ELECTRONIC SUPPLEMENTARY INFORMATION

Coal Waste Derived Synthesis of Yellow Oxidized Graphene Quantum Dots with Highly Specific Superoxide Dismutase Activity: Characterization, Kinetics and Biological Studies

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1. GENERAL METHODOLOGY AND METHODS

1.1 Pre-treatment of coal

Briefly, before using CW towards synthesizing Graphene oxide via improved Hummer's method. The raw CW was finely powdered using a mechanical grinder. The powdered coal was washed with ethanol and dried in a hot air oven for 6 h at 60℃ to remove trace impurities. CW's dried black fine powder was further used to synthesize GO.

1.2 Synthesis of GO from CW

Typically, large lumps of CW (from Dhanbad, India) are finely powdered using mechanical grinder and pre-treated with ethanol and dried, **Fig. S1**. H_2SO_4(CDH chemicals (P) Ltd, LR grade) and H_3PO_4 (Merck Millipore In, Suprapur) in the ratio (6:4 v/v), 100 mL, are added to the finely powdered CW (0.2 g), followed by mixing at 200 rpm at rt. $KMnO_4$ (6 equivalent) is slowly added below rt to avoid exotherm. The entire contents are heated to 50-60 ℃ for 12-13 h, then cooling to rt before adding 30% H_2O_2 (Fischer Chemicals India, AR grade) v/v. 100mL deionized (DI) water was added subsequently. Reaction workup was done by adding 20% HCl (CDH chemicals (P) Ltd, LR grade) and 50 ml C_2H_5OH (Fischer Chemicals, AR grade) to remove trace impurities. Further purification of formed graphene oxide GO was done using a dialysis bag MWCO 12 KDa (Himedia) and drying at 60℃ for 24 h.

1.3 HR-TEM sample preparation and EDS map of GO and YGQDs

Briefly required quantity GO (0.05 mg/ml) and YGQDs (0.02 mg/ml) in Isopropyl alcohol (SRL chemical India Ltd) was sonicated for a period of 30 mins under sweep sonication at 60

KHz frequency until a uniform particle distribution was obtained. 10 µL of the above sample was taken and poured onto Carbon-Cu Type B grid having mesh size 300 under normal visible light. The added components were dried initially under visible light and vacuum dried at 60°C for 48 h. The prepared grid was stored under an inert atmosphere to avoid cross-contamination before using it for HR-TEM analysis.

1.4 H_2O_2 induced damage-control in Caco2 and HepG2 cells by YGQDs

Glial, Colon, and liver cell lines were grown in the DMEM and RPMI-1640 medium, respectively, with 10% FBS and 1% antibiotics at 37^0C in a CO_2 incubator. $1x10^5$ cells/ml of both cell lines were seeded in the 96 well plates and allowed to grow overnight. Further, cells were treated with YGQDs (2.5, 5, 10, and 25 µM), and then 200 µM-H_2O_2 was added to each well and incubated for 24 h. The MTT assay-based cell protection efficiency of YGQD is evaluated for these cell lines.

1.5 H_2O_2 and LPS induced damage-control in C6 cells by YGQDs

C6 cells ($1x10^6$ cells/ml) grown in the 96 wells plate were pre-mixed with YGQDs (2.5, 5, 10, and 25 µM) and then treated with LPS-0.35µM and H_2O_2 (200 µM) for 24 hr. Untreated and LPS or H_2O_2-treated cells were taken as a negative and positive control, respectively. After incubation, an MTT assay was performed to evaluate the cell viability under different ROS-inducing agents.

1.6 SOD-like activity of YGQDs

For evaluation of superoxide scavenging activity, solution A, 0.1 mol/L Tris-HCl buffer solution with 1mmol/L EDTA at pH 8.2, and solution B, 0.1-10 mmol/ml pyrogallol solution in HCl was used. Mix 117 µl Solution A and 100µl DI water into a 96 wells plate. Then YGQD-17.5µM and natural SOD-0.61µM (EIASODC, Thermoscientific) were added in each well, solution B (7.5 µl) was added into all wells, and the solution was immediately mixed. The absorbance was measured at 325 nm. The absorbance difference between the two aliquots, ΔA325 (min^{-1}), indicates the rate of pyrogallol autoxidation[1]. Line weaver burk double reciprocal curve obtained by varying pyrogallol concentration to determine enzyme affinity. The Michaelis–Menten equation with a non-linear least square algorithm in Graph-Prism 5.0 was used to calculate kinetic parameters, K_m and V_{max}.

1.7 CAT-like activity of YGQDs

Natural CAT (C-1345, Sigma Aldrich) was diluted with 10 mM PBS-pH 7.4 to evaluate catalase activity. Different concentrations of CAT and YGQD, ranging (upto 25µM), were added to a 96-well plate. H_2O_2 was diluted in 10 mM PBS-pH 7.4 to a final concentration of 5 mM. A volume of 200 µL of substrate solution was rapidly added to each well of the microtiter

plate using a repeating pipette. The plate was then immediately scanned in a spectrophotometer (Carry, UV-visible spectrophotometer, Agilent) at $\lambda = 240$ nm after 15 min of reaction at room temperature [2].

1.8 Peroxidase-like activity of YGQDs

The peroxidase-like activity of YGQD was studied using 3,3′, 5,5' – tetramethylbenzidine (TMB), and H_2O_2 as reaction models[3]. In short, TMB (2 mM) 100 µl, H_2O_2 (100 mM) 100 µl and YGQD ranging (upto 25µM) were incubated at 37°C for 20 min. Natural HRP enzyme-1U (2µg) was used as positive control instead of YGQD. The volume of the reaction system was adjusted to 300µl using 0.1 M acetic acid buffer-pH 3. The colour formation of the complex was detected by UV-Visible spectrophotometer. The absorbance change at 595 nm was recorded.

1.9 RNA extraction, reverse transcription, and RT-PCR

The freshly grown mid-log phase C6 cells ($1x10^6$ cells/ml) were treated with YGQD (2.5µM and 5µM), SOD (0.61µM). H_2O_2 (200µM) treated cells were taken as a positive control, while untreated cells were taken as a negative control. After 12 h incubation, cells were processed for RNA isolation using the TRIZOL method. The isolated RNA pellet was dissolved in RNase-free, double distilled water and quantified by NanoDrop (Thermo Scientific, USA) for further processing. cDNA synthesis was performed using total RNA as a template using Transcriptor high fidelity cDNA synthesis kit (Roche) per manufacturer's instructions. The initial reaction mixture of 10 µl was prepared by adding 60 µM random hexamer primers, 1.3 µg of total RNA, and RNase-free water. The mixture was incubated at 65°C for 10 minutes and was chilled immediately on ice. Further, 8 mM $MgCl_2$, 20U RNase inhibitor, 1 mM dNTP mixture, 5 mM DTT, 10 U of reverse transcriptase, and reaction buffer were added to make the final volume of 20 µl. The mixture was incubated at 29°C for 10 minutes and then at 48°C for 60 minutes. The reaction was stopped by heating at 85°C for 5 minutes, and NanoDrop quantified synthesized cDNA. Using a cDNA template, genes (SOD, Catalase, GPx, and GAPDH) were amplified by RT-PCR. The PCR mixture (10 µl) contains 0.1 µM of forward and reverse primer, 0.2 µg of synthesized cDNA, and 5µl SYBR master mix. The PCR was run for 36 cycles, each consisting of 45 seconds of denaturation at 95°C, 45 seconds of annealing (between 56-62°C), and 1 minute of extension at 72°C. Change in gene expression was analyzed by fold-differences calculation using the ΔCT method.

2.0 Cytotoxic studies and hemolytic assay

Cytotoxicity of YGQDs is tested against human peripheral blood mononuclear cells (PBMC) by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell-

proliferation assay. Briefly, PBMC cells were isolated from blood by using histopaque, rinsed with PBS, and transferred in Roswell Park Memorial Institute (RPMI)-1640 medium in 6 well plates (1×10^6/well) and loaded with YGQDs with concentrations (25, 50, 100, 250, 500 μM) for 48 h. Amphotericin B (AmB) (100 μM) is a positive control. Cell feasibility is measured after successful incubation.

RBC hemolysis assay of YGQDs was carried out as per the earlier method with some modification[4]. Wherein human erythrocytes are isolated by using histopaque and incubated as (4×10^8 cells/ml in PBS) in the presence of varied concentrations of YGQDs (100, 250 & 500 μM) and with AmB (100 μM) for 4 h at 37°C. The incubated samples were centrifuged at 1500 g for 5 minutes. The absorbance of the supernatant was measured at 560 nm. Relative hemolysis was calculated by considering hemolysis of 100 μM AmB-treated erythrocytes as 100%.

2.1 Enzyme-linked immunosorbent assay for pro-inflammatory markers

Raw264.7, culture was treated with 200μM-H_2O_2 and 200μM-H_2O_2 + YGQDs (2.5 and 5μM), while only SOD-0.61μM treated, then supernatants were measured with ELISA kits for IL-1β, IL-6, TNF-α, IFN-γ, and IL-10 according to the manufacturer's directions (Invitrogen-88-771144). Briefly, 100μl of 1μg/ml (carbonate coating buffer-pH ~9.5) of capture antibody for all cytokines were coated in 96 wells plate in triplicates and incubated overnight at 4^0C. Further, wells were washed thrice with 1xPBS-0.1% tween-20, 100μl of 5%-BSA as blocking solution added to each well, and incubated for 2 hr at room temperature. Plates were washed, and 100μl of samples were added, then incubated for 2 hr at room temperature; further washing proceeded, and then 100μl of 1μg/ml (carbonate coating buffer-pH ~9.5) of detection antibody was added, incubated for 2hr at room temperature, washed thrice and TMB substrate was added to develop the blue color. A UV-visible spectrophotometer recorded the color changes at 595nm.

2.2 YGQD cellular uptake study

C6 cells were grown in the high glucose Dulbecco′s Modified Eagle′s Medium (DMEM) media, containing 10%-fetal bovine serum (FBS) with 1% penicillin/streptomycin antibiotics. $1x10^6$ cells/ml cells are seeded in the 12 wells plate and treated with and without YGQD-62.5 μM for intervals from 0, 30, and 60 min. After incubation, cells were centrifuged at 2500xg for 15 min, and the pellet was washed thrice with 1x-PBS, pH 7.4, to remove uninternalized YGQDs. The cell pellet was diluted in 250μl of 2.5% glutaraldehyde, and a smear was prepared

in the slides for all differently treated cells. Slides were covered with a cover slip and then confocal imaging.

2.3 ITC-based study for YGQD and SOD binding to $O_2^{\bullet-}$superoxide

YGQD or SOD interaction with $O_2^{\bullet-}$was analyzed by isothermal calorimetry (ITC), MICROCAL PEAQ ITC (Malvern). 0.1 mol/L Tris HCl buffer solution with 1mmol/L EDTA at pH 8.2 and 0.1-10 mmol/ml pyrogallol solution in HCl was used for the superoxide generation. The sample cell was loaded with 0.05mM-270 µL of Tris HCl-EDTA, pyrogallol buffer, and the injection syringe was filled with 70 µL of ~20µM YGQD or 0.5 µM SOD as superoxide scavenger. The titration response was completed with the following parameters: rotation speed 600 rpm, temperature 25 °C, beginning delay 60 s, injection volume 2 µl except for the first (0.4 µl), interval time between two injections 120 s, total 19 injections. The data were then fitted with Origin (Origin Lab Corporation, USA) instrument-integrated software to determine the binding constant (K_d), change in enthalpy (H), and change in entropy (S) like our earlier published results[5].

2. LIST OF FIGURE(S) AND TABLE(S)

2.1 Pre-treatment of CW

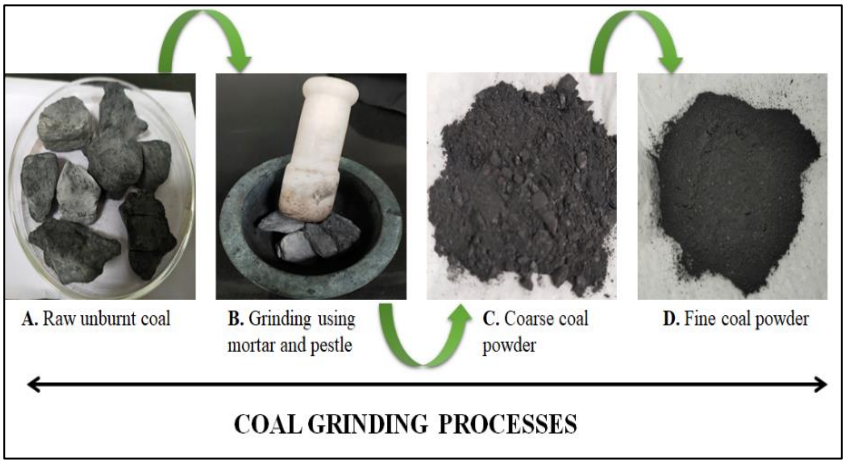

Fig. S1. Sequential steps required for pre-treatment CW before synthesizing graphene oxide viz., improved hummers method.

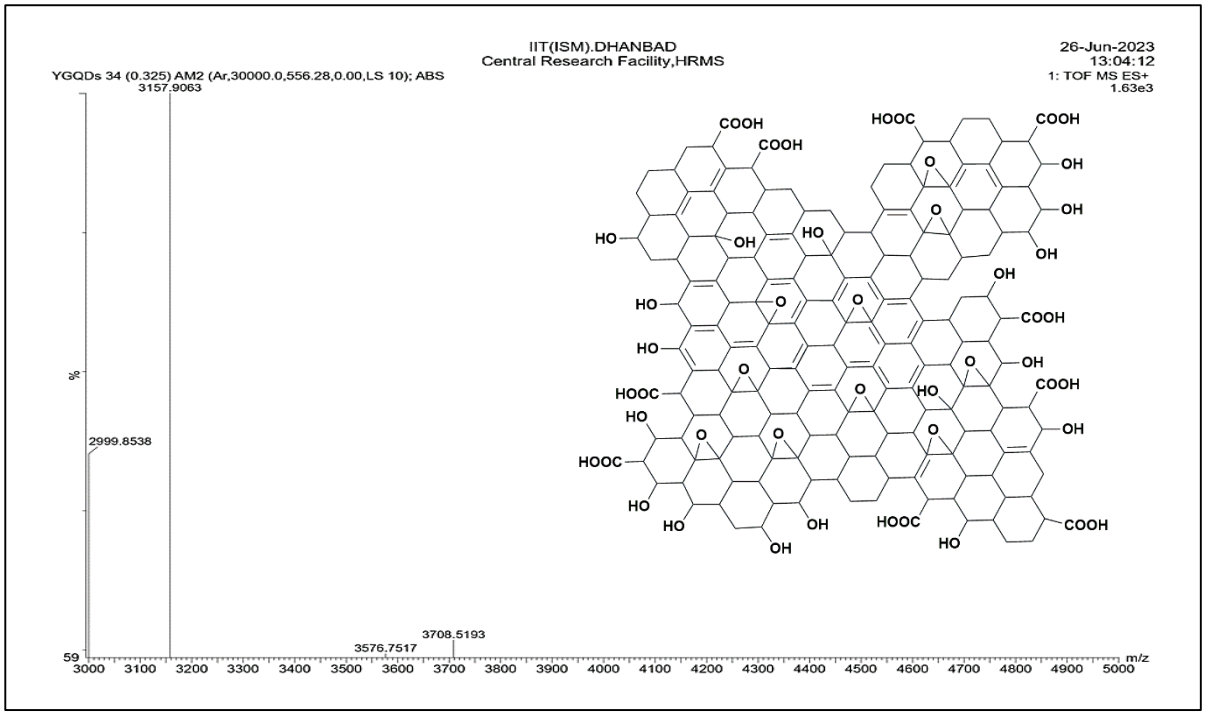

Fig. S2. TOF-mass of YGQDs taken in the range 3000-5000 Da showing different molecular components.

2.2 Optimization table for the synthesis of YGQDs

Table S1. Optimization table for the synthesis of YGQDs

S.no	Weight (mmol)		DI water	GO	Temp°C	Time	*Ca. yield	Colour
	NaOH	H_2O_2						
1	1	1	50 ml	100 mg	100	12 h	< 52 %	Dark green
2	1	2	50 ml	100 mg			57.8%	
3	2	1	50 ml	100 mg			58.3%	
4	2	2	50 mL	100 mg			60.8 %	
5	3	2	50 ml	100 mg			57.1 %	
6	2	3	50 ml	100 mg			66.7 %	
7	3	3	50 ml	100 mg			61.2 %	
1	1	1	50 ml	100 mg	121		67.8%	Bright yellow
2	1	2	50 ml	100 mg			66%	
3	2	1	50 ml	100 mg			63.7%	
4	2	2	50 mL	100 mg			64.5%	
5	3	2	50 ml	100 mg			75.3%	
6	2	3	50 ml	100 mg			87.6%	
7	3	3	50 ml	100 mg			82.2%	

1	1	1	50 ml	100 mg			79.4%	
2	1	2	50 ml	100 mg			82.7%	
3	2	1	50 ml	100 mg			77.3 %	
4	2	2	50 mL	100 mg			80.7 %	Green
5	3	2	50 ml	100 mg	150		76.3 %	
6	2	3	50 ml	100 mg			85.7 %	
7	3	3	50 ml	100 mg			86%	
Note: *Ca. The yield represents the number of YGQDs formed in comparison to GO taken.								

2.3 HR-TEM morphology and EDS graph of GO and YGQDs

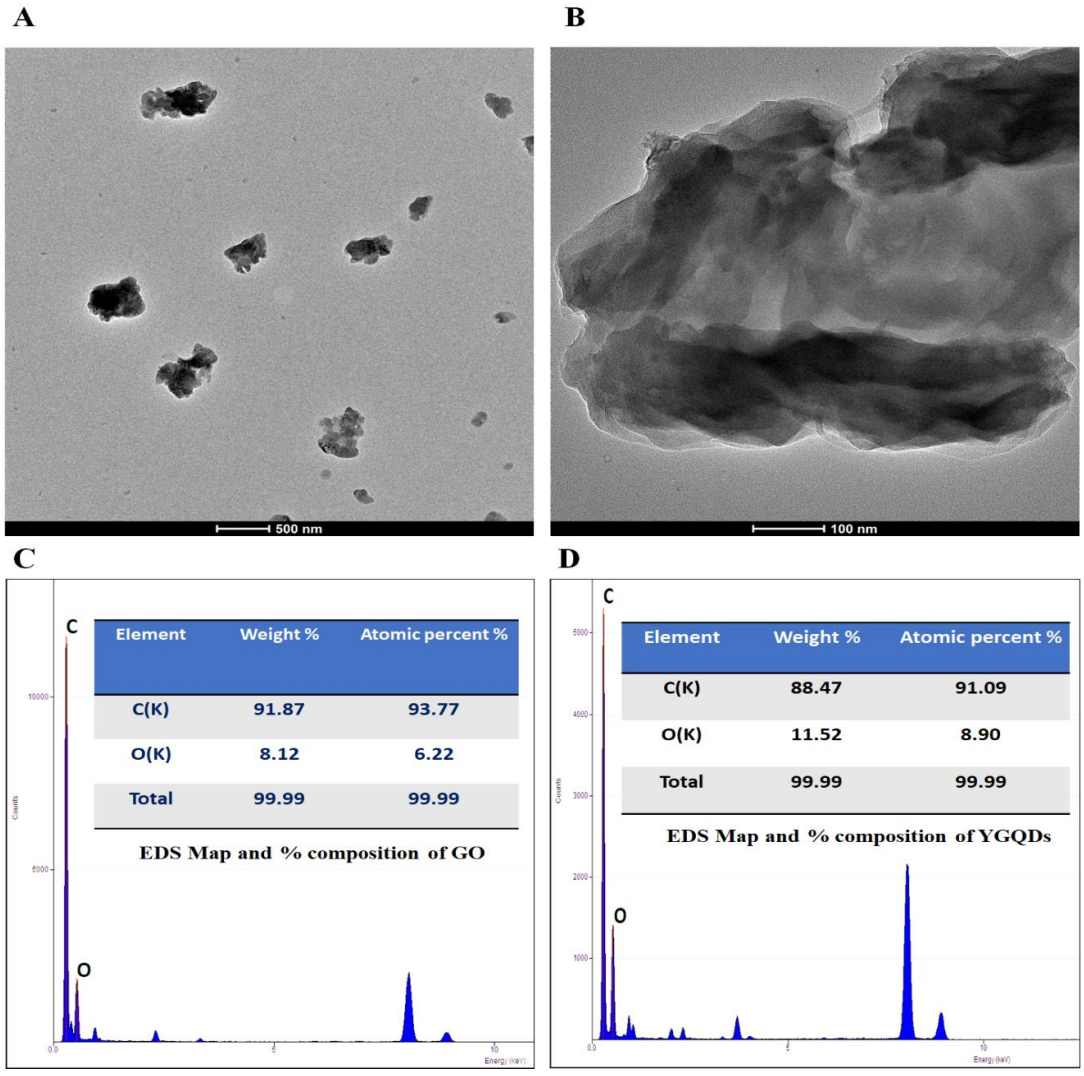

Fig. S3. (A) Picture Image representing the HR-TEM morphology of GO observed at 500 nm (B) Morphology of GO observed at 100 nm (C) Picture representing the acquired Energy dispersive spectroscopy (EDS) map for GO along with elemental percentage. (D) A picture representing the acquired Energy dispersive spectroscopy (EDS) map for YGQDs and elemental percentage.

2.4 Dispersibility of YGQDs in different solvents

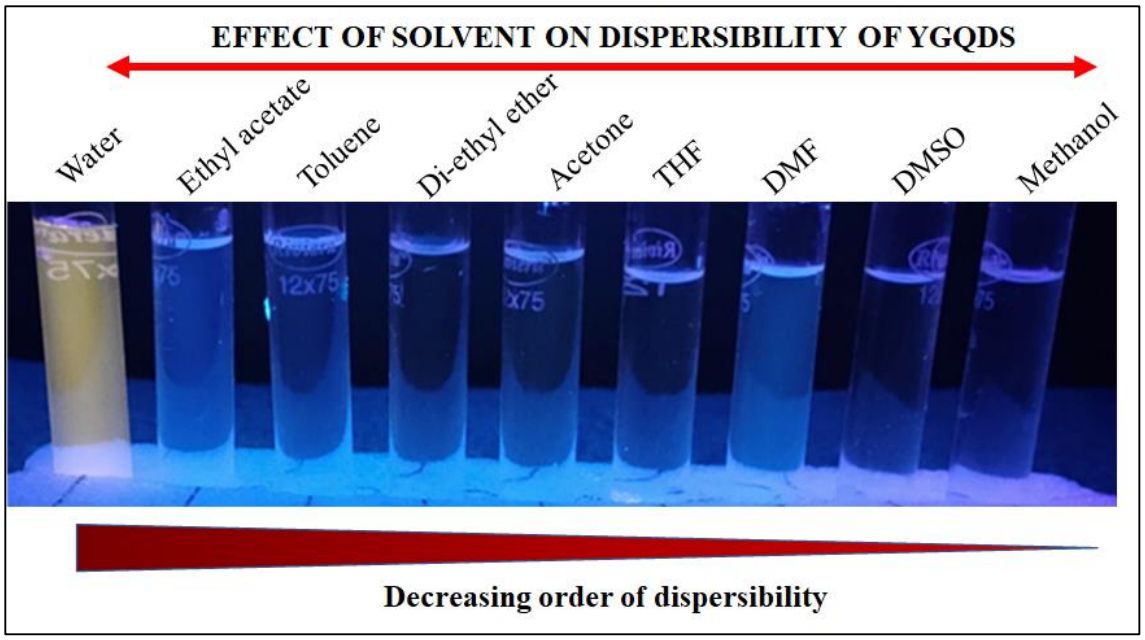

Fig. S4. Picture representing dispersibility of YGQDs in different solvents

2.5 UV-vis spectra of GO, pH-dependent photophysical studies of YGQDs and its lifetime details

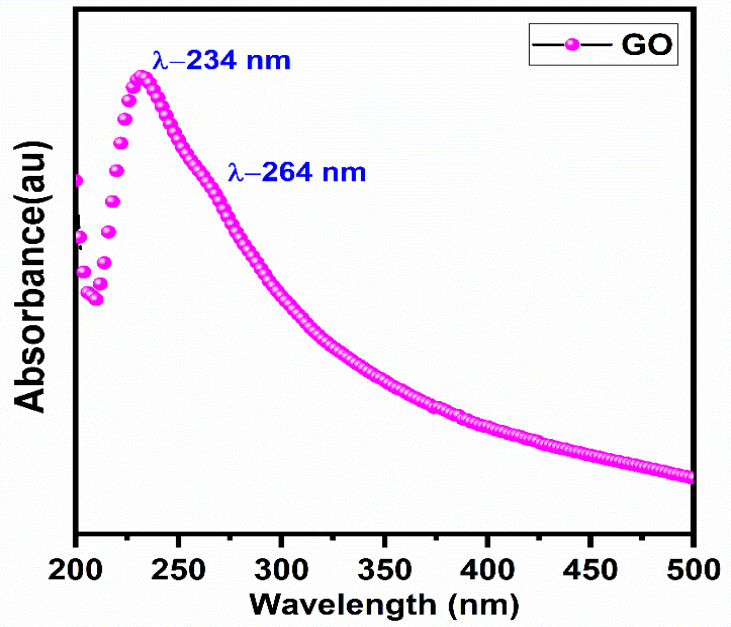

Fig. S5. UV-vis spectra of GO showing two different characteristic transition peaks at 234 and 264 nm representing Π-Π* and n- Π* transitions from sp^2 and sp^3 centers.

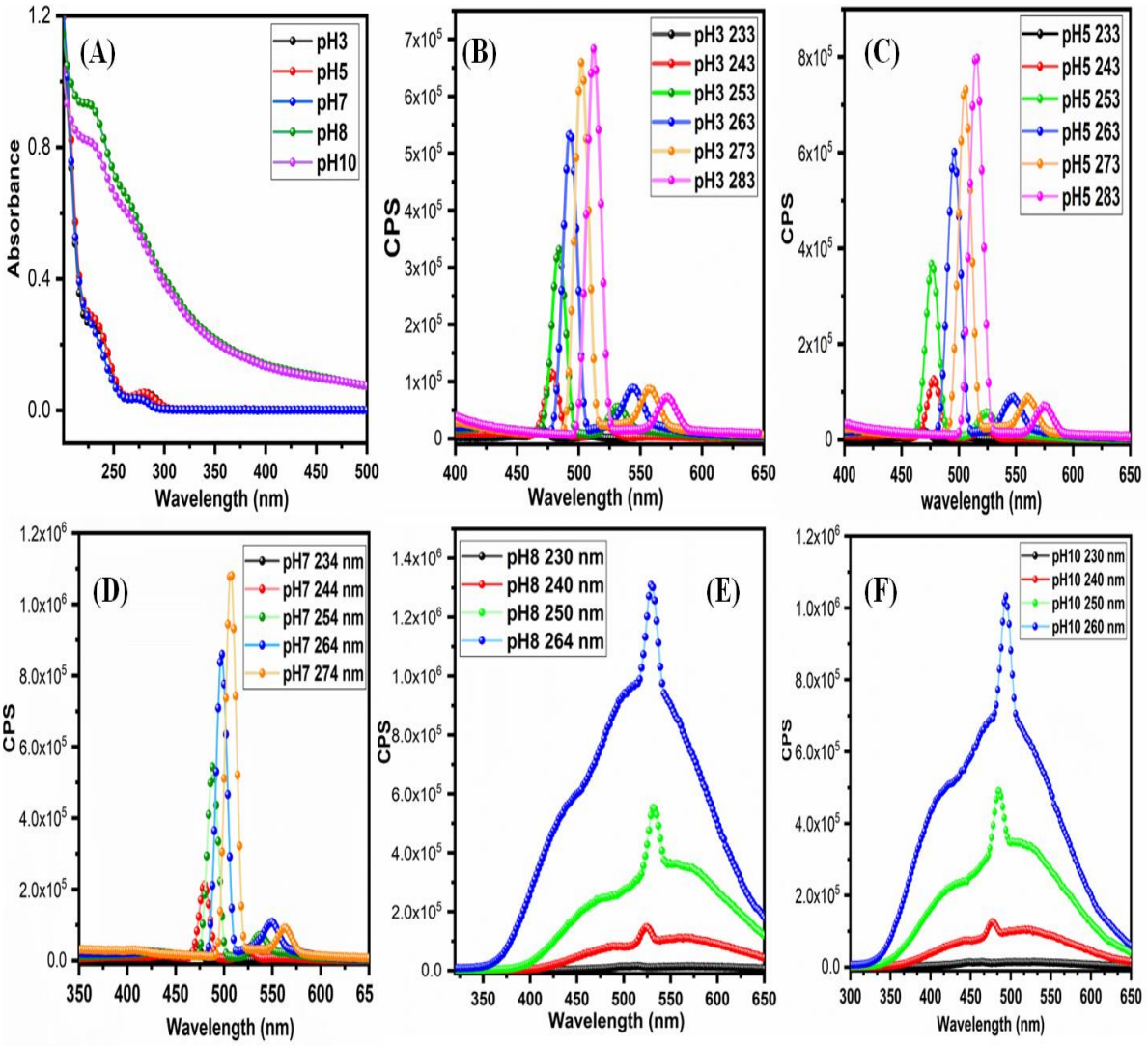

Fig. S6. The pH-dependent change in PL spectra for YGQDs was recorded over different excitation wavelengths. (A) representing the UV spectra of YGQDs at pH 7, 8, 9 and 10 respectively (B) Change in the Pl spectra of YGQDs at different excitation ranges (233, 243, 253, 263, 273, and 283 nm respectively) at pH 3. (C) Change in the Pl spectra of YGQDs at different excitation ranges (233, 243, 253, 263, 273, and 283 nm, respectively) at pH 5. (D) Change in the Pl spectra of YGQDs at different excitation ranges (234, 244, 254, 264, 274 nm, respectively) at pH 7. (E) Change in the Pl spectra of YGQDs at different excitation ranges (230, 240, 250, and 260 nm, respectively) at pH 8. (F) Change in the Pl spectra of YGQDs at different excitation ranges (230, 240, 250, 260 nm, respectively) at pH 10.

Table S2. Calculated values of τ1/B1, τ2/B2, and τ3/B3 calculated using tri-exponential curve function along with Avg lifetime and χ^2

S. No	Ex/Em	χ^2	τ1/B1	τ2/B2	τ3/B3	Avg. Lifetime
1	440 nm	1.06	0.96/ 30.44	3.85/ 51.61	0.19/ 17.96	0.724
2	528 nm	1.11	0.97/ 63.02	3.60/ 63.02	0.25/ 10.24	1.17

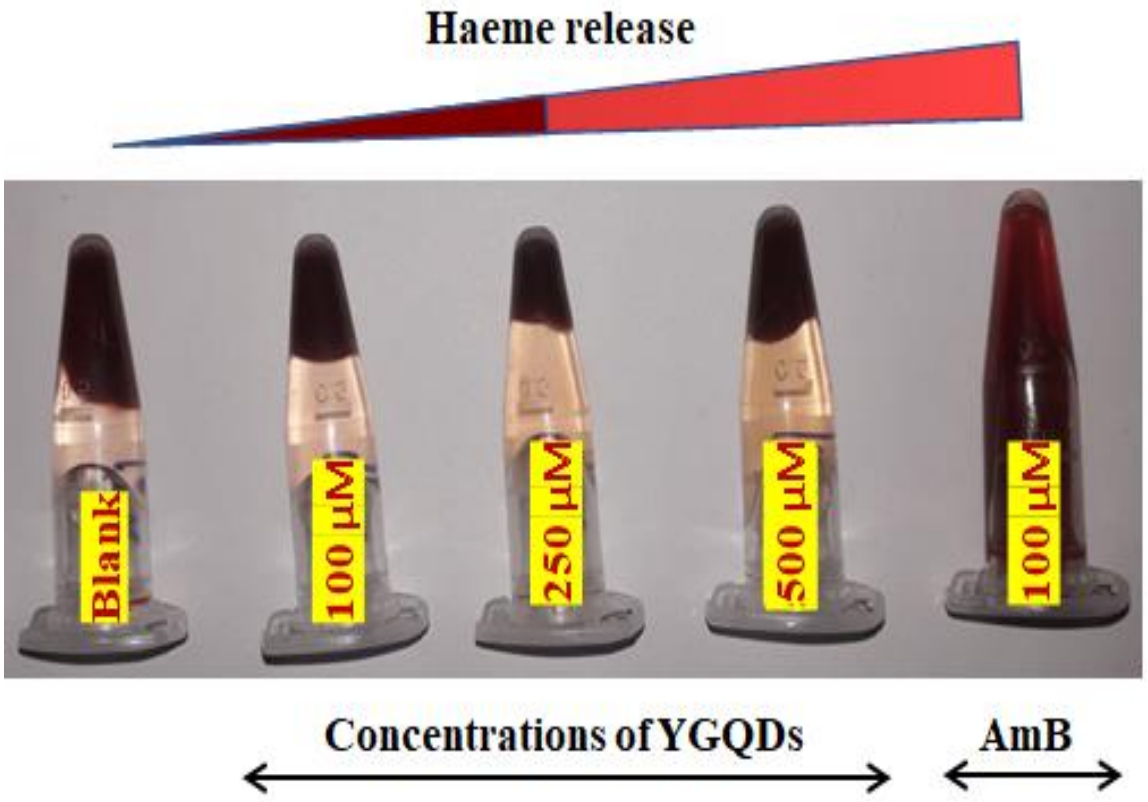

Fig. S7. Picture representing the hemolytic effect of YGQDs tested at different concentrations and compared to AA used as a standard.

2.6 Cell viability assay of YGQDs

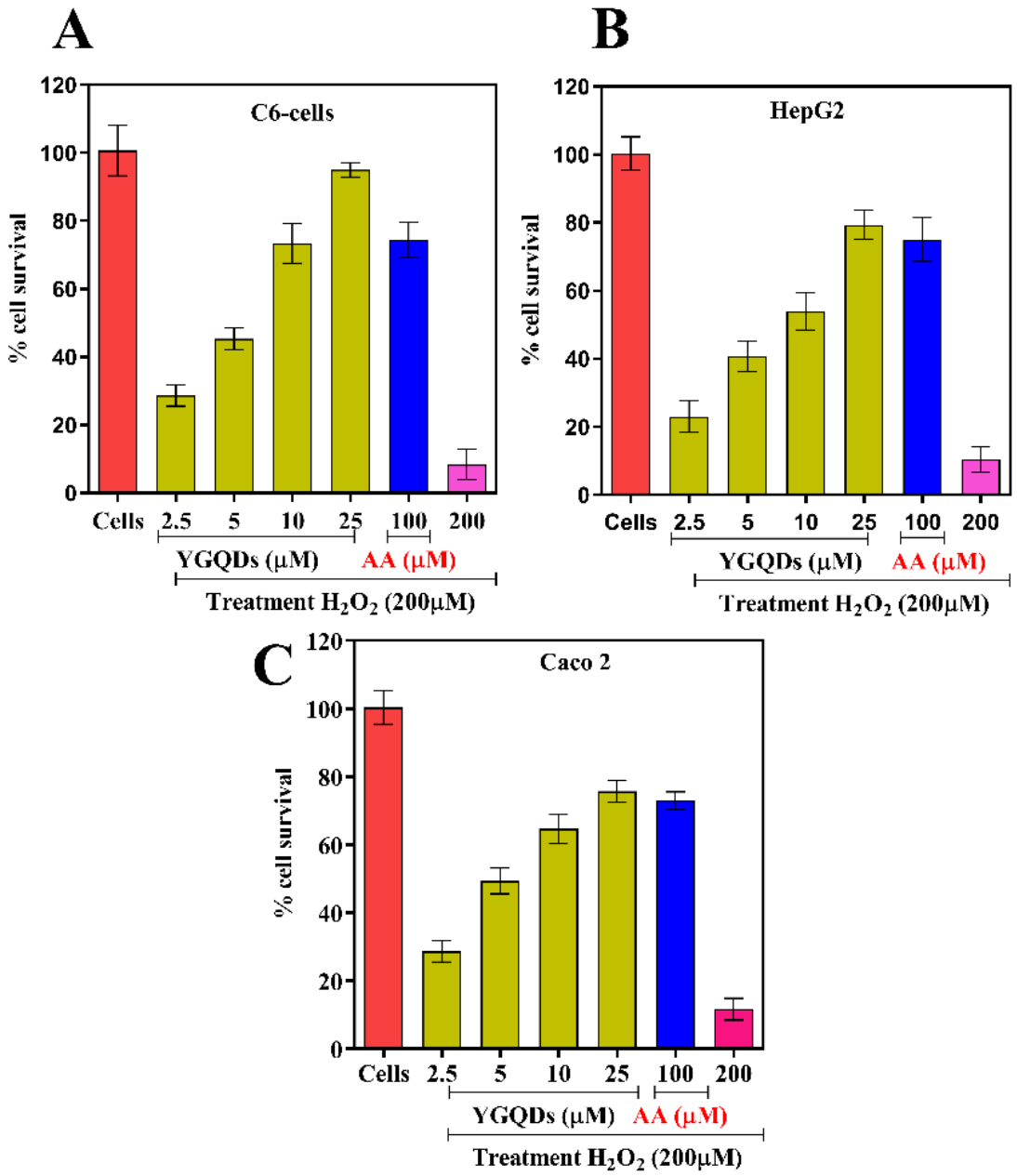

Fig. S8. Cell viability assay evaluated for YGQD against H_2O_2 induced ROS in (A) C6-cells, (B) Caco2 cell line, and (C) HepG2 cell line.

2.7 MTT assay color changes for LPS-treated and untreated YGQDs

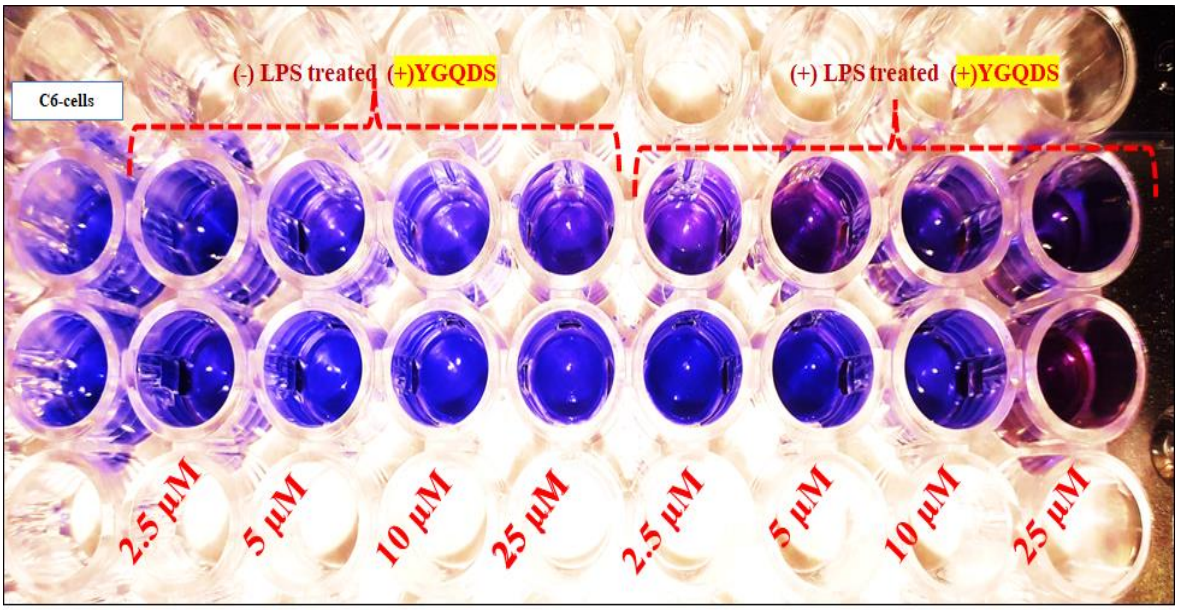

Fig. S9. Picture representing MTT assay color changes for LPS+YGQD treated and untreated cells with blue color indicating more viability, reddish-purple less viable

2.8 Enzyme assay response of YGQDs

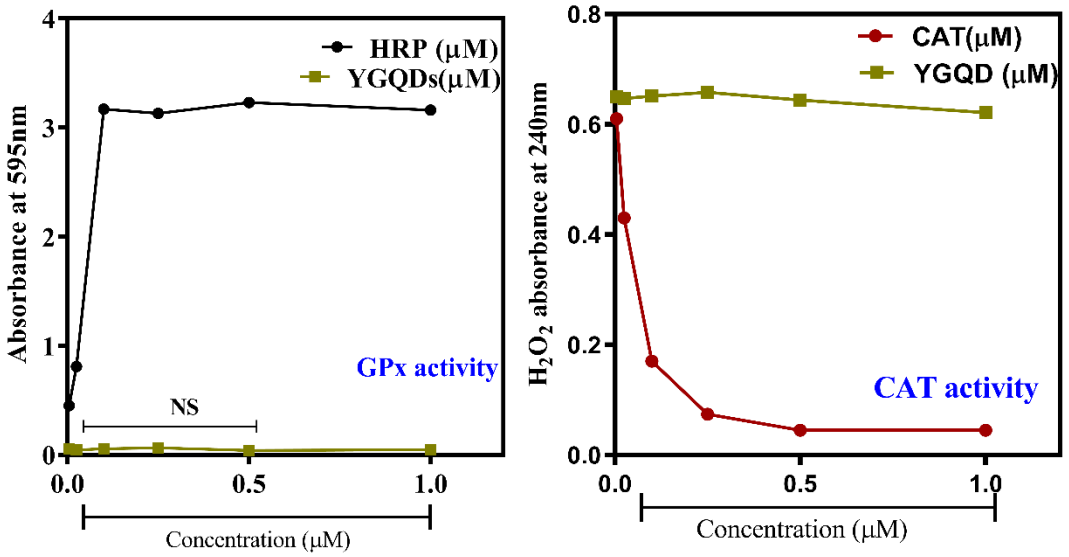

Fig. S10. Picture representing (A) GPx and (B) CAT activity of YGQD and the results compared with natural enzymes. The results show no effect for both enzyme activities with YGQDs. The figure needs to be changed

2.9 Gene expression studies with YGQDs

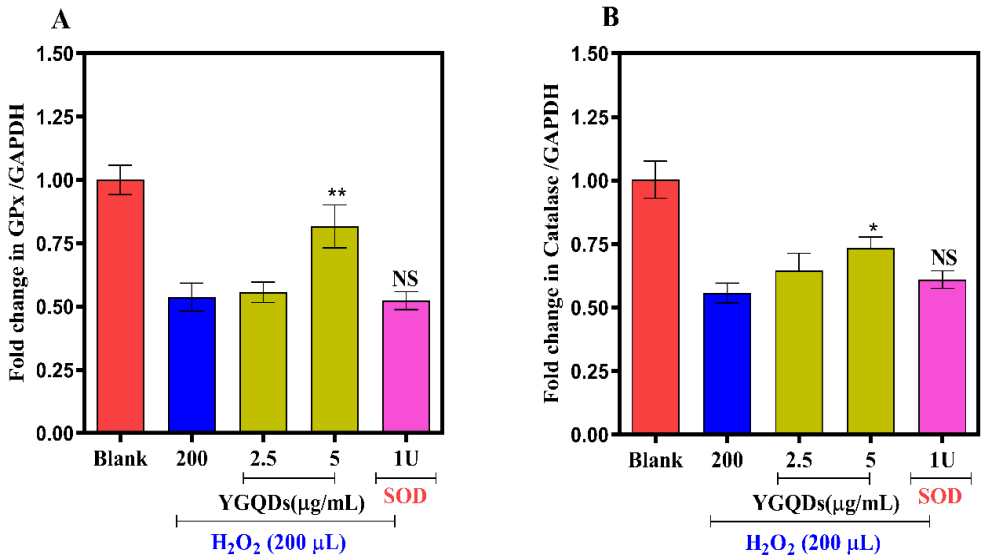

Fig. S11. Picture showing the gene expression of (A) GPx and (B) Catalase activity, having up-regulated in the case of YGQD, while with bSOD, it did not change.

3.0 MDA levels estimation

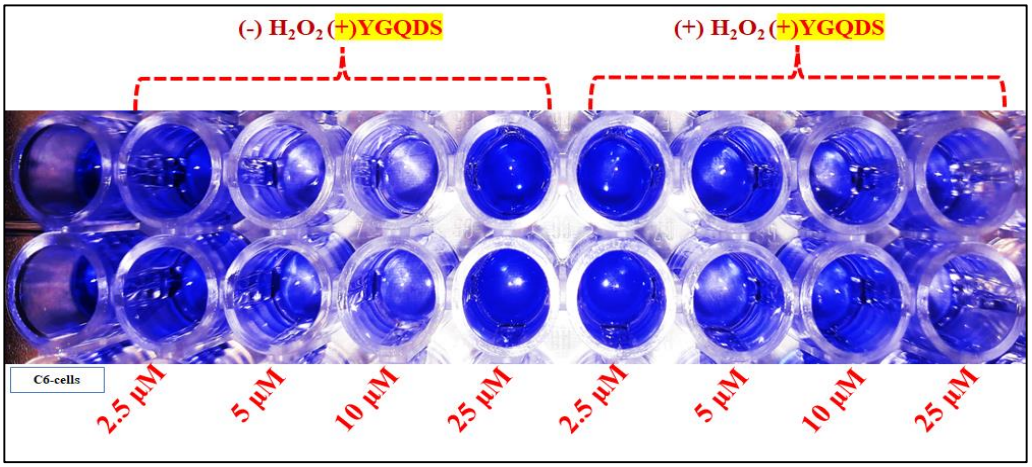

Fig. S12. ELISA picture representing color intensities for MDA levels estimation

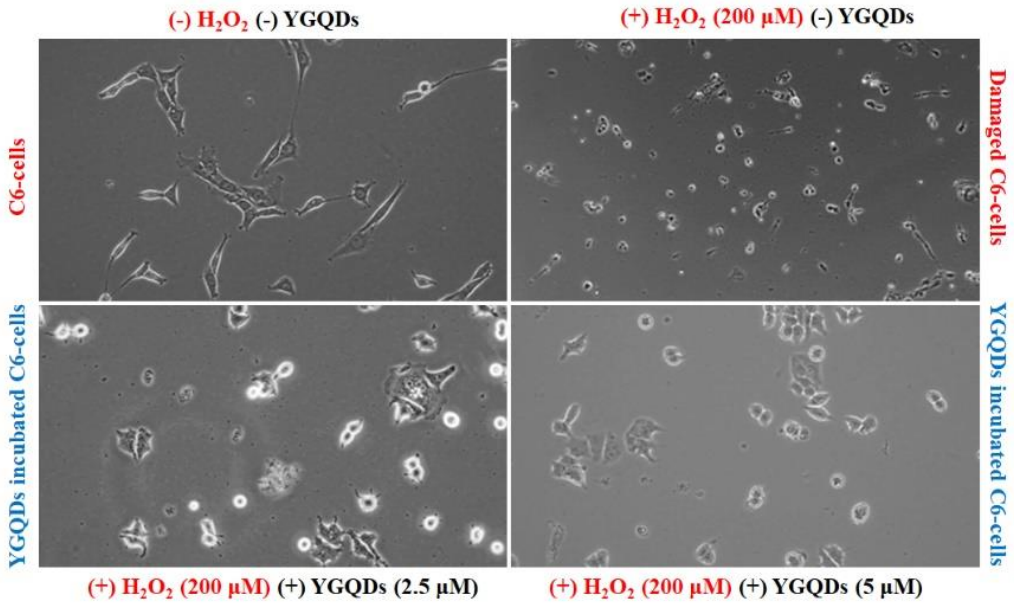

Fig. S13. C-6 cell protection against H_2O_2 induced ROS by YGQDs at different concentration

References

1 X. Li, *Journal of Agricultural and Food Chemistry*, 2012, **60**, 6418–6424.

2 B. Weiss, R. Lehne and S. Strada, *Analytical Biochemistry*, 1972, **45**, 222–235.

3 H. Guo, H. Guo, Y. Xie, Y. Chen, C. Lu, Z. Yang, Y. Zhu, Y. Ouyang, Y. Zhang and X. Wang, *Redox Biology*, 2022, **56**, 102441.

4 M. A. Dobrovolskaia, J. D. Clogston, B. W. Neun, J. B. Hall, A. K. Patri and S. E. McNeil, *Nano Letters*, 2008, **8**, 2180–2187.

5 S. Mandal, S. R. Prasad, D. Mandal and P. Das, *ACS Appl Mater Interfaces*, 2019, **11**, 33273–33284.