Supporting Information

Green synthesized copper oxide nanostructures for potential multifaceted biomedical applications

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1. Materials and methods

1.1. Materials

Annona squamosa (AS) leaves were plucked from a local botanical garden and authenticated in Andhra University with herbarium voucher no. A.U. BDH 22290. Copper acetate, Carbopol 934, Folin Ciocalteau reagent were purchased from Sigma-Aldrich, Bangalore, India. *Staphylococcus aureus* NCIM, 2079, *Bacillus subtilis* 2063, *Escherichia coli* 2065, *Pseudomonas aeruginosa* 5514 were purchased from National Collection of Industrial Microorganisms (NCIM), NCL, Pune, India. *Enterococcus faecium* 5153 was procured from the Microbial Type Culture Collection, Chandigarh, India. Agar medium, nutrient broth, Dulbecco's modified Eagle's medium (DMEM) and neomycin were purchased from Hi-media, Mumbai, India. MTT reagent was procured from Thermo Fisher Scientific, M-6494. HEK-293 and HeLa cell lines were obtained from National Centre for Cell Science (NCCS) Pune, India. Albino Wistar rats were purchased from Mahavir Enterprises, Hyderabad, India.

1.2. Preparation and quantification of Annona squamosa aqueous leaf extract

The AS leaves were washed thoroughly, shade dried and were powdered in a mixer. 5g of powder was mixed with 100 mL of water and allowed to macerate in distilled water in a mechanical shaker for 48 h at room temperature (~24°C). The extract was then concentrated in rotary evaporator and filtered using Whatman no. 1 filter paper followed by centrifugation for 10 minutes at 5000 rpm. The supernatant was collected and autoclaved for 15 min at 121°C under 15 lbs pressure. A greenish yellow colored transparent solution was collected and stored in screw capped bottles at 4-8°C for phytochemical analysis. The phytochemical analysis was done to detect the presence of alkaloids, carbohydrates, glycosides, proteins, oils, tannins, phenols, acids

in AS leaf extract using standard chemical tests. The quantification of phenols was determined by the Folin Ciocalteau method.

1.3. Preparation of CuO nanostructures

Copper acetate (0.005- 0.02 M), stirring time (5 min-2 h) and AS leaf extract (2-10 mL) were optimized by fixing two components and altering one component each time to produce uniformly small sized nanoparticles. 30 mL (0.02 M) of copper acetate was poured in a 50 mL round bottom flask and AS leaf extract (10 mL) was added and refluxed under vacuum in presence of oxygen atmosphere (50 cm^3/min) for the required time period (10 min) at 90 °C with continuous stirring at 600 rpm. The dispersed medium was scanned for the absorbance peak in all experimental conditions using UV-Vis spectroscopy at a range of 300-800 nm, with 1 nm resolution placing Millipore water as blank. The nanoparticle dispersion was centrifuged at 16,000 rpm at 4°C for 10 min and dried at 100 °C in a hot air oven. The schematic representation for CuO nanostructure synthesis is shown in **Fig. S1.**

1.4. Characterization of CuO nanostructures

The morphology, structure and composition analysis was investigated by field emission scanning electron microscopy-electron diffraction spectrum (FESEM-EDS) (JEOL JSM-6610-LV- with OXFORD EDS) at an accelerated voltage of 10 kV by placing small amount of sample on nickel grid and transmission electron microscopy images (JEM1200EX, JEOL Ltd., Japan equipment) were obtained at an accelerated voltage of 200 kV. The crystallinity was analyzed by XRD (PANalytical: XPERT-PRO) operated at 40 kV and 30 mA under Cu Kα radiations (λ= 1.54060°A) using nickel monochromator at a range of 2Ɵ from 20° to 90°. The IR spectrum of AS leaf extract and AS-CuO NLs was evaluated by FTIR (Shimadzu FTIR 21) prestige equipment using potassium bromide pellet in a wavelength range of 4000 cm^{-1} to 400 cm^{-1} at a

resolution of 4 cm^{-1} to analyze the functional groups responsible for nanostructure formation. Raman analysis was performed with Horiba HR 8000 equipment at an excitation wavelength of 514.5 nm to investigate the composition and crystallinity of nanomaterials. The Brunauer Emmett-Teller (BET) surface area was measured from the N2 adsorption-desorption isotherm at 77.3 K by using Quanta chrome Nova 2200 E system. The hydrodynamic size distributions of nanoparticles and zeta potential of CuO NLs were determined using the HORIBA- SZ-100 instrument to explore stability and surface potential of CuO NLs.

1.5. Stability of CuO NLs

CuO NLs were re-dispersed in pH 7.4 phosphate buffer saline and the solutions were stored in airtight containers at 25±2°C and 75±5% relative humidity and stability was examined by UV-Vis studies. The UV-Vis spectra of CuO NLs were traced in range of 300 nm and 800 nm at different time periods until 6 months. Further, the stability was estimated using zeta potential analyzer (HORIBA- SZ-100) by addition of 5 µL of CuO NLs dispersion in the cuvette by setting temperature at 25 °C and conductance of 355 µS and with an electrode voltage of 14.5 V/cm.

1.6. Determination of Minimum inhibitory concentration (MIC) and evaluation of synergetic antibacterial activity

The MIC of CuO NLs and neomycin were tested against *Escherishia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomanas aeruginosa and Enterobacter faecium* by agar well diffusion method. Antibacterial activity was done based on recommended standards of the Clinical and Laboratory Standards Institute by making suitable dilutions required for assessing MIC. With the help of micropipette, 100 µL of each concentration of neomycin solution/CuO NLs dispersion was poured into each well of agar plate and incubated for 24 h at 37°C.

Subsequently, the plates were observed for zone of inhibition (ZOI) to evaluate MIC.

A composite dispersion of CuO NLs and neomycin was prepared as follows; CuO NLs (50 µg/mL) dispersion and neomycin (50 µg/mL) solution were kept under continuous stirring at 800 rpm at room temperature (~27°C) for 24 h to make a composite without aggregation. The composite was assessed for interaction between CuO NLs and neomycin using UV-Vis spectroscopy in the wave length range of 200 nm to 800 nm. The CuO NLs dispersion (50 µg/mL), neomycin solution (50 µg/mL), and composite (50 µg/mL) each at a quantity of 100 µL were incorporated into wells of the plates prepared by pour plate technique as described in MIC, followed by incubation at 37°C for 24 h placing DMSO as a control. The combinational antibacterial activity was carried out thrice and the values are expressed as mean±s.d. The increase in percentage area was calculated by using the formula;

$$\frac{Area\ of\ ZOI\ (NP + antibiotic) - Area\ of\ ZOI\ of\ \ Antibiotic}{Area\ of\ ZOI\ of\ antibiotic} * 100$$

1.7. *In vivo* wound healing activity of gel formulations of CuO NLs and combinations

CuO NLs and the combination of CuO NLs with neomycin were formulated into gels using Carbopol 934 (Sigma Aldrich) based on the standard procedure and were evaluated for appearance, homogeneity, pH, spreadability, viscosity, spreadability, and extrudability. Four groups of Wistar albino healthy rats with 6 rats in each group were designed for the wound healing studies and treated with control (no treatment), commercial neomycin (standard), CuO NLs gel and CuO NLs with neomycin (combination). The experiments were performed in accordance with ethical norms as approved by CPSCEA guidelines and Institutional Animal Ethical Committee Ref. no. IAEC No.439/PO/01/a/CPCSEA. Prior to 24 h of the test, hair on the dorsal side of each rat was shaved cleanly for each rat, exposing 6 cm^2 area of skin approximately. The animals were anaesthetized by xylazine HCl (50mg/Kg) and ketamine

hydrochloride (10mg/Kg). An excision wound of area around 500 mm^2 was made using surgical knife on the dorsal side for all the test animals and treatment with respective formulation was done for 14 days. The consequent changes in the wound area have been monitored at regular time intervals for skin irritation, percentage of wound contraction.

$$\%\, Wound\ contraction\ = 1 - \frac{A_d}{A_0} * 100$$

Where, A_0=wound area on day zero, A_d=wound area on corresponding days

The skin tissue samples were collected on 14^{th} day for histopathological studies. Specimens of biopsy were preserved in 10% buffered formalin and pathology studies were performed by staining with haemotoxylin and eosin using an optical microscope (OLYMPUS BX 51) under 10X magnification.

1.8. Anti-cancer activity and biocompatibility assessment of CuO NLs by MTT assay

1 mg of CuO NLs was dispersed in 5 mL of Dulbecco's Modified Eagle's Medium (DMEM) medium, which contains 500 mL DMEM solution, 50 mL of 10% fetal bovine serum, 5.5 mL penicillin/streptomycin antibiotics. The stock solution was subsequently diluted to obtain different concentrations (6.25-100 µg/mL) using DMEM medium. The obtained concentrations were subjected to sonication for 15 min at 45W prior to cell exposure to assure the uniform dispersion of nanoparticle in the solution. 1 mL of respective cell suspension (HeLa/HEK cells) was seeded into wells of 6 well plate (surface area of a single well ~9.6 cm^2 with volume of media ~5 mL) and cells were evenly spread by rocking the plate back and forth. 1 mL of CuO NLs of different concentrations (6.25-100 µg/mL) was placed in each plate with untreated cells as control. The plates were placed in a CO_2 incubator for 24 h at 37°C with 5% CO_2 and 95% R.H. After 24 h incubation, the cell images were taken using an optical microscope (OLYMPUS CK-30 and OLYMPUS C-4000). The cell cytotoxicity was analyzed by MTT assay by adding

MTT reagent (200 µL) in each well and incubated at 37°C for 4 h, which resulted in formation of formazan crystals. Subsequently, 500 µL of DMSO was added and from each well 200 µL of solution was transferred to 96 well plate and taken the absorbance readings using Spectra Max 190 microplate reader (Molecular Devices, USA) at 570 nm. The % cell viability was calculated using the formula;

$$\%\ cell\ viability\ = \frac{O.D\ of\ sample}{O.D\ of\ \ control} * 100$$

1.9. Estimation of intracellular ROS amount

Concentrations of intracellular ROS was measured using ROS assay kit (Abcam, India), which contains an oxidation-sensitive dye, a cell permeable 2′,7′-dichlorofluorescein diacetate (DCFH-DA) that deacetylate by esterases into dichlorofluorescein (DCFH, a impermeable compound). DCFH further oxidizes into fluorescent 2′,7′-dichlorofluorescein (DCF) by intracellular ROS to depict concentrations of intracellular ROS. After 24 h exposure of HeLa cells to different concentrations of CuO NPs, the cells were collected and thoroughly washed using PBS solution and then stained using 1 mL of 100 µM 7-dichlorofluorescein diacetate (DCFDA) solution for a period of 45 min. Lysis of the cells was done using 0.3 mm glass beads through vortexing for 10 min. Further, homogenate was centrifuged at 8000 g for 5 min at 4 °C. 200 µL of supernatant was collected from each concentration and transferred in to 96 well plate to measure the readings using a microplate reader (FLx 800, Biotekinstrument Inc., Winooski star) at an excitation wavelength and emission wavelength of 485 nm 530 nm respectively.

1.10. Statistical significance

All the experiments were carried out in triplicate and the mean ± s.d was calculated. The statistical data was estimated using one-way analysis of variance (ANOVA) method to establish

statistical significance, the values were assessed by using Graph Pad Prism version 5.03 (Graph Pad Software, Inc.CA, USA trail version). The results have shown to be statistically significant with p value < 0.05.

Table S1: Phytochemical screening of *Annona squamosa* aqueous leaf extract

Phyto chemicals	Test/ Reagent	AS aqueous leaf extract
Alkaloids	Mayer's test	+
	Wagner's test	+
Glycosides	Legal's test	+
Flavonoids	Shinoda test	+
Tannins	Ferric chloride test	+
Saponins	Foam test	++
Oils	Spot test	+
Carbohydrates	Benedict's test	+
Phenols	Ferric chloride test	++
	Lead acetate test	++
Sterols and Triterpenoids	Liebermann- Burchard test	-
	Salkowski test	-
Proteins	Xanthoproteic test	+
Acids	Sodium bicarbonate test	-

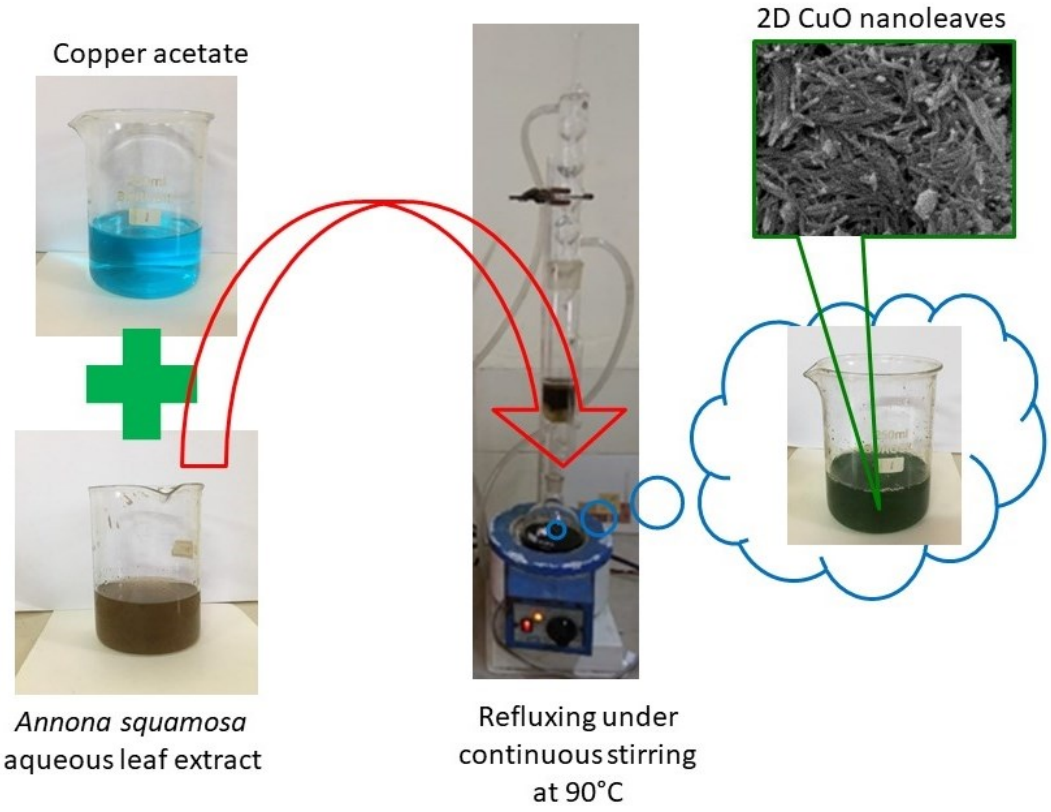


Fig. S1: Synthesis reaction procedure for 2D CuO nano leaves

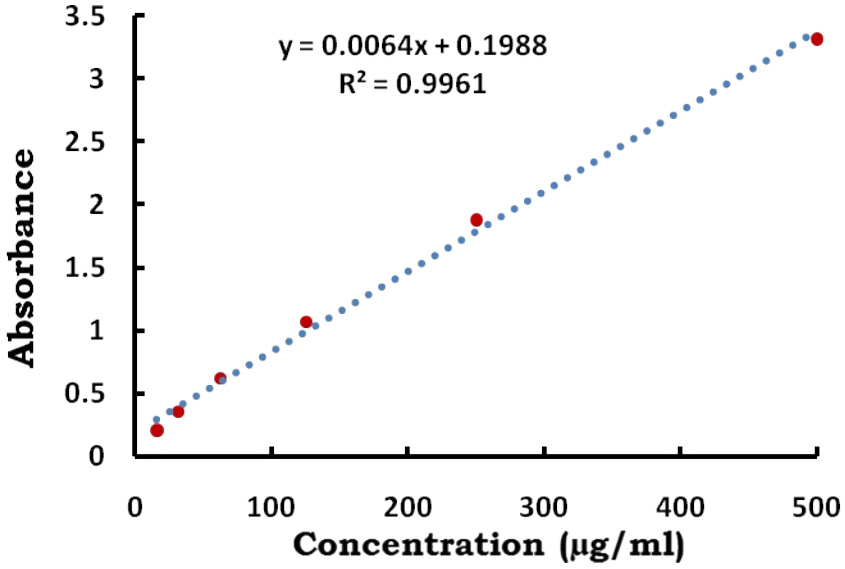
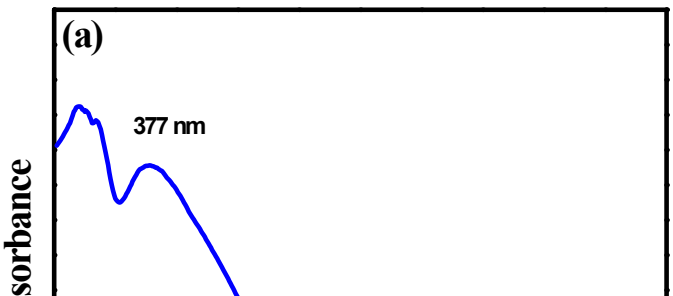


Fig. S2: Calibration curve of gallic acid



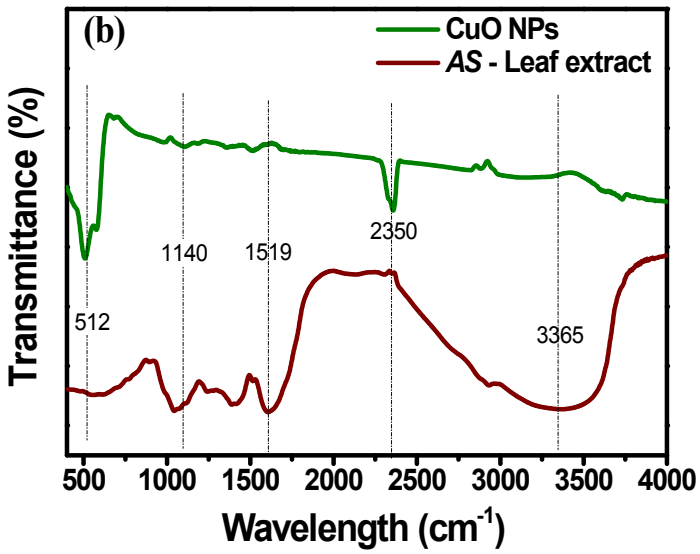


Fig. S3. (a) UV-Vis spectra of CuO nanoleaves under optimized conditions (b) FTIR spectra of CuO nanoleaves

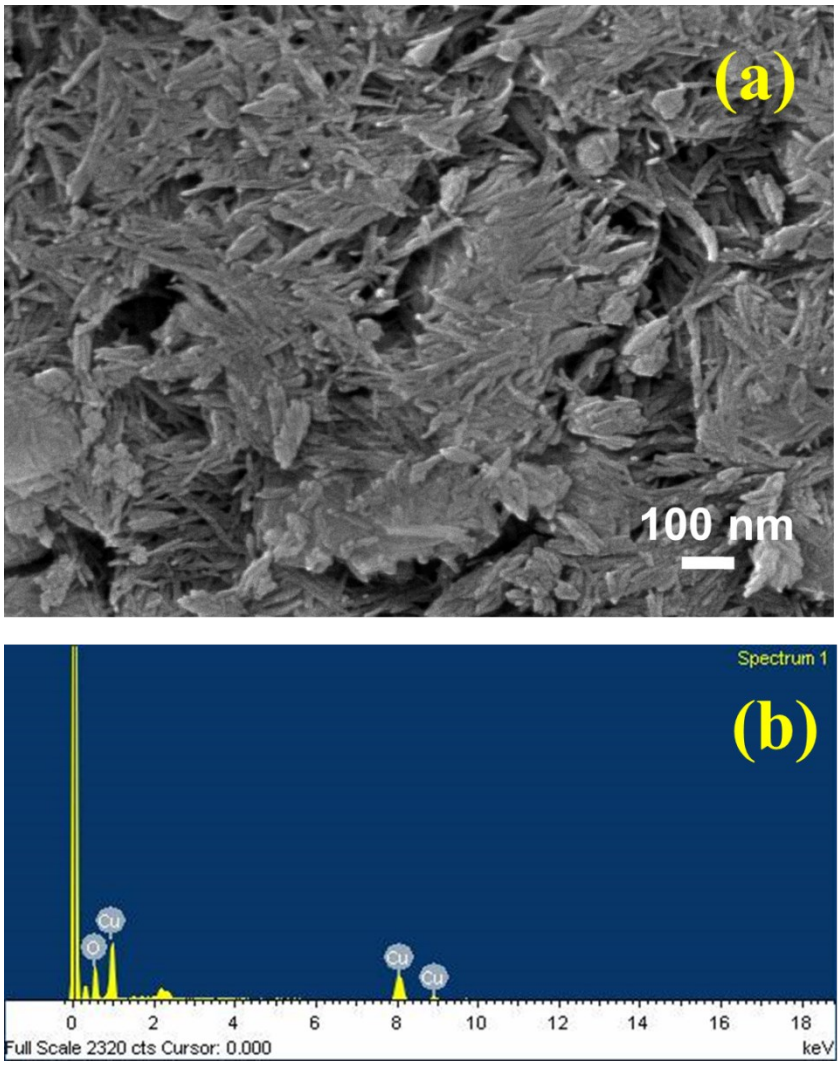


Fig. S4: SEM images, EDS analysis of CuO nanostructures

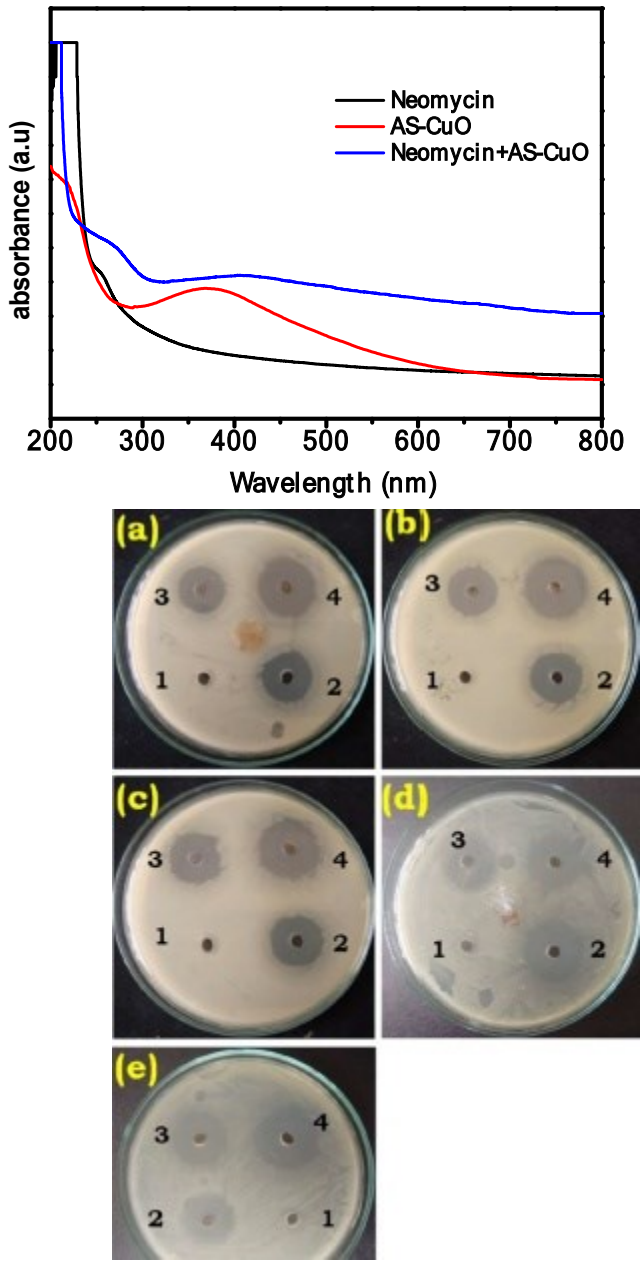


Fig. S5. (a) UV-Vis spectra of CD of neomycin with CuO NLs(b) Zone of inhibitions against (a) *E. coli*; (b) *B. subtilis*; (c) *S. aureus*; (d) *P. aeruginosa*; (e) *E. faecium* treated with (1) DMSO; (2) neomycin; (3) CuO NP; (4) CuONP+neomycin.