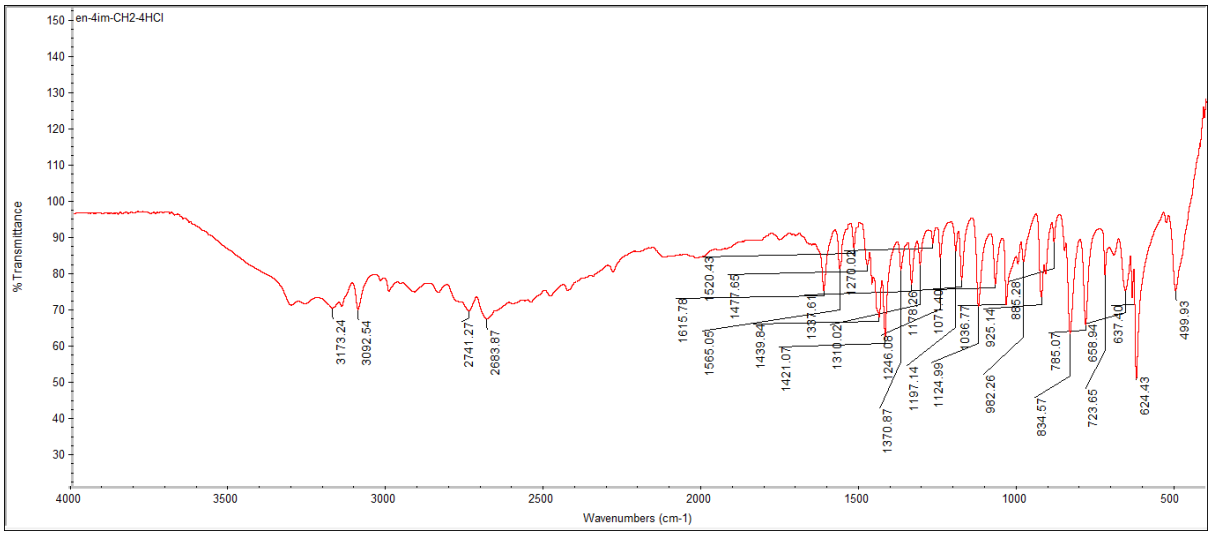
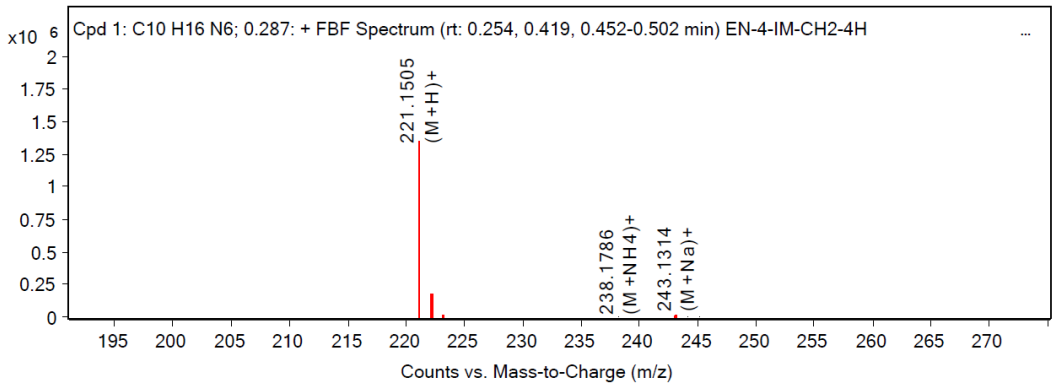


Figure S6. IR spectrum of **H_2L·4HCl**.



MS Spectrum Peak List

m/z	z	Abund	Ion
221.1505	1	1341978.25	(M+H)+
222.1529	1	135285.8	(M+H)+
223.1551	1	6638.54	(M+H)+
238.1786	1	260.95	(M+NH4)+
243.1314	1	13732.77	(M+Na)+
244.1347	1	1596.37	(M+Na)+
245.142	1	161.83	(M+Na)+

Figure S7. HRMS of **H_2L·4HCl**.

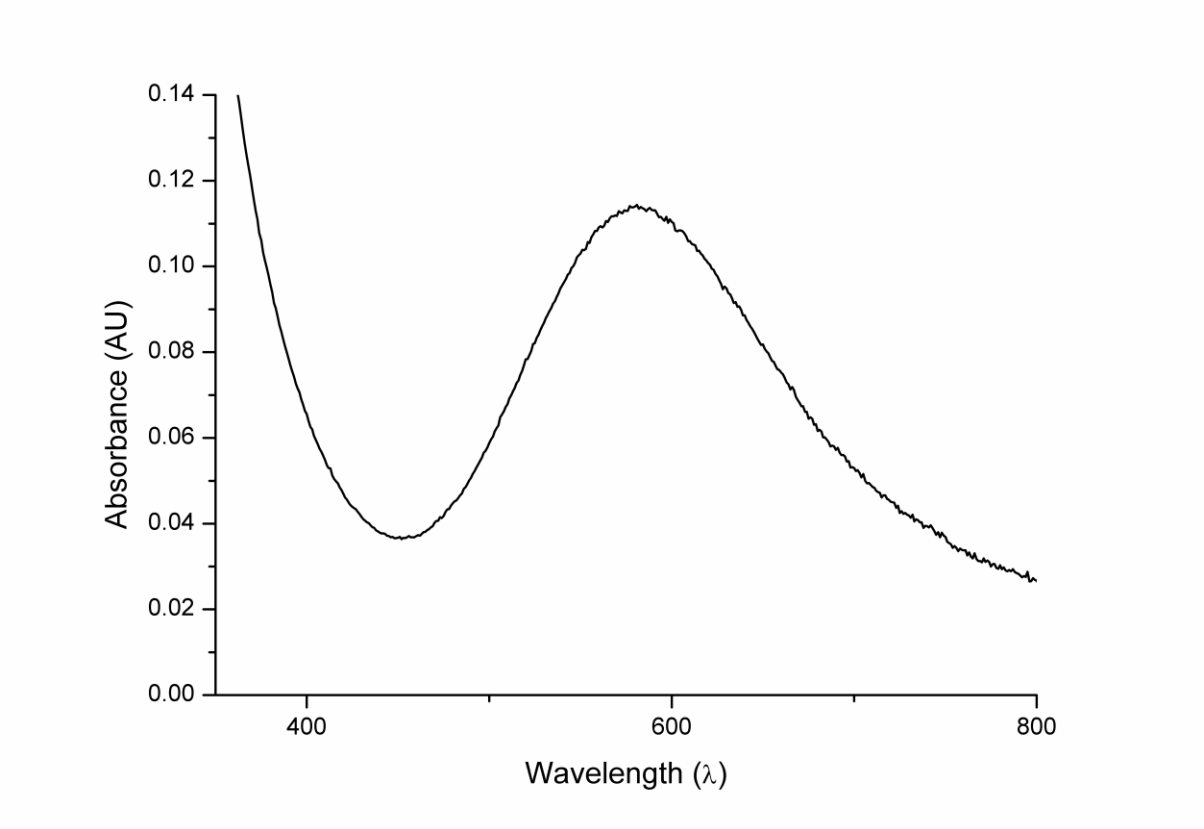


Figure S8. UV-visible spectrum of **1** λ_{max} (H_2O)/nm 580 (ε = 114).

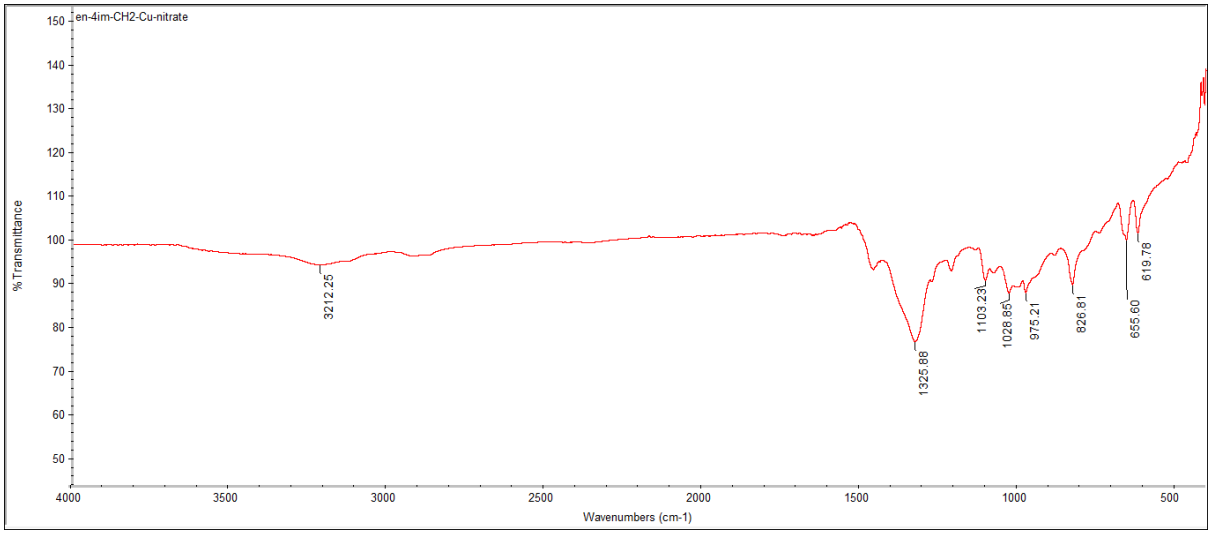


Figure S9. IR spectrum of **1**.

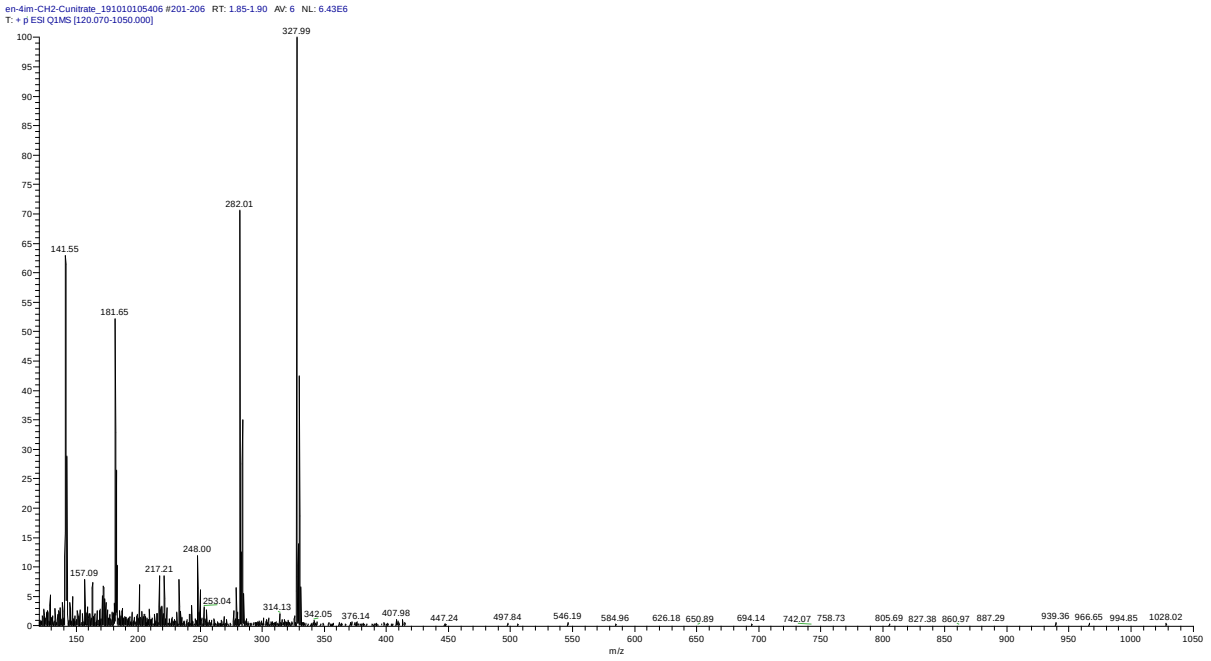


Figure S10. ESI-MS of **1**.

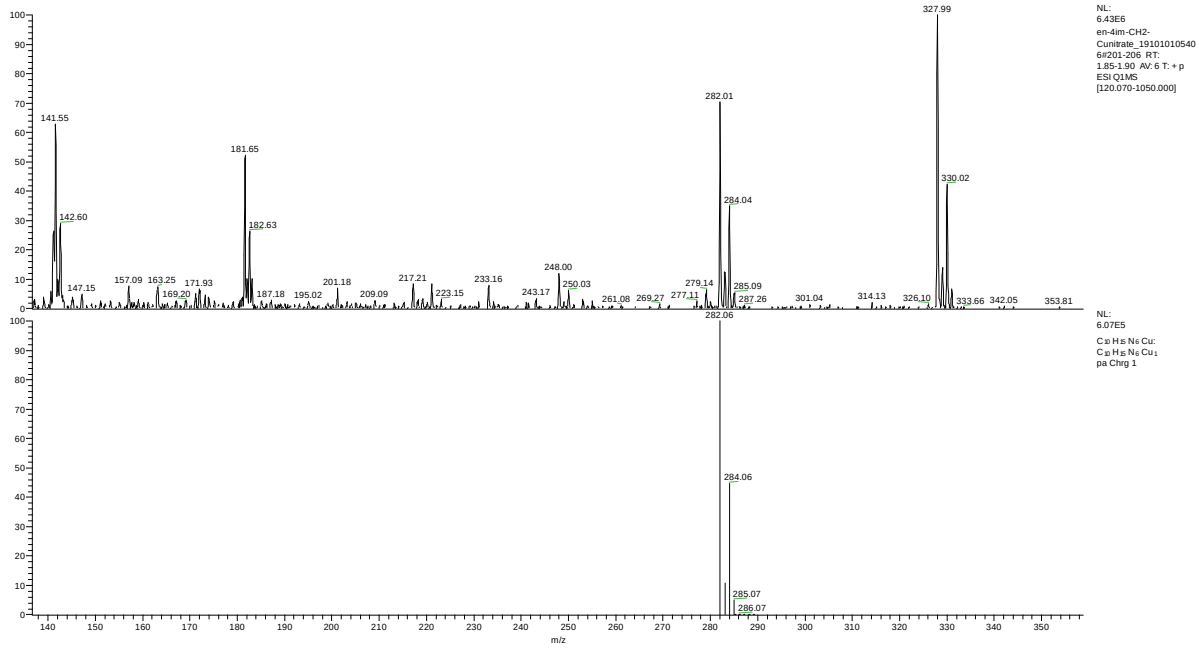


Figure S11. Comparison of ESI-MS data of **1** with theoretical values.

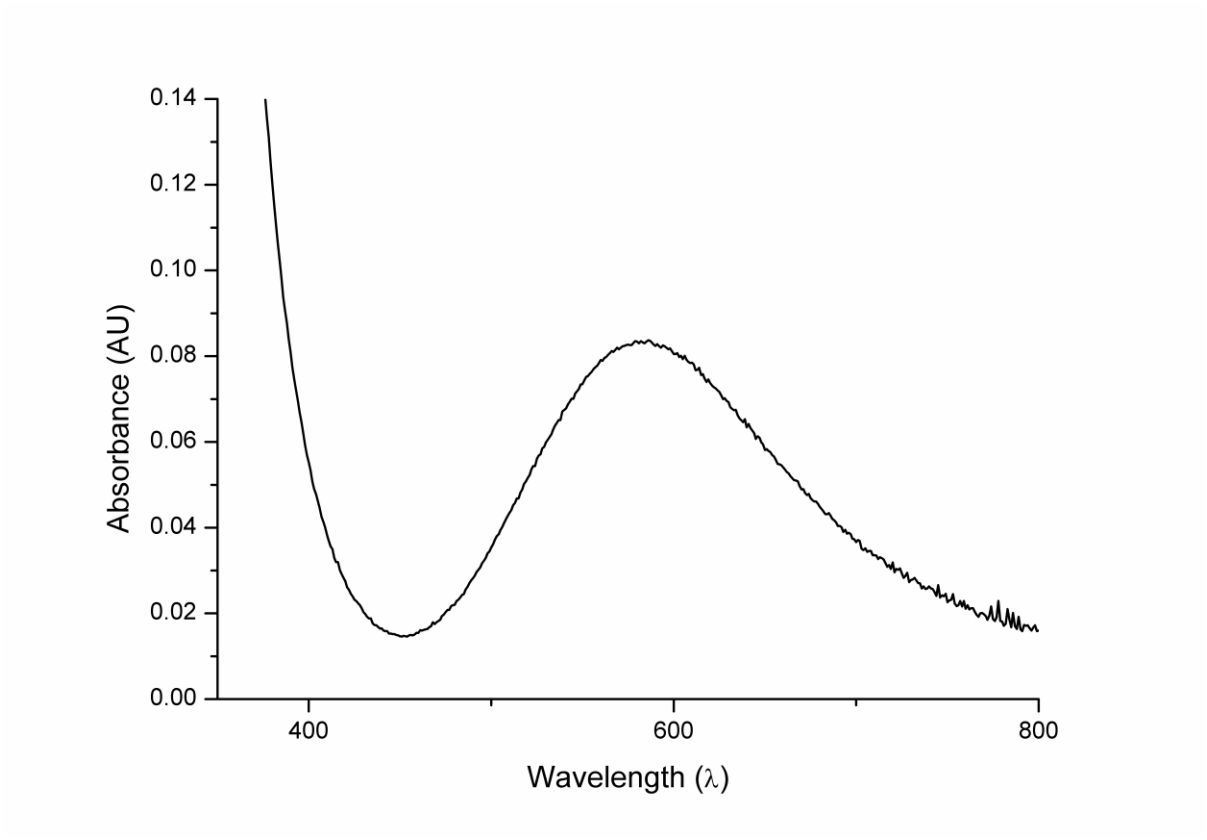


Figure S12. UV-visible spectrum of **2** λ_{max} (H_2O)/nm 580 ($\varepsilon = 83$).

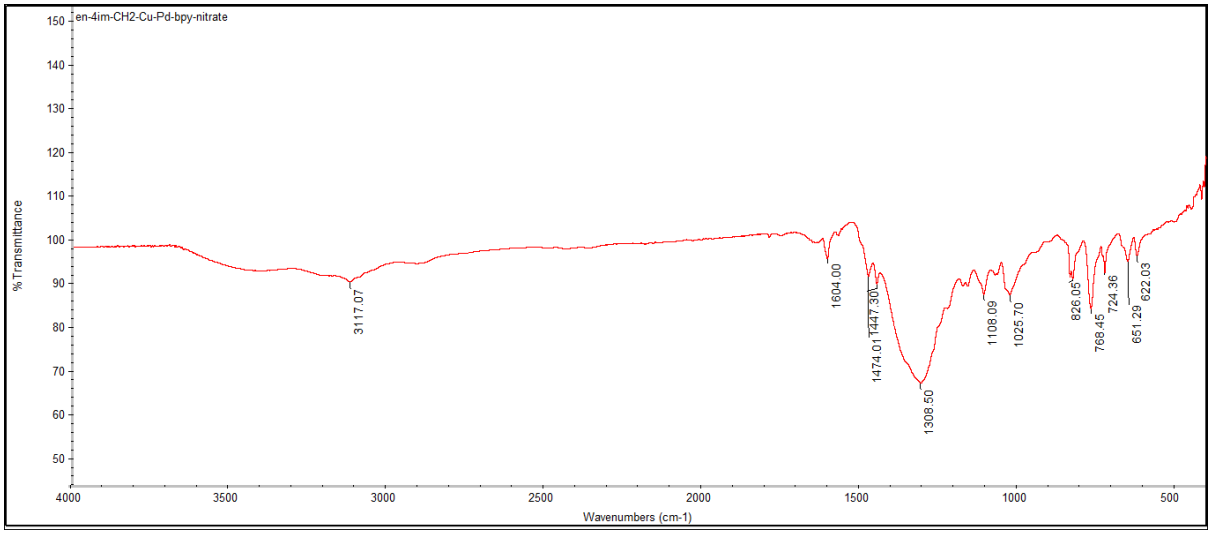


Figure S13. IR spectrum of **2**.

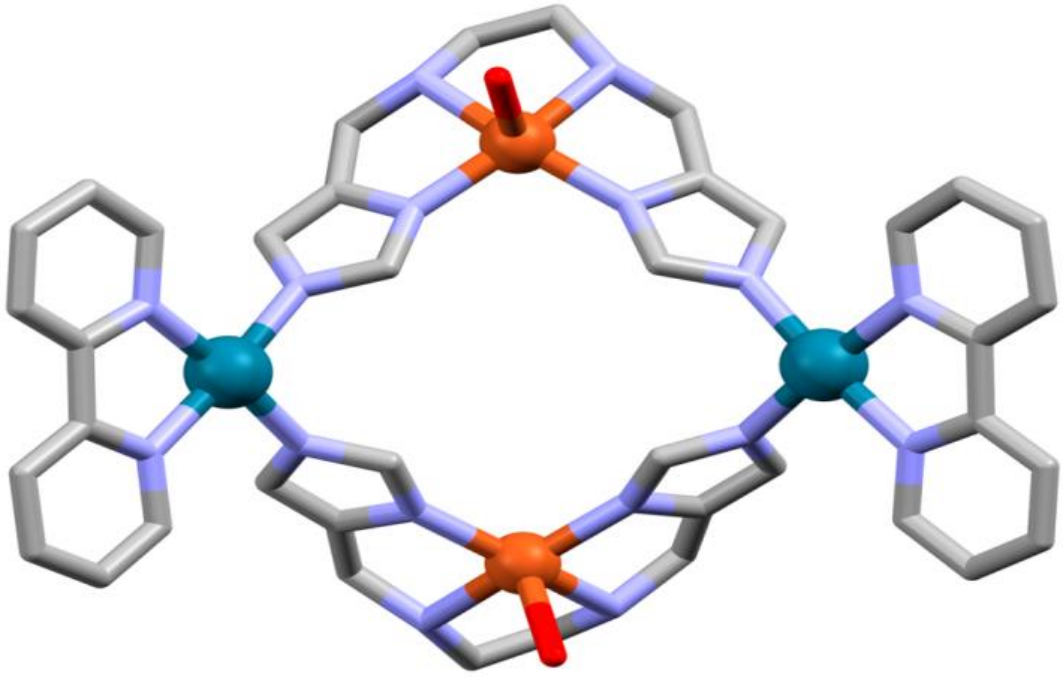


Figure S14. Single crystal X-ray structure of compound **2**·2H_2O. Counter anions, hydrogen atoms and solvent molecules are omitted for clarity. Orange and cyan coloured balls represent Cu(II) and Pd(II) respectively; Grey, blue and red coloured capped sticks represent C, N and O respectively.

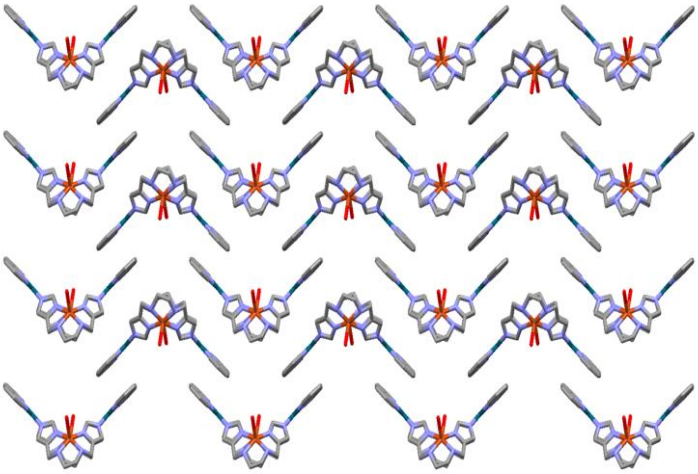


Figure S15. Packing of compound **2**·2H_2O showing the π-π and CH- π interactions in crystal structure. Counter anions, hydrogen atoms and solvent molecules are omitted for clarity. Orange and cyan coloured balls represent Cu(II) and Pd(II) respectively; Grey, blue and red coloured capped sticks represent C, N and O respectively.

Table S1. X-ray diffraction experimental details

Identification code	**2**·2H_2O
Empirical formula	$C_{40}H_{48}Cu_2N_{18}O_9Pd_2$
Formula weight	1264.84
Temperature/K	293(2)
Crystal system	Monoclinic
Space group	I2/a
a/Å	21.6397(5)
b/Å	10.4800(2)
c/Å	26.8204(6)
α/°	90
β/°	104.348(2)
γ/°	90
Volume/Å^3	5892.7(2)
Z	4
ρ_{calc}g/cm^3	1.426
μ/mm^{-1}	6.165
F(000)	2544.0
Radiation	CuKα (λ = 1.54184)
2Θ range for data collection/°	6.804 to 153.232
Index ranges	-27 ≤ h ≤ 27, -11 ≤ k ≤ 13, -33 ≤ l ≤ 33
Reflections collected	24139
Independent reflections	6031 [R_{int} = 0.0758, R_{sigma} = 0.0474]
Data/restraints/parameters	6031/0/322
Goodness-of-fit on F^2	1.062
Final R indexes [I>=2σ (I)]	R1 = 0.0592, wR2 = 0.1747
Final R indexes [all data]	R1 = 0.0710, wR2 = 0.1848
Largest diff. peak/hole / e Å^{-3}	1.29/-0.69

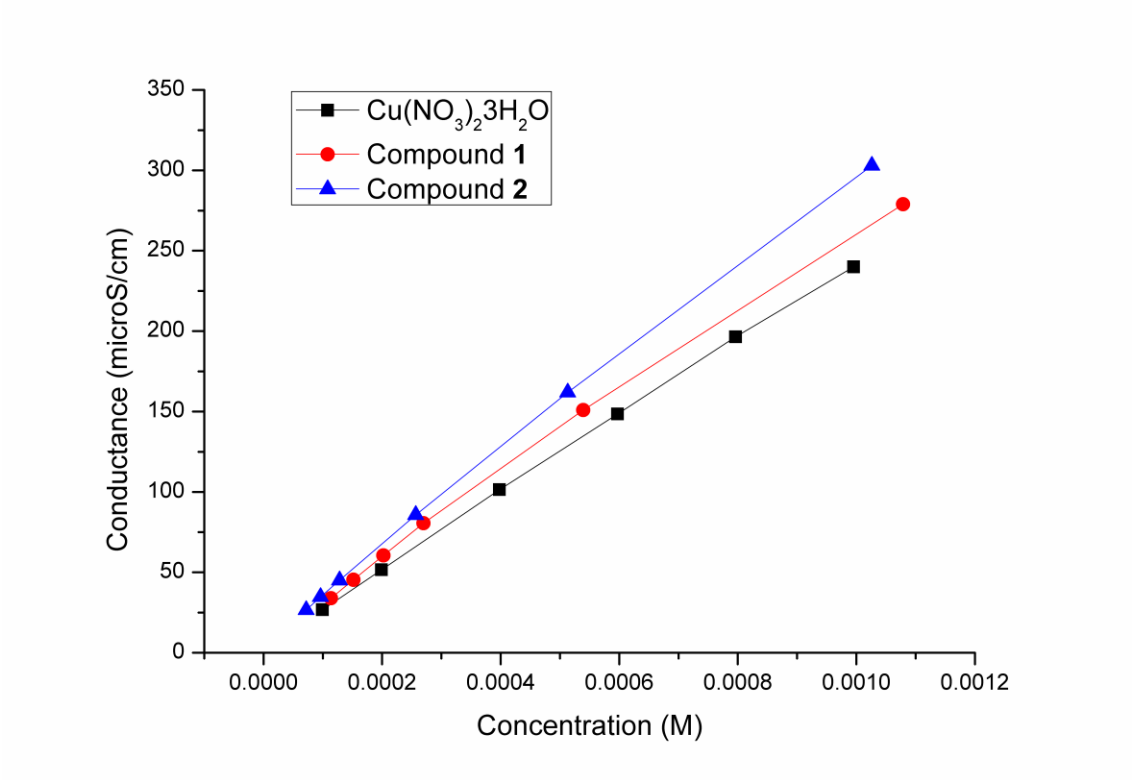


Figure S16. Comparison of conductance measurements between copper nitrate trihydrate, **1** and **2**.

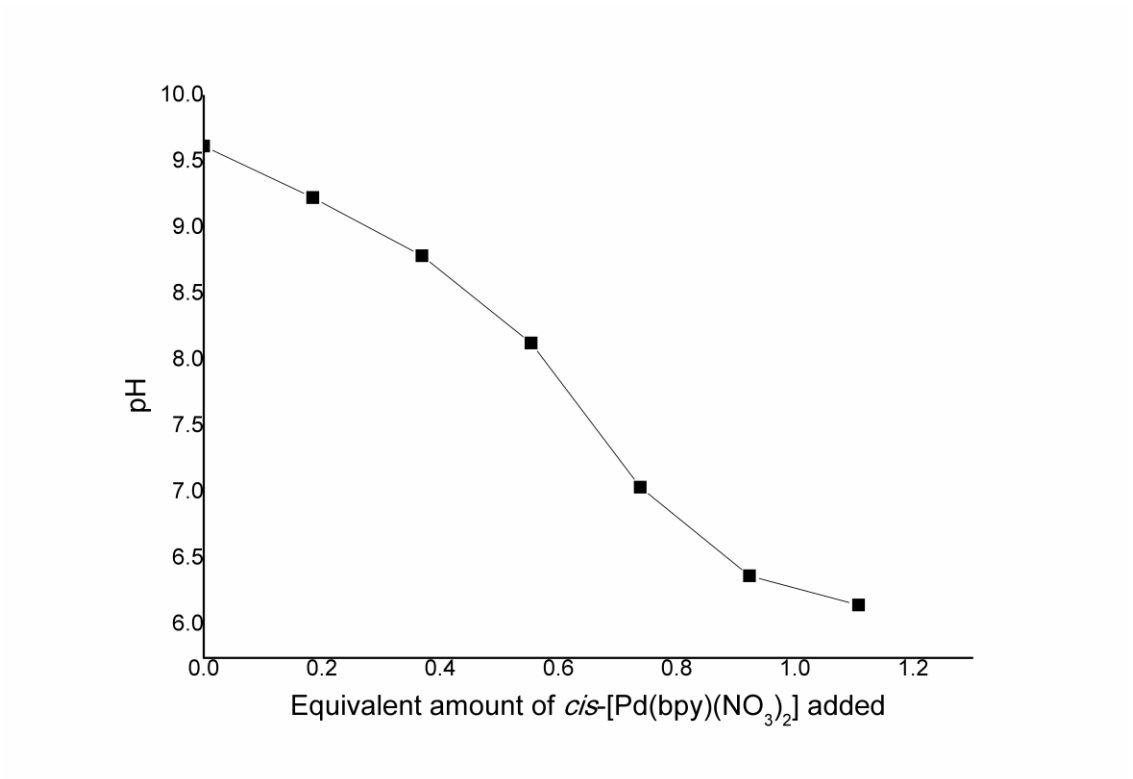


Figure S17. pH measurements at various equivalent of *cis*-[Pd(bpy)(NO_3)$_2$] added to 1 mM of compound **1**.

Live subject statement

This study was performed in accordance with the provisions of the Prevention of Cruelty to Animals Act 1960, and the Breeding of and Experiments on Animals (Control and Supervision) Rules 1998, Government of India. These experiments were approved by the Institutional Ethical Committee of Odisha University of Agriculture and Technology (Bhubaneswar, Odisha, India)

Animal sources

Adult male mud crab (*S. serrata*) at intermoult stage weighing 63.95 ± 4.67 g of body weight were collected from Arakhakuda region of Chilika lagoon (19º 28' and 19º 54' N and 85º 05' and 85º 38' E) of Orissa, India during May 2018 and were transported to the laboratory in gunny bags containing see weeds. The transport time from the site of collection to the laboratory was around 4 hrs. Salinity of Chilika lagoon varies throughout the year ranging nearly 10 ppt in rainy season (July to September), 17 ppt during winter (December and January) and 35 ppt during summer (April to June) with a temperature variation from 18 $^{\circ}$C (winter) to 32 $^{\circ}$C (summer).[2] Fresh chicken liver was collected from slaughter house in normal saline kept in ice box.

Experimental protocol for animals

The crabs were disinfected by dipping them into 17 ppt saline water having 500 ppm (parts per million) potassium permanganate ($KMnO_4$) for 5-7 minutes and then were acclimated in 17 ppt saline water without $KMnO_4$ for 24 hours in the laboratory.[3] During this acclimatization period in aquarium (75 x 30 x 30 cm^3), about 08 % mortality rate was observed. Only active crabs were selected and used for experiments. Aquarium had 5 cm high bed of sand which was pre-treated with $KMnO_4$ followed by extensive washing with tap water and was high enough to keep the crabs in merged condition. All aquaria were continuously aerated and covered with plastic opaque covers to make least disturbances to the animals. Saline water was changed daily at 18.00 hours and crabs were exposed to 12h: 12h natural light and day periods throughout the experimentation. The crabs were fed once daily during night (at 19.00 hours) with fresh chick liver pieces. Temperature and salinity of water of aquaria were recorded once daily (at 8.00 hours) with the help of specific electrodes (µP based soil and water analysis kit, Esico. Co., New Delhi, India). The mean salinity and temperature of aquarium water was 17.51 ± 0.73 ppt and 29.31 ± 1.99 ºC and 35.39 ± 0.66 ppt and 29.7 ± 1.6 ºC, respectively.

Tissue collection and sub cellular fractionation

Animals were sacrificed by removing their carapace from their abdomen by a jerk and hepatopancreas, gills and abdominal muscle tissues were dissected out quickly. Tissues were washed in ice cold normal saline (0.67%, w/v), blotted, flash frozen in liquid nitrogen and stored at -20 ºC for further analyses. A 20% (w/v) homogenates of muscle, hepatopancreas of crabs and liver of chicken were prepared at 4 ºC in homogenizing buffer (50 mM Tris-Cl, 1 mM EDTA, I mM DTT, 0.5 mM sucrose, 150 mM KCl and 1mM PMSF, pH 7.8) with the help of Potter-Elvejhem type, motor driven glass Teflon homogenizer at 250 rpm speed with 7-8 up and down strokes whereas due to rigidity, gill tissues were homogenized in pre-cooled mortar and pestle. The crude homogenates were centrifuged at 1000 x g for 10 minutes at 4 ºC in a cooling centrifuge (Eppendorf, Germany, Model 5430 R) to sediment nuclei and tissue debris. The supernatant fractions were centrifuged at 10,000 x g for 10 min at 4 ºC to obtain the clear supernatant which was referred as post mitochondrial fraction (PMF) which was used for the assay of SOD.[4]

Biochemical determinations

Protein estimation in PMF samples were made according to the method of Lowry *et al.*,[5] by taking BSA as standard.

Superoxide dismutase assay

SOD activity in samples (both in compound **2** and tissue samples) was measured according to the method of Das group.[6] The principle of the assay is based on the estimation of superoxide scavenging capacity of samples. Superoxide radicals were generated in the assay system by photo-reduction of riboflavin. In brief, the assay mixture (1.58 ml) contained hydroxylamine hydrochloride (0.47 mM), L-methionine (0.9 mM), triton-X 100 (0.026%), riboflavin (2.5 µM) in 100 mM Tris buffer, pH 8. The reaction was started by exposing the mixture to two 20 W fluorescent lamps (fitted parallel to each other inside an aluminium coated wooden chamber) for 10 minutes at 25 ºC. Nitrite produced by superoxide radicals via hydroxylamine hydrochloride was fixed by adding 1 mL of Griess reagent and intensity of the red azo compound produced was measured at 543 nm against appropriate blank. Enzyme activity was expressed in U per mg protein. Final SOD activity was calculated by subtracting the value obtained for boiled (95 ºC for 30 minutes) samples from the corresponding un-boiled samples to get rid of any back ground superoxide radical scavenging molecules such

as metallothioneins in tissues. Two different concentrations (80 and 100 µL) of compound **2** were used in the above assay system to check its SOD like activity.

SOD like activity of compound 2 on acrylamide-bisacrylamide gel

One assay system was developed to check the SOD like activity of compound **2**. In brief, a 15% mini acrylamide-bisacrylamide gel with 0.5 cm thick was casted and seven 2.5 mm diameter and 2 mm deep holes were made on its surface. Three holes were made on its upper (2, 3 and 4) and three were made on its lower (5, 6 and 7) side keeping about 0.5 cm distance from the respective edges. One hole was made at the middle of 2^{nd} and 5^{th} holes. Compound **2** samples with 5, 10, 20, 40, 60 and 80 µL volumes were added into, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} and 7^{th} holes, respectively and the gel was dried under fan for 15 min at room temperature in order to allow the added samples to be soaked onto the gel completely. The gel was then soaked in the staining solution (0.2 mM nitrobluetetrazolium, 0.2 mM EDTA, 0.03 mM riboflavin, and 200 µl TEMED/100 ml solution) at 37 ºC for 15 min with constant shaking at room temperature. The gel was then exposed to fluorescence light until the gel turned blue with achromatic zones exhibiting superoxide radical scavenging activity of compound **2**. Such staining method gives a totally purple blue back ground of gels if superoxide radical scavengers are absent in the gel. And, such assay system was found to work to determine the SOD like activity of compound **2**. Following the procedure, illumination was discontinued when maximum contrast between achromatic zones around the holes and the general purple blue color of the gel showing un-use of was achieved. Gel was then photographed in Bio-Rad Gel Documentation System (California, USA, Model EZ^{TM} Doc Imager).

Calculation of isoenzyme bands and their intensity

The intensity achromatic zone in the gel was counted using computer assisted densitometry Image-Quant TL, Image Analysis Software version 2003. Measurement was also performed from the chromatic zone to use the value as back ground error. The corresponding back ground intensity of the gel was reduced from the intensity achromatic zones produced by the compound **2**. The relative value obtained was simple expressed as the units corresponding to the area of the bands and their back ground intensity.[7]

Statistical analyses

Mean of the relative achromatic densities or biochemical assay (n=3 in each experiment) were compared whenever necessary by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range tests to find out the level of significance among the mean values. T-test was employed using Microsoft excel version 10.0 to find difference when group numbers were two. The minimal statistical significance was considered at $p < 0.05$ level.

Validation of the SOD activity assay system and SOD activity of compound 2

The present method uses generation of $O_2^{\cdot -}$ by photo (by exposing florescence light) reduction of riboflavin via semiquinone in which L-methionine and EDTA are used as electron donors. The generated $O_2^{\cdot -}$ then reacts with hydroxylamine hydrochloride to produce nitrite. After the reaction is over at the end of light exposure, Griess Reagent (*sulfanilamide* and N-1-napthylethylenediamine, 1:1, v/v) is added to produce a diazonium compound (by sulfanilamide) which is converted into red azo compound (N-1-napthylethylenediamine) that has λ_{max} at 543 nm. The kinetics of the whole reaction usually works as a function of EDTA concentration. In 1.56 mL assay mixture, usually 100 mM EDTA is standardized to be used to assay SOD activity in biochemical systems. Therefore, the assay system was validated with 10, 20, 40, 60, 80 and 100 mM EDTA (Fig. S18 i). The increase in optical density was linear with a collinear coefficient of 0.99 (Fig. S18 ii). Hence, it was confirmed that the assay system was finely working. Further, different tissue samples were used to show the SOD activity. Results indicate that muscle, gills and hepatopancreas of crabs had the SOD activity in the increasing order ($p<0.001$). A lower dose of PMF (~ 400 µg) and their corresponding double doses of PSF proteins were taken to ensure the SOD activity. Accordingly, the SOD activity was doubled from the lower to their corresponding higher doses ($p\leq0.0001$, Fig. S19 a, a' b, b', c and c'). Similarly, chicken liver tissue PMF was also used to double check the assay system and it was noticed that a similar trend of doubling ($p<0.0001$) SOD activity was noticed with double amount of proteins (Fig. S19 d and d'). The SOD activity in above tissues was in the order of muscle<gill<hepatopancreas<liver. Finally, 1 mM compound **2** was used in similar way and it was observed a strong SOD mimicking activity was present in it. Two different concentrations i.e. 80 µL (51 µM) and 100 µL (102 µM) of the compound had a very strong SOD activity showing 20 and 25 unit of SOD activity, respectively (Fig. S19 e and e'). Surprisingly, the SOD like activity of compound **2** was the highest among all the above tissues studied. The amount of compound **2** required to

inhibit 50% of the OD from control was 28 µM. Hence, we tried to develop a new assay system to observe its SOD activity in naked eye. We employed acrylamide-bisacrylamide gel system to investigate the SOD mimicking activity of the compound **2**.

SOD mimicking activity of compound 2

For the first time, we employed acrylamide-bisacrylamide gel system to visualize the SOD like activity of the compound using NBT reduction method. Results of the present experiment indicate that the compound **2** had a strong SOD like activity as each well had a clear achromatic zone around them except the control. Although, results of the biochemical assay system use in above section (validation of the SOD activity assay system and SOD activity of compound **2**) will not be comparable with the results of this section, the present system can be used as additional confirmation of the SOD activity of the compound that can be visualized in naked eye. The SOD like activity of the compound (1 mM) with 5, 10, 20, 40, 60 and 80 µL volume in each well (Fig. 2A) was 7.5, 11.24, 20.22, 32.83, 44.90 and 48.02 units (Fig. 2B), respectively. It is noteworthy to mention that this unit corresponds to the achromatic zone ($O_2^{\cdot -}$ scavenging activity) that corresponds to create on the acrylamide-bisacrylamide gel by the applied compound **2** and was measured by the pixel counts. Therefore, the units obtained from the gel can't be comparable with the biochemical SOD activity of the compound observed in Fig. S19e. The increasing ($p<0.001$) trend of SOD activity of compound **2** with 5, 10, 20, 40, 60 and 80 µL volume was 4.56, 6.82, 12.27, 19.93, 27.26 and 29.15%, respectively (Fig. 2B).

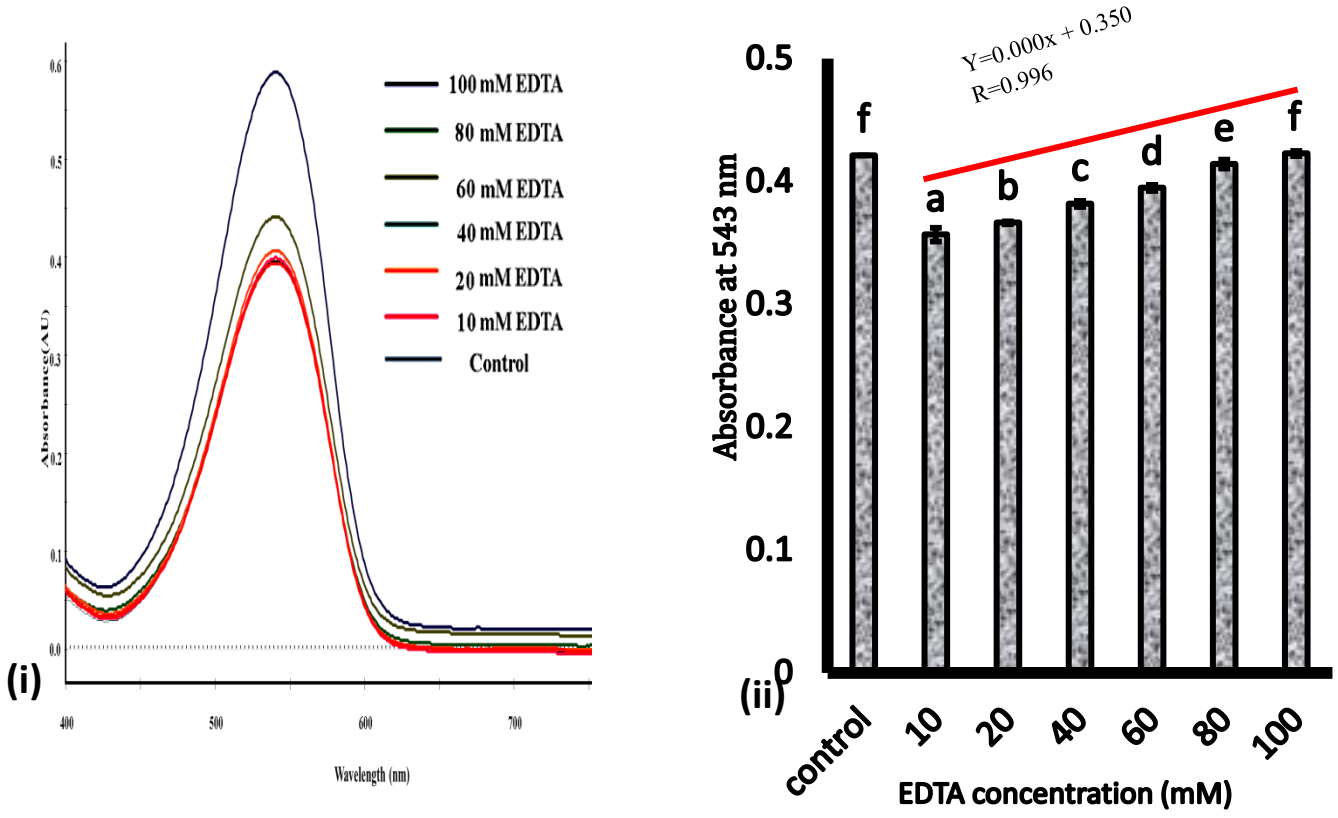
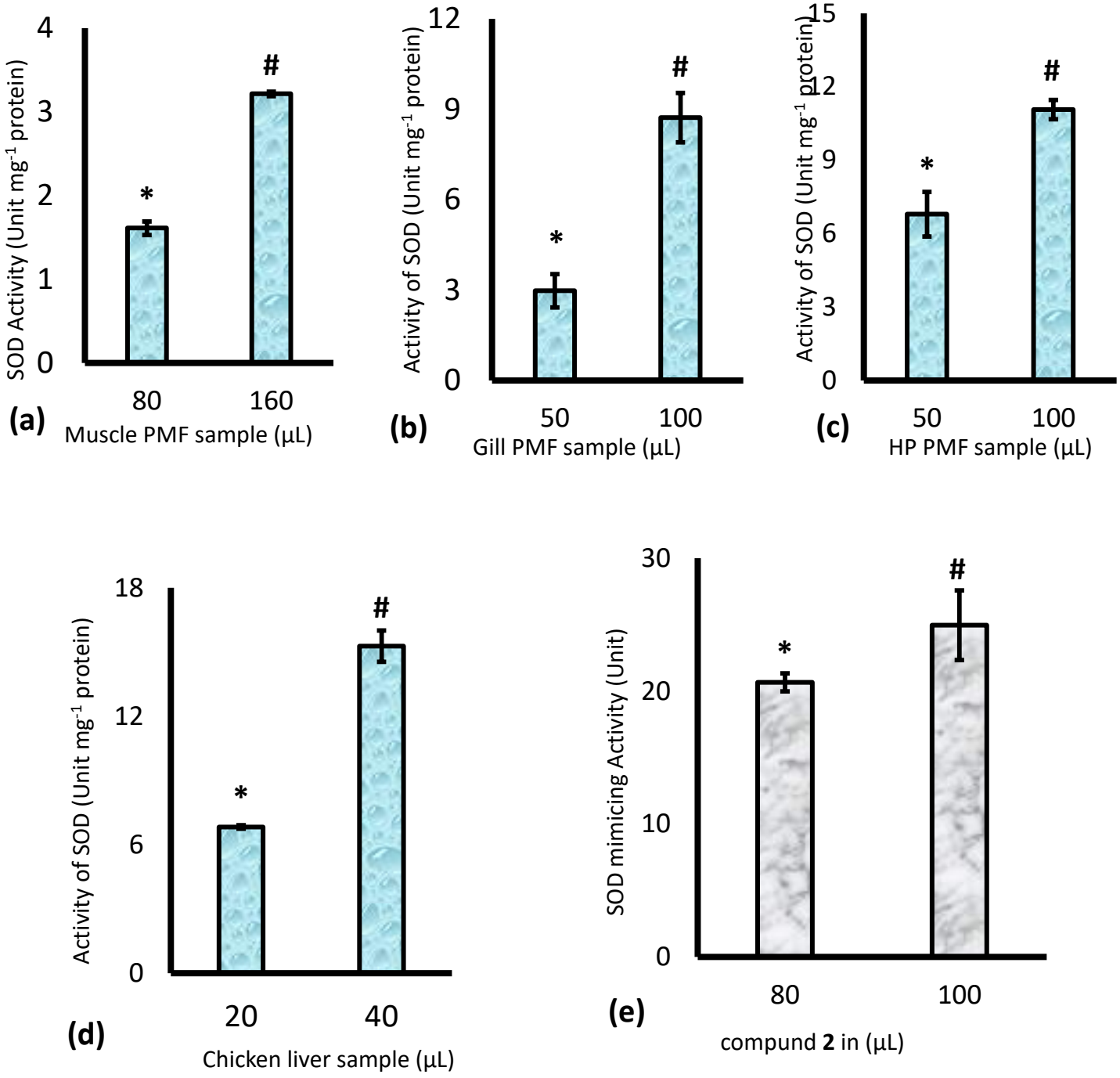


Figure S18. Graphical representation of $O_2^{\cdot-}$ radical anion generation: Increase of $O_2^{\cdot-}$ radical anion generation as a function of EDTA (10 to 100 mM) in the assay mixture to ensure the working condition of the assay system *i.e.* generation of superoxide radical. Statistical difference between different concentrations (n=3) was denoted with different superscripts such as a, b, c etc. (i.) Spectral representation of the assay mixture after light exposure and their corresponding values (ii) are represented. In figure (ii) 100 mM EDTA was optimally used (control) for all corresponding SOD assay. Minimum statistical (one way ANOVA) difference between any two different concentrations was considered at $p<0.05$ level.


SOD Activity (Unit mg-1 protein)
4
3
2
1
0
*
#
80
160
(a)
Muscle PMF sample (µL)
Activity of SOD (Unit mg-1 protein)
12
9
6
3
0
*
#
50
100
(b)
Gill PMF sample (µL)
Activity of SOD (Unit mg-1 protein)
15
12
9
6
3
0
*
#
50
100
(c)
HP PMF sample (µL)
Activity of SOD (Unit mg-1 protein)
18
12
6
0
*
#
20
40
(d)
Chicken liver sample (µL)
SOD mimicing Activity (Unit)
30
20
10
0
*
#
80
100
(e)
compund 2 in (µL)

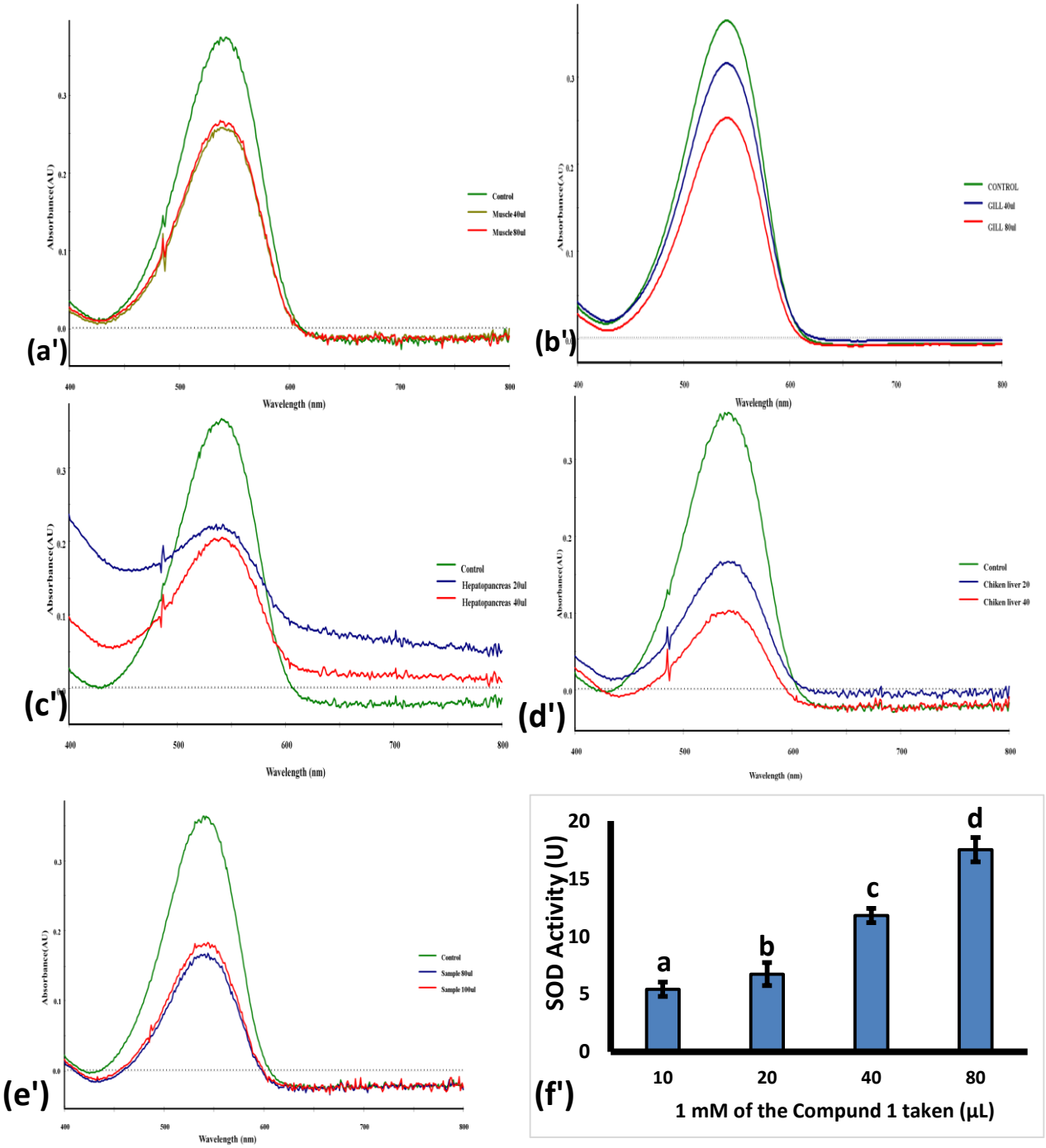


Figure S19. SOD activity studies in various systems: Positive controls showing SOD activity in different concentrations of post mitochondrial fractions (PMF) in muscle (a and a′), gill (b and b′), hepatopancreas (HP, c and c′) of the crab and in the liver of chicken PMF samples (d and d′). Simultaneously, compound **2** in different concentrations (~50 and 100 µM had also exhibited SOD like activity in similar manner (e and e′). Different symbols (* and #) above graphs indicate statistical (t-test) difference between two different concentrations. Minimum statistical difference was considered at $p<0.01$ level. The values of SOD activity in figures (a, b, c, d and e) represent the OD values obtained from the corresponding UV-visible scanning (a′, b′, c′, d′ and e′) of the assay mixtures after light exposure. SOD activity of compound **1** using riboflavin reduction method (f′). It was found that its activity was comparable but significantly low than compound **2**.

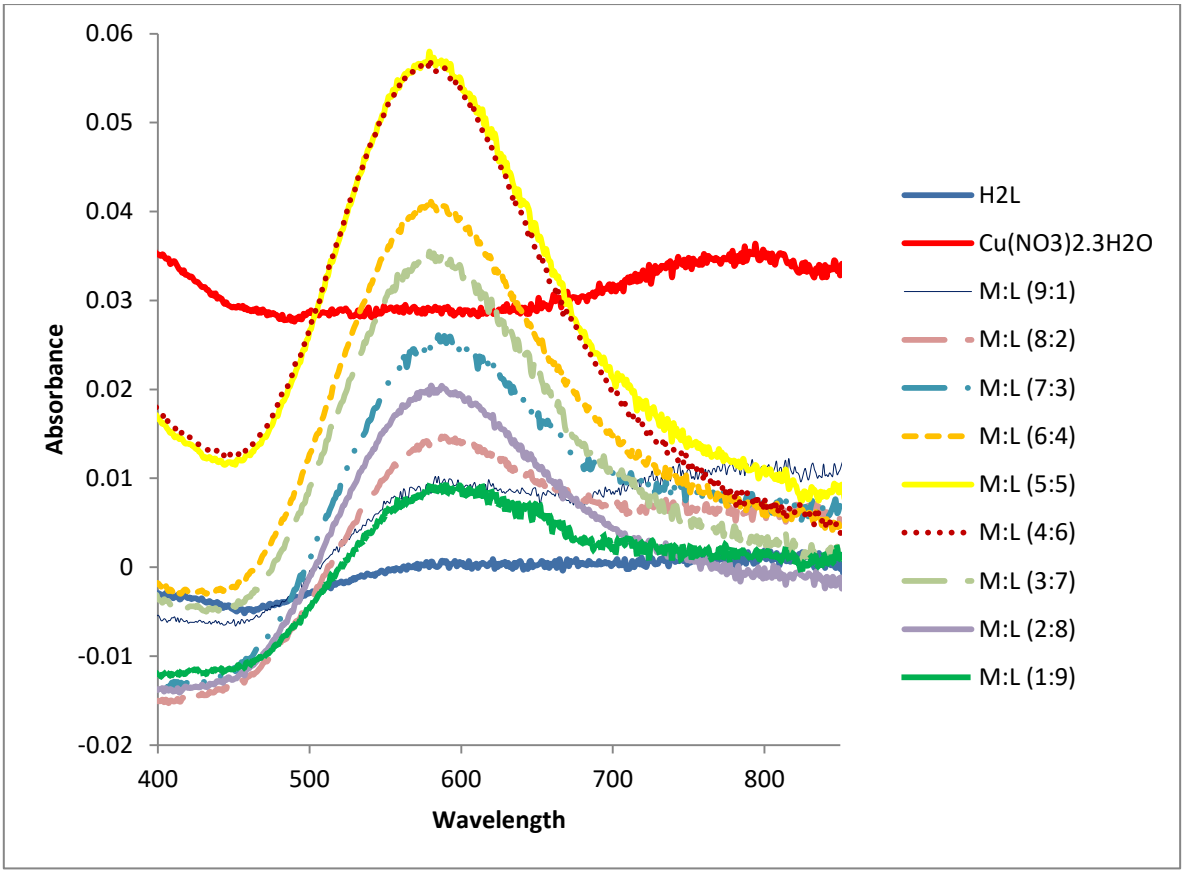


Figure S20. UV-visible spectra of **H_2L**, $Cu(NO_3)_2 \cdot 3H_2O$ and their combination at different ratio.

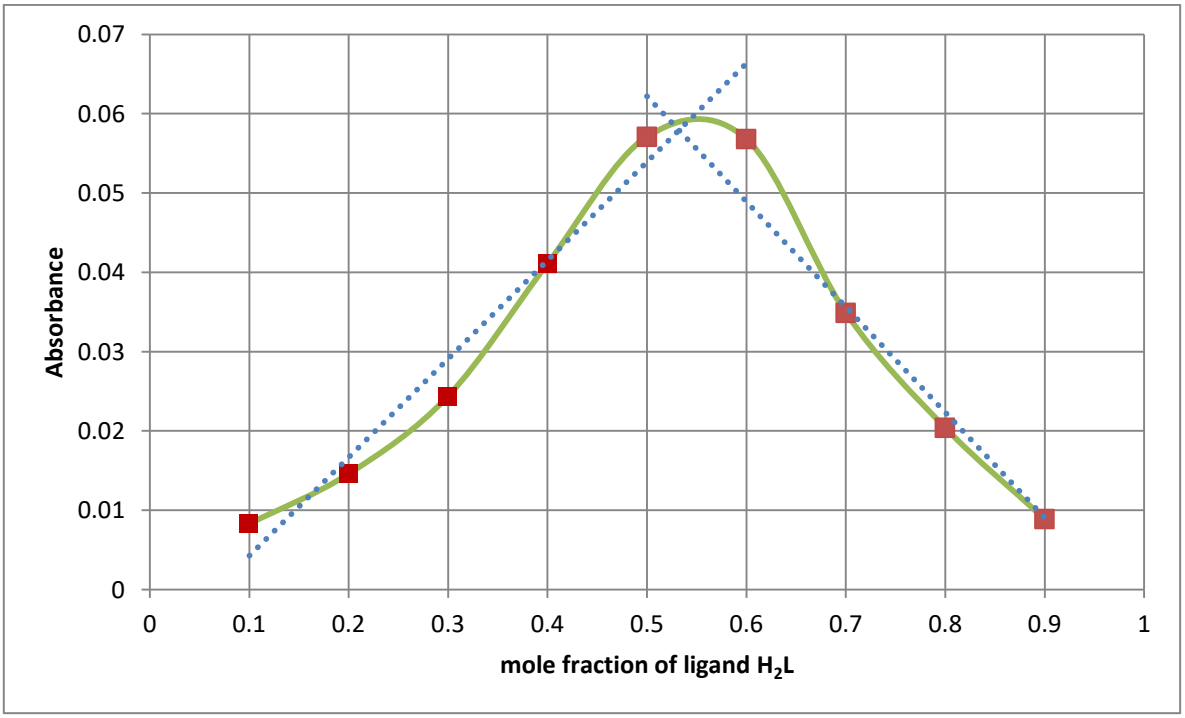


Figure S21. Job's plot for compound **1**.

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