

Indian long pepper leaves-derived dual fluorescent carbon nano dots for environmental and health aspects

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Abstract

In this article, we present the synthesis of *Piper longum* leaves-derived ethanolic carbon dots (PLECDs) using the most simplistic environmentally friendly solvothermal carbonization method. The PLECDs fluoresced pink color with maximum emission at 670 nm at 397 nm excitation. Additionally, the dried PLECDs dissolved in water showed green fluorescence with higher emission at 452 nm at 370 nm excitation. The UV spectra showed peaks in the UV region (271.25 nm & 320.79 nm) and a noticeable tail in the visible region, signifying the efficient synthesis of nano-sized carbon particles and the Mie scattering effect. Various functional groups (-OH, -N-H, -C-H, -C = C, -C-N, and -C-O) were identified using Fourier-transform infrared spectroscopy (FTIR). Its nanocrystalline property was revealed by the sharp peaks in the X-ray diffraction (XRD). High-Resolution Transmission Electron Microscopy (HRTEM) photomicrograph displayed a roughly-spherical structure with a mean size of 2.835 nm. The Energy Dispersive X-ray (EDAX) spectra showed the presence of C, O, N, S, and P. The High-performance thin layer chromatography (HPTLC) fingerprint of PLECDs showed an altered pattern than its precursor (*Piper longum* leaves ethanolic extract or PLLEE). The PLECDs sensed Cu^{2+} selectively with a limit of detection (LOD) of 0.11 μM . It showed excellent cytotoxicity towards MDA-MB-231 (human breast cancer), SiHa (human cervical carcinoma), and B16F10 (murine melanoma) cell lines with excellent *in-vitro* bioimaging outcomes. It also has free radical scavenging activity. The PLECDs also showed outstanding bacterial biocompatibility, pH-dependent fluorescence stability, photostability, physicochemical stability, and thermal stability.

1. Introduction

In the last few decades, nano-scale materials have intrigued emerging fields of interest in multiple disciplines of science. Among them, the serendipitously discovered carbon nano dots (CNDs) or carbon dots (CDs) are multidisciplinary investigated ultra low-dimensional (zero) apparently-spherical fluorescent nanomaterials that gained immense popularity in recent years over traditional semiconductor fluorescent quantum dots (QDs) (Roy et al. 2015; Shen et al. 2016; Hu et al. 2017; Peng et al. 2017; Sciortino et al. 2018). QDs were previously used as fluorescent nanomaterial, composed of tellurium, cadmium, or selenium, which require multistep for synthesis, are high price, and highly toxic (Shahshahanipour et al. 2019). In contrast, CDs are novel nanomaterials having a dimension below 10 nm and possess properties such as excellent photoluminescence, tunable emission characteristic, excellent biocompatibility, chemically inertness, photobleaching resistant, nontoxic, safe, highly soluble, better physicochemical and photochemical stability, the possibility for surface functionalization with multiple functional groups, marked electron accepting and donating capabilities, facile and cost-effective one step synthetic potential, etc. (Roy et al. 2015; Jaleel et al. 2018; Shahshahanipour et al. 2019). CDs are mainly composed of C, H, and O; however, N, S, and B can be doped onto their surface to modify their fluorescent behaviors and physical properties (Jaleel et al. 2018). They possess amorphous or nanocrystalline cores with sp^2 carbon; however, some exceptions with sp^3 hybridized carbon are also noticed (Peng et al. 2017). CDs possess multiple properties, such as fluorescence, phosphorescence, electro-chemiluminescence (ECL), adsorption property, electrical properties, and biological properties (Namdari et al. 2017).

CDs are widely studied for bioimaging, cancer theranostic, drug delivery, diagnosing various pathological conditions, gene therapy, photodynamic therapy, sensing, optoelectronic devices, catalysis, and many biological activities (Roy et al. 2015; Jaleel et al. 2018; Sciortino et al. 2018; Mohapatra et al. 2022). They can be produced mainly by two different approaches; top-down and bottom-up approaches. In the top-down process, large particles are converted to nano-sized CDs via arc discharge, chemical oxidation, laser ablation, and electrochemical oxidation, whereas, in the bottom-up scheme, the CDs are formed from molecular dispersed material via hydrothermal, microwave-assisted

pyrolysis, ultrasonic methods (Roy et al. 2015; Peng et al. 2017; Jaleel et al. 2018; Sciortino et al. 2018). Among multiple synthetic methods, the solvothermal carbonization method is widely accepted due to its cost-effective, environmentally friendly, nontoxic, and highly efficient technique (Namdari et al. 2017; Wang et al. 2017).

The top-down approaches have disadvantages in participating in harsh conditions, necessitate expensive apparatus, and include various steps. Contrary to the above technique, the bottom-up technique-based solvothermal synthesis provides the benefits of low cost, simplicity, easy scale-up potential, eco-friendly, and use of varied available carbon precursors (Mohapatra et al. 2022). This method is widely explored for the preparation of CDs for multiple applications. A green chemistry strategy can be adopted to synthesize CDs using natural biomass as carbon precursor and eco-friendly food-grade solvents (Shahshahanipour et al. 2019). These cost-effective ecofriendly, highly biocompatible CDs do not require post-treatment for functionalization or surface passivation (Mehta et al. 2015; Jaleel et al. 2018). Natural substances are being recognized for their structural and functional miscellany and complexity (Shreya et al. 2022). Plant materials contain primary and secondary metabolites comprising C, H, O, and N that act as a precursor of CDs. Again, due to the accessibility of numerous chemical groups, the precursor itself acts as a doping agent to produce multifunctional CDs. CDs were prepared from multiple green carbon sources, such as stems and leaves of *Tinospora cordifolia* (family: Menispermaceae) (Mohapatra et al. 2021; Mohapatra et al. 2021), leaves of *Andrographis paniculata* (family: Acanthaceae) (Naik et al. 2020; Naik et al. 2020), essential oil of *Thymus vulgaris* (family: Lamiaceae) (Bayat et al. 2019), fruits of *Manilkara zapota* (family: Sapotaceae) (Bhamore et al. 2019), peels of *Citrus sinensis* (family: Rutaceae) (Gudimella et al. 2021), and many natural precursors (Naik et al. 2021). During hydrothermal carbonization under high temperature and pressure conditions, the natural biomass undergoes dehydration, decomposition, condensation, aromatization, and carbonization with subsequent in situ surface passivation (Vandarkuzhali et al. 2017; Shahshahanipour et al. 2019).

Indian long pepper (*Piper longum*, family: Piperaceae) is a familiar plant in the traditional Indian system of medicine. There are reports of using the entire plant as well as other parts of it to cure a variety of pathological illnesses, including diabetes, cancer, obesity, inflammation, depressive disorders, and hepatotoxicity. The leaves of the plant are traditionally used to treat a variety of conditions, including pancreatic lipase stimulation, digestive problems, eye problems, asthma, coughing, stopping milk secretion, avoiding infections, constipation, etc. (Manjusha et al. 2018). The existence of various phytoconstituents accounts for the leaves' varied pharmacology. Compounds such as β -Myrcene, bis(2-ethylhexyl) ester, 2,2-Dimethoxybutane, and 1,2-Benzenedicarboxylic acid are present in the leaves (Das et al. 2012). Compounds such as β -Pinene, limonene, α -pinene, linalool, caryophyllene, α -muurolene, γ -elemene, and δ -cadinol are present in the essential oil of leaves. (Varughese et al. 2016). The leaves are employed as an easily available, affordable natural precursor for the fabrication of CDs due to their broad chemical diversity.

Heavy metals are environmental pollutants, and their poisonousness is an issue of increasing concern for nutritional, ecological, evolutionary, and environmental reasons. They pose significant risks to health, with a variety of toxicities resulting in serious illnesses and even death (Duan et al. 2019). Through food, water, or air, a number of heavy metals may simultaneously enter the body. They don't easily break down in the body or be eliminated, which builds up quickly and cause death. Heavy metals like Pb, Hg, Sb, Cr, As, and Co are highly toxic heavy metals. Some heavy metals, including Fe, Zn, and Cu, are recognized as vital micronutrients for all living things. A certain amount of these heavy metals has a favorable effect on life activities. However, their absence or overabundance in the body causes the pathogenesis of numerous fatal illnesses. Being an essential component of living systems, copper performs critical physiological roles in a variety of biological events, and its excessive amounts may result in health problems (Ali et al. 2020). Too much accumulation of copper in the body can cause kidney problems, harm to the central nervous system, gastrointestinal distress, renal and hepatic failures. Further, there is some connection

between Cu^{2+} and several diseases like Alzheimer's, prion, and Wilson diseases (Ganiga et al. 2016; Ali et al. 2020). In the case of plants, Cu acts as a structural element in certain metalloproteins and a cofactor in many enzymes. It plays a vital role in signaling the transcription protein, iron mobilization, cell wall metabolism, biogenesis of molybdenum cofactor, and oxidative phosphorylation (Ganiga et al. 2016). Thus, plants need Cu for normal growth and development, and in case of its deficiency, plants develop certain deficiency symptoms. However, it has the capacity to induce oxidative damage and interfere with photosynthesis, plasma membrane permeability, pigment synthesis, and other metabolic activities, which inhibit plant development. Its excess cause toxicity, stunting, chlorosis, necrosis, and inhibit root and shoot growth (Ganiga et al. 2016). Moreover, both Cu deficiency and excess can cause disorders in plant growth and development. Hence, the tissue and cellular levels of Cu need to be within an optimal physiological range. To avoid negative effects on biological systems, it is crucial to efficiently and precisely measure its amount in environmental and biological specimens. Till date, various sophisticated chromatographic and spectroscopic techniques have been utilized for the qualitative and quantitative estimation of metal ions in various samples (Kailasa et al. 2019; Ali et al. 2020). Nevertheless, these methods require sophisticated instrumental setup, expensive reagents, and multistep sample preparation. Therefore, the development of easily available, cost-effective probes are required that can selectively sense and quantify Cu^{2+} . The CDs-based fluorescent nano probes have gained immense popularity for selective sensing many heavy metal ions, including Cu^{2+} ions (Ganiga et al. 2016; Ali et al. 2020).

The biggest issue that causes the development of cancer is the initial diagnosis of tumors. Fluorescence-based bioimaging is one of the successful approaches for cancer cell imaging and target sensing because of its great sensitivity and spatial resolution (Du et al. 2019). QDs, fluorescent organic dyes, and green fluorescent proteins (GFP) were formerly used as bioimaging probes. Long-term imaging is difficult with GFPs because of their poor photostability and weak fluorescence, which limits their broad application in biomedical domains. Most QDs are inappropriate for viable-cell bioimaging owing to their poor aqueous solubility and high intrinsic toxicity. (Peng et al. 2017; Li et al. 2020). Therefore, finding novel fluorescent probes that are easily accessible and biocompatible is important for *in-vivo* and *in-vitro* bioimaging. CDs are good alternatives as innovative fluorescent probes for cellular bioimaging (Peng et al. 2017). They are eco-friendly, cost-effective, biocompatible, less toxic, membrane permeable, and photostable (Boakye-Yiadom et al. 2019). Additionally, multicolor bioimaging is feasible by using the excitation-dependent emission characteristic of CDs (Shen et al. 2016). The utility of CDs for bioimaging against various cancer cells like breast cancer (MDA-MB-231 and MCF-7), melanoma (B16F10), cervical carcinoma (SiHa, HeLa), osteoblast (MC3T3-E1), glioma (C6), lung cancer (A549), etc. has been previously studied (Peng et al. 2017; Li et al. 2020; Mohapatra et al. 2021; Mohapatra et al. 2021).

Free radicals are extremely unstable and reactive molecules, playing a significant role in metabolism and aerobic life and are connected to several metabolic pathways. In biological systems, these free radicals have a dual role (i.e., they possess both positive and harmful effects) (Pham-Huy et al. 2008; Pavithra et al. 2015; Maddu 2019). They elicit positive effects on cellular responses and immune systems at low or intermediate amounts. In contrast, at high amounts, they produce oxidative stress (Pham-Huy et al. 2008). Oxidative stress induces ailments like autoimmune disorders, aging, ulcerative colitis, cancer, neurodegenerative diseases, arthritis, and cardiovascular diseases (Pham-Huy et al. 2008; Pavithra et al. 2015). Antioxidant substances scavenge the free radicals, which are excreted from the body and ultimately reduce the risk of pathological conditions (Pham-Huy et al. 2008). In this context, CDs from stems and leaves of *Tinospora cordifolia* (Mohapatra et al. 2021; Mohapatra et al. 2021), fruits of *Citrus sinensis* (Gudimella et al. 2021), leaves of *Andrographis paniculata* (Naik et al. 2020), *Glycine max* (Jia et al. 2019), have been well explored to be used as an antioxidant. In this study, we have synthesized and evaluated the dual

fluorescent PLECDs as a probe for heavy metal sensing, *in-vitro* cancer cell bioimaging, and free radical scavenging for environmental and health aspects.

2. Experimental

2.1. Materials

The leaves of Indian long pepper were obtained from a rural area of Odisha (Village: Jayachandrapur), and the voucher specimen (DM/PLL/PHYMEDLAB/2020) was placed in Phytomedicine Research Lab., Department of Pharmaceutical Engineering & Technology IIT (BHU), Varanasi. Quinine sulfate, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS.⁺), 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), DPPH (1, 1-diphenyl-2-picrylhydrazyl), ciprofloxacin, Vitamin C, 4',6-diamidino-2-phenylindole (DAPI), and potassium persulfate were obtained from Sigma-Aldrich, Germany. Rhodamine B was purchased from Otto chemie Pvt. Ltd. Ethanol was collected from Merck (Darmstadt, Germany). Silica gel 60 F₂₅₄ pre-coated thin layer chromatographic aluminum plates (20×20, 0.2 mm layers), ethyl acetate, and toluene were purchased from Merck. B16F10 (murine melanoma), SiHa (human cervical carcinoma), and MDA-MB-231 (breast cancer) cell lines were purchased from National Centre for Cell Science (NCCS), Pune, India. Dimethyl Sulfoxide (DMSO), fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM/F-12), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Mueller-Hinton agar (MHA) media were acquired from Himedia (Himedia Laboratories, Mumbai, India). The bacterial strains; *Klebsiella pneumoniae*, *Escherichia coli*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *Enterococcus faecalis*, and *Staphylococcus aureus* were kindly provided by the Department of Microbiology, Institute of Medical Science (IMS), Banaras Hindu University (BHU), Varanasi, India. The NH₄Cl, KCl, NaCl, CaCl₂, CuCl₂, MgCl₂, were purchased from Sisco Research Lab. Pvt. Ltd. (SRL, Mumbai, India); HgCl₂, and BaCl₂ were obtained from Fisher Scientific Pvt. Ltd. (Mumbai, India); NaAsO₂, Na₂HAsO₄ purchased from SD Fine Chemical Ltd. (Mumbai, India), AgNO₃ received from Merck (Darmstadt, Germany), and FeCl₃ purchased from Finar Chemicals Pvt. Ltd. (Gujarat, India). Ultrapure water (Milli-Q[®] Type 1, Merck, Darmstadt, Germany) was used in various experiments whenever needed.

2.2. Preparation of ethanolic extract and synthesis of carbon dots.

Piper longum leaves ethanolic extract (PLLEE) was made by soaking coarsely milled fresh leaves with ethanol at a ratio of leaves to solvent 1:3.5w/v. To facilitate the extraction, the mixture was placed on a shaker (REMI, RS 12 plus) for 24 hours. Then the ethanolic dispersion was strained, and the filtrate (PLLEE) was utilized for the preparation of *Piper longum* leaves ethanolic carbon dots (PLECDs). Briefly, the PLLEE (100mL) was poured into a 200mL solvothermal autoclave and heated at 160°C for 8 hours in a muffle furnace. The carbonized product was centrifuged at 12000 rpm for 15 minutes, filtered (0.2 µm syringe filter), and the resulting PLECDs was stored in a refrigerator protected from light until further use. Additionally, the PLECDs was evaporated in a vacuum oven to get dried PLECDs and redispersed in water to obtain aqueous PLECDs (A-PLECDs).

2.3. Instrumentation and characterizations

Absorption bands were obtained by UV-Visible spectrophotometer (Cary 60, Agilent, USA) with Cary Win UV software (version 5.1.3.1042). The shape, morphology, energy dispersive analysis of X-rays (EDAX), and selected area electron diffraction (SAED) patterns were investigated at 200kV by high-resolution transmission electron microscopy (HRTEM) (TECNAI G2 20 TWIN, FEI, USA) furnished with high angle annular dark field detector (HAADF) and Gatan's Digital Micrograph software (version 3.7.4). The sample was diluted twice with ethanol before being put onto a carbon-coated copper grid (Ted Pella, USA) and dried at ambient temperature. The HRTEM images were analyzed

and processed through ImageJ software (NIH, Bethesda, Maryland, version 1.53e, Java 1.8.0_172) to obtain the average size and size distribution. A fluorescence spectrophotometer (HORIBA, France) was used to investigate the fluorescence spectra with 5 nm slit width and FluorEssence™ software (version 3.5.1.2). A vacuum-dried sample was utilized for the determination of surface functionality by Fourier Transform Infrared Spectroscopy (Nicolet iS5, Thermo Fisher Scientific, USA) from 4000 to 500 cm⁻¹ wavelength at 4 cm⁻¹ spectral resolution using OMNIC™ spectrum software (Version 9.9.473). X-ray diffraction study was carried out by Rigaku Miniflex 600 desktop X-Ray diffractometer using MiniFlex Guidance software (Version 2.0.2.1) from 2° to 60° with 0.02°/step and a scan speed of 7°/min at 25°C. The sample was prepared on a clean microscopic glass slide (1.7×1.7 cm) via the drop-casting method by dropping a few drops of PLECDs on a clear glass plate (area of 2.89 cm²) and drying to produce a thin film. The interplane distance (d-spacing) was measured using Bragg's equation, and interatomic distance (a-spacing) was calculated utilizing the d-spacing value and Miller indices. The refractive index (RI) of the PLECDs was measured by a digital Abbe-type refractometer (Labman Scientific Instruments Pvt. Ltd., India) furnished with refractometer prisms, monocular system, tungsten lamp, telescopic adjustment system, and temperature controller at 25 ± 1°C. Briefly, the prisms of the refractometer were cleaned carefully, and a few drops of the undiluted PLECDs were spread uniformly on the surface of the lower illuminating prism, and RI was measured. The pH of prepared CDs was measured utilizing a pH meter (PC 700, Eutech, Singapore). High-performance thin layer chromatography (HPTLC) was performed by HPTLC system (CAMAG) equipped with a sample applicator (Linomat V), scanner (TLC Scanner IV), and WinCATS software (version 1.4.7.2018). The thermal stability of the CDs was evaluated by Thermogravimetric analysis (TGA) analysis using Shimadzu TGA-50 (Shimadzu, Japan), platinum pan, and TA-60WS Collection software (Version 2.21) in a nitrogen environment at 100mL/min flow rate at a temperature increment rate of 10.00°C/minute up to 800°C. Briefly, the PLECDs were dried in a vacuum oven (BTI-52, D. Haridas and Co., India) at 70°C, and the resulting dried PLECDs were used for TGA analysis. *In-vitro* bio imaging was carried out by a fluorescence microscope (EVOS Invitrogen fluorescence microscope, Life technologies). The absorbance measurement in the cytotoxicity study was performed by a microplate reader (Bio-Rad Laboratories, Germany).

2.4. Quantum yield (QY)

Rhodamine B (Rh-B) and quinine sulfate (QS) were used as standard reference fluorescent chemicals for the relative QY measurement. The QY of PLECDs was estimated using rhodamine B (having QY of 89% in ethanol), and that of A-PLECDs was estimated using quinine sulfate in 0.1 M of H₂SO₄ (having QY of 54%) utilizing Eq. 1 (Mehta et al. 2014; Gedda et al. 2016; Han et al. 2018).

$$QY_{PLECDs \text{ or } A-PLECDs} = Q_R \frac{I_{PLECDs \text{ or } A-PLECDs}}{I_{Rh \text{ or } QS}} \times \frac{A_{Rh \text{ or } QS}}{A_{PLECDs \text{ or } A-PLECDs}} \times \frac{\eta^2_{PLECDs \text{ or } A-PLECDs}}{\eta^2_R} \quad (1)$$

where, 'QY_{PLECDs or A-PLECDs}' is the Quantum yield of synthesized PLECDs or A-PLECDs, subscript "PLECDs or A-PLECDs" represents the carbon dots. The subscript "Rh-B" and "QS" represents Rh-B and QS, respectively. "I" is the intensity of fluorescence, "A" is the absorbance at maximum excited wavelength, and "η" refers to the RI of the solvent (1.33 for water or 0.1M H₂SO₄ and 1.36 for ethanol). The QYs were calculated at the maximum excitation wavelength of 397 (for PLECDs) and 370 nm (for A-PLECDs).

2.5. High-performance thin-layer chromatography (HPTLC) fingerprinting

The HPTLC finger printing of PLLEE and PLECDs was carried out to check the chemical variation among them using CAMAG HPTLC instrument (Switzerland) associated with WinCATS software (version: 1.4.7.2018). Fingerprinting was performed on silica gel 60 F254 pre-coated TLC plates (Size: 10×10 cm, E. Merck, Darmstadt, Germany). Each sample (5µL) was applied as the band (bandwidth 8mm) in triplicate in a 100 µL CAMAG syringe using a Linomat V applicator (CAMAG, Switzerland). A triplicate band of standards PLLEE and PLECDs were applied on the pre-coated aluminum plate. Multiple mobile phases were tried to resolve the chemical groups into distinguished bands, and a combination of toluene: ethyl acetate (60:40% v/v) was selected for the development of the plate due to their satisfactory resolving capacity. After the application of the sample, the plate was developed in a mobile phase pre-saturated developing glass chamber (twin trough, CAMAG, 10 x 10 cm) up to 80 mm height using mobile phase (10 ml). Then the plate was dried with a hair drier to remove the mobile phase and scanned by HPTLC scanner (CAMAG TLC Scanner IV, slit dimension: 6.00 × 0.45 mm micro) at a scanning speed of 20 mm/s. The fingerprint of the developed plate was visualized in CAMAG TLC Visualizer 2, and images were captured by the equipped digital charge-coupled device (CCD) camera (SONY, Super HAD, HDR) under 254 nm (short UV), 366 nm (long UV), and white light.

2.1. Metal Ions Sensing Study

The metal ion sensing potential of the PLECDs was examined as per the previously reported method with few modifications (Venkatesan et al. 2019). Aqueous solutions of various metallic cations (Pb^{2+} , Cu^{2+} , Hg^{2+} , Fe^{3+} , Mg^{2+} , Ba^{2+} , Ag^{3+} , As^{3+} , As^{5+} , and NH_4^+) were prepared from their respective salts. In order to study the selectivity of PLECDs for sensing different metal ions, accurately 2 mL of various metal ion solutions was incubated with an equal volume of PLECDs (concentration: 0.541 mg/mL). The final metal ion concentration of various metals in the total 4 mL of the final volume was kept at 250µM. Then the metal ion-PLECDs solution mixture was kept for 15 minutes at 25°C and observed for the changes in photoluminescence intensity at 397 nm.

The sensitivity of PLECDs to a particular metal ion was estimated using the fluorescence titration method (Edison et al. 2016). Accurately 2mL of different concentrations of selected metal cation was added to an equal volume of PLECDs solution (concentration: 0.541 mg/mL) to produce final concentrations of metal from 10 to 250µM and incubated for 15 minutes to determine the changes in the fluorescent intensities. By utilizing the Stern-Volmer equation (Eq. 2), the quenching effectiveness of PLECDs was calculated (Pal et al. 2018).

$$\frac{F_a}{F_p} = 1 + K_{sv} [Q] \quad (2)$$

Where, F_a and F_p are the emission intensities of PLECDs without and with the selected metal ions, respectively, K_{sv} is the Stern–Volmer quenching constant, and $[Q]$ is the metal ion concentration.

The lower limit of detection (LOD) was found using Eq. 3.

$$LOD = \frac{3\sigma}{s}$$

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Where, 'σ' is the standard deviation (SD) of the y-intercept (F_a/F_p values), and 's' is the slope of the linear plot (Edison et al. 2016).

2.2. MTT assay and cellular imaging study

MTT assay was carried out on breast cancer (MDA-MB-231), cervical cancer (SiHa), and melanoma (B16F10) cell lines as per our previously reported methods with some modifications (Mohapatra et al. 2021; Mohapatra et al. 2021; Mohapatra et al. 2022). The cell lines at a density of 1×10^6 /well were seeded in a polypropylene-based 96 microtiter plate in DMEM/F-12 culture media with fetal bovine serum (FBS, 10%) solution, penicillin-streptomycin solution (50 unit/mL), and incubated with 5% CO₂, 95% air at 37°C for 24 hours in a humidified chamber. The cells are allowed for adherence and grow up to 70% of confluency before the treatments with samples. To avoid the cytotoxicity effect of ethanol medium in the PLLEE sample towards the used cell lines, the PLLEE was evaporated by vacuum drying and reconstituted with media (containing 0.1% DMSO to solubilize the samples). In the case of PLECDs, the ethanol media was removed by vacuum drying, and the dried PLECDs was solubilized in ultrapure water (the product is the same as A-PLECDs) and treated against cell lines. The media was discarded, and the adhered cells were washed with PBS (phosphate buffer saline, pH 7.4) and further incubated with various concentrations (0-300 µg/mL) of sample solutions for 24 hours. After incubation, the media was withdrawn carefully, followed by incubation with fresh media containing 200 µL of MTT reagent (5 mg/mL) and allowed to stay for 4 hours. The DMSO was added to solubilize formazan crystals, and the absorbance was recorded by a microplate reader at 570 nm. The cell viability percentage was calculated by using Eq. 4.

$$cell\,viability(\%) = \frac{Absorbance\,of\,test}{Absorbance\,of\,control} \times 100 \quad (4)$$

The *in-vitro* bioimaging potential of PLECDs (PLECDs reconstituted with ultrapure water) was explored by fluorescence microscope. Initially, 1×10^5 cells/well were cultured in the media overnight in a six-well plate and incubated in a CO₂ incubator at 37°C. After incubation, the media was substituted by fresh media containing PLECDs (25–75µg/mL) and incubated for 24 hours. After the achievement of sufficient staining, the media was discarded, and cells were washed twice with PBS, and fixed with 4% paraformaldehyde, followed by nuclear counterstaining by DAPI. The bioimaging was achieved by an EVOS® FL cell inverted fluorescent microscope (Thermo Fisher Scientific, USA) at 20X magnification.

2.3. Scavenging of free radical

The scavenging potential of PLLEE and PLECDs to the free radicals was estimated by DPPH and ABTS.⁺ assay.

2.3.1. DPPH assay

In this experiment, one ml of test solution (PLLEE & PLECDs) of various concentrations was incubated with 2ml of fresh DPPH ethanolic solution (100 µM) for 30 minutes in a dark environment. An aqueous solution of Vitamin C was utilized as a standard radical scavenger (antioxidant), and the assay was executed in a similar manner as that of the test solution. The decrease in the absorbance at 517nm was determined by absorption spectroscopy. The percentage scavenging of DPPH was calculated by Eq. 5.

$$\%Scavenging_{DPPH} = \frac{A_{DPPH} - A_{DPPH+S}}{A_{DPPH}} \times 100 \quad (5)$$

where, where A_{DPPH} and A_{DPPH+S} are the absorbance of DPPH without any sample (control) and with sample or standard, respectively. Plots of percentage DPPH scavenging v/s concentration of samples or standard (in µg/mL)

were made to calculate the concentration of samples or standard required to cause 50% of scavenging (IC₅₀).

2.3.2. ABTS.⁺ radical scavenging

The ABTS.⁺ radical is not exist naturally. So it was prepared by mixing 7 mM ABTS aqueous solution with 2.45 mM potassium persulfate aqueous solution at 1:1 v/v and letting it stay for 16 hours away from light until the completion of the reaction. By examining the stability in the absorption intensity (by UV spectroscopy), the end point of the reaction was confirmed. Further, the ABTS.⁺ solution was diluted in such a way as to achieve the absorbance of 0.740 ± 0.036 at a wavelength of 734 nm. Precisely 3 mL of diluted aqueous solution ABTS.⁺ was mixed with 1mL of different concentrations of samples (PLLEE and PLECDs). The samples with the ABTS.⁺ solution was placed in a dark environment for 5 minutes, and the absorption spectra were recorded at 734nm. As a positive control, Trolox was utilized, and the experiment was conducted concurrently and similarly. The percentage scavenging of ABTS.⁺ was calculated by Eq. 6.

$$\%Scavenging_{ABTS^+} = \frac{A_{ABTS^+} - A_{ABTS^++S}}{A_{ABTS^+}} \times 100 \quad (6)$$

where, A_{ABTS^+} and A_{ABTS^++S} are the absorbances of ABTS.⁺ without any sample (control) and with sample or standard, respectively. The IC₅₀ for scavenging of ABTS.⁺ radicals was estimated from the plots of percentage radical scavenging v/s concentration of analytes.

2.4. Microbial biocompatibility study

The disc diffusion method was used to evaluate bacterial biocompatibility (Shahshahanipour et al. 2019). Various Multi-Drug Resistance (MDR) type pathogenic Gram-negative (*Klebsiella pneumoniae*, *Aeromonas hydrophila*, *Escherichia coli*, *Edwardsiella tarda*), and Gram-positive strains (*Enterococcus faecalis* and *Staphylococcus aureus*) were used in the study. The microbial suspension (0.5 McFarland) was dispersed in NaCl solution (isotonic), cultured over MHA media by spread plate technique, and incubated at a constant temperature (37°C) in an incubator (Orbital Shaking Incubator, REMI, Mumbai, India) for 30 minutes. The vacuum-dried samples of PLEE and PLECDs were used for the study by diluting with DMSO. Six-millimeter diameter sterile filter discs were suitably placed on the surface of cultured microorganisms, and 10µl of each sample (PLLEE and PLECDs) at a concentration of 14.08 mg/mL was dropped on the discs. A solution of ciprofloxacin (2 mg/mL) in DMSO was used as a +ve standard. Additionally, a sterile filter paper disc soaked with DMSO was considered as a -ve control. The bacterial biocompatibility of the tested samples was estimated by measuring the zone of inhibition.

2.5. Stability of PLECDs

Stability was checked at 3 sets of storage conditions: storing the PLECDs for a prolonged time (9 months) in a refrigerator, after 3cycles of a heating-cooling cycle (One cycle: 40°C for 24 hours and 4°C for 24 hours), and after 3cycles of freeze-thaw (One cycle: -20°C for 24 hours and 25°C for 24 hours). Various stability-indicating parameters, such as a change in physical properties (appearance, color, homogeneity, agglomeration, sedimentation, precipitation, pH, percent transmittance (at 600nm), and refractive index (RI)), chemical integrity (FTIR), optical properties (UV visible absorption & fluorescence behavior) were accessed at the end of the study. Further, the PLECDs was also evaluated for their photostability (UV irradiation at 254nm up to 3 hours), pH-dependent fluorescence stability (exposing PLECDs with an aqueous solution of different pH made by different proportions of NaOH or 0.1N HCl) and thermal stability (by TGA analysis).

3. Result And Discussion

3.1. Synthesis of PLECDs

A green synthetic pathway was followed to prepare the PLECDs via a solvothermal technique using ethanol as an eco-friendly nontoxic solvent and fresh leaves of long pepper as a carbon source. The solvothermal carbonization method was selected due to its simple, cost-effective, nontoxic, environmentally friendly, and highly efficient technique (Namdari et al. 2017; Wang et al. 2017). During continuous exposure of ethanolic extract (PLLEE) to constant temperature and pressure, the material undergoes decomposition, condensation, aromatization, and carbonization with simultaneous passivation to produce dark brown-colored PLECDs. During solvothermal carbonization, the aromatic centers with photoluminescence carbon cores are formed, and their PL property depends on hybridization and collaboration between the functional groups and the carbon core (Zhu et al. 2015). The synthetic procedure of PLECDs and their investigated applications is schematically presented in Fig. 1. Long pepper leaves are highly abundant in C, O, and N atoms, acting as a precursor for the fabrication of PLECDs. Multiple phytochemicals and their functional groups in ethanolic extract (PLLEE) serve as passivating agents for the production of carbon dots. Consequently, we have prepared CDs (PLECDs) from PLLEE utilizing one-step solvothermal carbonization technique. The production of dark brown colored liquid after 8 hours of solvothermal digestion signifies the effective synthesis of PLECDs which was further confirmed by emission of pink luminescence at 365 nm. Also, the reconstituted PLECDs in water (i.e., A-PLECDs) was found to be green fluorescence in nature.

3.2. Physicochemical properties

3.2.1. Organoleptic evaluations

The prepared PLECDs was found to be dark brown colored homogeneous liquid (Fig. 2a) with a typical burnt smell with no sign of agglomeration, sedimentation, or precipitation.

3.2.2. pH, percent transmittance (%T), and Refractive index (RI)

To avoid the concentration quenching, the PLECDs was diluted suitably PLECDs (60 μ L of PLECDs with 3mL of ethanol or water that provides the highest fluorescent intensity) with ethanol or water. The pH of the undiluted and diluted PLECDs was found to be 5.23 ± 0.043 and 4.06 ± 0.053 , respectively. The %T of undiluted PLECDs assessed by UV-vis spectroscopy at 600 nm was found to be $0.954 \pm 0.005\%$, whereas, for diluted PLECDs, it was found to be $91.532 \pm 0.005\%$. The % transmittance represents the optical clarity of the liquid materials. The low % T of the undiluted PLECDs is due to their high concentration (Fig. 2a), whereas the diluted PLECDs (Fig. 2a) represent the transparency of the product. The RI of the undiluted and diluted PLECDs were found to be 1.361 ± 0.006 and 1.335, respectively. The RI of diluted PLECDs was found to be close to the RI of water (1.333), signifying the transparency of the diluted PLECDs. Further, the RI of undiluted PLECDs was found to be uniform throughout the liquid medium (samples taken from various parts of PLECDs solution), representing its isotropic & homogeneous nature.

3.2.3. Fluorescence Spectroscopy

CDs possess wide emission spectra with greater Stokes shifts than that organic dyes. They possess wide photoluminescence (PL) bandwidth in comparison to quantum dots (QDs), which is due to the existence of random chemical structures and diverse PL centers (Zhu et al. 2015). To investigate the fluorescent property of ethanolic PLECDs, they were exposed to multiple excitation wavelengths to generate fluorescent spectra (Fig. 2b). Initially, a

regular rise in the emission intensity (at 670 nm) was detected from 320 nm to 397 nm excitation without shifting emission peak; afterward continuous decrease in the intensity and the hypsochromic shift was noticed between 400 nm to 440 nm. The PLECDs showed maximum PL intensity (154050.509 a.u.) at 670 nm at 397 nm excitation (Fig. 2c). Additionally, at various PLECDs concentrations, the fluorescence performance was investigated. A regular increase in fluorescence intensity was noticed between 93.24 to 276.07 $\mu\text{g/mL}$ (Fig. 2d), and concentration quenching was observed at 365.71 $\mu\text{g/mL}$.

Similarly, the PLECDs diluted in water (A-PLECDs) showed a continuous rise in the fluorescent intensity between excitation of 310 nm to 370 nm; afterward, a continuous decrease in the intensity and a bathochromic shift was noticed between 370 nm to 430 nm excitation (Fig. 2e). The A-PLECDs shows maximum PL intensity (53820.034 a.u.) at 452 nm at 370 nm excitation (Fig. 2f). Such excitation dependent emission behavior also previously found by various research groups (Mehta et al. 2014; Edison et al. 2016; Jhonsi et al. 2016; Shahshahanipour et al. 2019; Mohapatra et al. 2021; Mohapatra et al. 2021; Mohapatra et al. 2022). This property is attributed to the distribution of differently sized particles (quantum effect), salvation effects (presence of multiple emissive traps), their surface functionality, radiative recombination of excitons, and the presence of free zigzag sites (Mehta et al. 2014; Zhu et al. 2015). This type of typical behavior makes them suitable for application as a fluorescent probe for multicolor imaging (Namdari et al. 2017). The wide emission spectrum of PLECDs displays their probable *in-vitro* and *in-vivo* imaging applications. A continuous increase in emission intensity was noticed between 93.24 to 276.07 $\mu\text{g/mL}$ (Fig. 2g); afterward, the concentration quenching was observed at 365.71 $\mu\text{g/mL}$. The relative QY of PLECDs with respect to Rh-B was found to be 17.095%, whereas the relative QY of water-dissolved PLECD (A-PLECDs) with respect to QS was found to be 5.09%. The presence of strong fluorescent centers, a synergistic association between core carbon and surface functional groups, or the existence of fluorophores may be the reason behind the fluorescent behavior of the PLECDs and A-PLECDs.

3.2.4. UV- Visible Spectroscopy

The optical properties of PLECDs are represented by UV-Visible spectra (Fig. 2h) as complementary to fluorescent spectra. Most of the CDs possess common optical properties in terms of absorption spectra. They express strong absorption spectra within the UV range, with the tail outspreading toward the visible range (Zhu et al. 2015). The absorption spectra of PLECDs (Fig. 2h) and A-PLECDs (inset Fig. 2h) displayed an obvious tail in the visible region, signifying the efficient synthesis of nano-sized carbon particles and the Mie scattering effect (Shahshahanipour et al. 2019). The existence of strong absorption bands at 271.25 nm is attributed to the transition of $\pi-\pi^*$ of aromatic C = C bonds, and a very low with broad absorbance at 320.79 nm extends towards the visible region is due to the $n-\pi^*$ transition of C = O bonds (Mohapatra et al. 2021).

3.2.5. FTIR

The vibration spectrum of PLECDs (Fig. 2i) showed a strong and broad absorption band at 3425.44 cm^{-1} due to the phenolic or alcoholic -OH stretching. The characteristic peaks at 2932.28 & 2858.869 cm^{-1} are ascribed to -CH stretching of alkanes. The band at 1642.72 cm^{-1} is due to -C = C- stretching of alkenes or N-H bending of primary amines. Peak at 1385.772 cm^{-1} is assigned to -CH in-plane bending of alkene. A very small peak at 1252.51 cm^{-1} is ascribed to the C-N stretching of aliphatic amines. The sharp band at 1072.16 is due to the C-N stretching of aliphatic amine or C-O stretching of esters, carboxylic acids, alcohols, or ethers.

3.2.6. XRD

The results of XRD shown in Fig. 2j shows sharp, intense peaks at $2\theta = 28.021^\circ$ attributed to the characteristic diffraction of extract (PLLEE) (inset Fig. 2j). The characteristic diffraction at 41.83° assigned to C (100) plane of graphitic carbon, weak diffractions at $2\theta = 50.453^\circ$ and 59.156° are assigned to C (102) and C (103) planes of graphitic C, respectively (Atchudan et al. 2015). The sharp diffraction peaks reflect the nanocrystalline nature of the synthesized PLECDs. The d-spacing (inter-plane distance) was calculated by Bragg's equation Eq. 7 (Atchudan et al. 2016).

$$d = \frac{n\lambda}{2\sin\theta}$$

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where, "d" is the inter plane distance, "n" is a +ve integer (1), "λ" is the incident X-rays wavelength ($\lambda = 1.54 \text{ \AA}$) and θ is the angle between the surface of the sample and incident rays.

The calculated d-value of PLECDs is approximately 0.216 nm for C (100), 0.18 nm for C (102), and 0.156 nm for C (103) graphitic plane. The inter-atomic distance (a) can be calculated from Eq. 8.

$$\frac{a}{\sqrt{h^2 + k^2 + l^2}} = d_{(h,k,l)} \quad (8)$$

Where, "a" is the inter atomic distance, h, k, and l are the Miller indices, and "d" is the inter plane distance.

The estimated values of "a" were found to be 0.216 nm for C (100), 0.402 nm for C (102), and 0.493 nm for C (103) graphitic plane.

3.2.7. Morphology and EDAX

The morphology of PLECDs examined by HRTEM at 100 nm and 20 nm scales were presented in Fig. 3a and Fig. 3b, respectively. The results indicate the quasi-spherical morphology of PLECDs within the range of 2 nm to 4 nm (inset figure Fig. 3a) and an average size of 2.835 nm. The elemental abundance and composition of PLECDs were investigated by EDAX analysis (Shahshahanipour et al. 2019). The result exposed the abundance of C, O, N, S, and P in the PLECDs (Fig. 3c). The presence of Cu is due to the Cu grid used for HRTEM analysis.

3.2.8. HPTLC fingerprinting

The results of HPTLC fingerprints under 254nm, visible light, and 366nm were shown in Fig. 3d. The mobile phase (toluene and ethyl acetate-60:40% v/v) produced the best results out of all the mobile phases tested. The appearance of well-differentiated bands in the HPTLC fingerprint represents the existence of various groups of phytoconstituent. The HPTLC fingerprint of PLECDs is found to be altered from the PLLEE, which might be due to the modification of the chemical profile during the solvothermal carbonization. However, some of the bands of PLLEE are still present in PLECDs, reflecting the preservation of chemical integrity.

3.3. Metal ion sensing property

Various analytes such as cations, anions, macromolecules, bacteria, and cells have been widely explored to be sensed by CDs (Sun et al. 2017). Among them, the metal ion sensing of CDs is an interesting field of research. The sensing of metal ions can be ascertained by analyzing the quenching of the emission intensity upon incubation of metal ions. Metal ion sensing property of CDs has been reported previously (Gong et al. 2015; Bandi et al. 2016;

Huang et al. 2017; Pal et al. 2018; Mohapatra et al. 2021; Mohapatra et al. 2021). Various mechanisms involved in fluorescent quenching during metal ion addition include aggregation quenching, energy/electron transfer quenching, and dynamic quenching (Dong et al. 2015). In the aggregation mechanism, the quenching occurs due to the aggregation of CDs; in the electron transfer mechanism, the quenching is ascribed to metal–fluorophore complexation with the transform of an electron from excited CDs to the metal ion; in dynamic quenching, excited CDs go back to the ground state owing to a collision between CDs and metal ion (Dong et al. 2015; Molaei 2020).

To determine the selectivity of PLECDs towards metal cations (Pb^{2+} , Cu^{2+} , Hg^{2+} , Fe^{3+} , Mg^{2+} , Ba^{2+} , Ag^{3+} , As^{3+} , As^{5+} , and NH_4^+), the fluorescent intensity was investigated by observing emission peaks of PLECDs at 466 & 670 nm (Fig. 4a). The peak at 466 nm almost remains constant with different metal ions; however, the quenching of the second peak at 670 nm was detected. The Pb^{2+} , Fe^{3+} , Mg^{2+} , Ag^{3+} , As^{3+} , and As^{5+} showed slight quenching, whereas Cu^{2+} caused the strongest quenching (inset Fig. 4a), and this is exploited for selective Cu^{2+} ion sensing application.

Cu^{2+} ion is a vital trace constituent in the human body that is involved in various physiological events, such as metabolism regulation, oxidation, and protein activation (Huang et al. 2019). Small consumption of Cu^{2+} is beneficial for health, but consumption of too much can cause toxicity and develop multiple diseases (Huang et al. 2019). An excess amount of copper in the body leads to renal disorders and negatively affects the central nervous system (Ganiga et al. 2016). Additionally, Cu^{2+} and molecular oxygen can interact to form reactive oxygen species (ROS), that worsen nucleic acids, lipids, and proteins. High levels of Cu^{2+} can produce gastrointestinal distress within a short period of time, and prolonged exposure can result in renal and liver failure. Further, there is a link between Cu^{2+} and diseases such as Alzheimer's, and Wilson diseases (Ali et al. 2020). The Cu^{2+} serves as a structural component in a few metalloproteins in plants, some of which are involved in electron transport in mitochondria and chloroplasts as well as in plant defense mechanisms against oxidative stress. Numerous enzymes, including cytochrome c oxidase, ascorbate oxidase, Cu/Zn-superoxide dismutase, laccase, amino oxidase, plastocyanin, and polyphenol oxidase, use Cu^{2+} as a cofactor. It is essential for signaling the transcription protein, cell wall metabolism, iron mobilization, the biosynthesis of the molybdenum cofactor, and oxidative phosphorylation at the cellular level (Ganiga et al. 2016). Thus, plants need copper for healthy growth and development, and when they don't get enough of it, they show several indications of a deficit, the majority of which affect young leaves and reproductive organs. However, it has the potential to cause oxidative damage and interfere with key cellular functions like photosynthesis, plasma membrane permeability, pigment synthesis, and other metabolic processes, which impede plant growth. Its excess results in toxicity, chlorosis, stunting, necrosis, and root and shoot-growth inhibition. An excessive Cu buildup at the cellular level might inactivate and disrupt protein structure (Ganiga et al. 2016).

Moreover, both Cu excess and shortage can lead to abnormalities in plant growth and development. Therefore, Cu^{2+} levels in tissues and cells must be within a desirable physiological range. To minimize the undesirable effects of Cu^{2+} on biological systems, it is crucial to efficiently and precisely quantify its amount in environmental and biological specimens. A number of analytical tools, including inductively coupled plasma mass spectrometry, atomic absorption spectrometry, and electrochemical methods. Although the analysis through the above methods is highly accurate, precise, and sensitive, they use costly equipments, need complicated sample preparations, have high operating prices, and involve multistep analytical events. It is, therefore, necessary to develop easy, highly selective, sensitive, and low-cost tools for detecting Cu^{2+} ions. Fluorescence-based sensors are highly sensitive, selective, and easier to operate compared with other electrochemical or spectroscopic sensors. Various CDs were already exploited for sensing Cu^{2+} ions previously (Ganiga et al. 2016; Huang et al. 2019; Ali et al. 2020).

A fluorescent titration assay was carried out to investigate the sensitivity of green synthesized PLECDs towards Cu^{2+} . An inverse relationship found between fluorescence property and concentration of Cu^{2+} (Fig. 4b & Fig. 4c). The linearity exists within the concentration range of 40–70 μM with regression equation $y = 23402.611 - 164.25x$ & a correlation coefficient of 0.999 (inset Fig. 4c), representing stronger sensitivity towards Cu^{2+} ions. The quenching efficiency of PLECDs was derived from the Stern-Volmer relationship by plotting the value of relative fluorescence intensities (F_a/F_p) against the concentration of Cu^{2+} ions (40–70 μM) (Fig. 4d) (Pal et al. 2018). A correlation coefficient of 0.998 and y-intercept 4.626 with linear equation $F_0/F = 0.111x + 4.626$ was estimated from the plot. From the above equation, the obtained K_{sv} or slope was 0.111, and the LOD was 0.11 μM . The above results describe the potential of green synthesized PLECDs as a sensor for Cu^{2+} sensing.

Because of their outstanding sensitivity, low cost, and faster fluorescence-based sensors, they have gained a lot of interest recently. (Sun et al. 2017). Numerous mechanisms, including photo-induced charge transfer (PCT), inner filter effect (IFE), photo-induced electron transfer (PET), and Forster resonance energy transfer (FRET) govern the fluorescence changes of CDs utilized for sensing applications. The fluorescence quenching phenomena of PLECDs by Cu^{2+} might be due to the nonradiative transfer of an electron from PLECDs to the vacant d-orbitals of Cu^{2+} (Ganiga et al. 2016). Ligand-to-metal charge transfer transitions are frequently seen in metals with higher oxidation states and ligands with filled orbitals. Such a mechanism is well documented in semiconductor-based QDs (Ganiga et al. 2016).

3.4. *In-vitro* cytotoxicity and bioimaging

The *in-vitro* cytotoxicity of PLLEE and PLECDs was investigated through MTT assay on B16F10, SiHa, and MDA-MB-231 cell lines. The % viability of B16F10 cells was plotted against the log of concentrations of PLLEE or PLECDs to find IC_{50} values through non-linear regression analysis (Fig. 5a & Fig. 5b). The IC_{50} values of PLLEE and PLECDs against B16F10 cell line were obtained to be 57.49 $\mu\text{g/mL}$ and 46.21 $\mu\text{g/mL}$, respectively. The comparative results of the percentage cell viability v/s concentration graph (Fig. 5c) demonstrated the high cytotoxic potential of PLECDs compared to PLLEE. The nanostructure of PLECDs, which allows for better penetration into the cells than PLLEE, may be the reason for their slightly greater cytotoxic potential. Our team previously reported a similar outcome (Mohapatra et al. 2022).

The PLECDs were examined for *in-vitro* cellular imaging because of their good fluorescence behavior and adequate water solubility (after drying and dissolving in water). It showed green fluorescence in B16F10 cell lines (Fig. 5d). In contrast, the cells without PLECDs as negative control did not display any fluorescence under the same UV excitation. The fluorescence behavior of PLECDs was found to be increased from 25–75 $\mu\text{g/mL}$ in a dose-dependent manner. Nuclear counterstaining with DAPI showed that the green-synthesized PLECDs (25 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$) were successfully internalized in the malignant cells to the cytoplasmic area (green fluorescence). However, a further increase in PLECDs concentration (75 $\mu\text{g/mL}$) displayed complete fluorescence with a cytotoxic effect. The binding sites at the cytoplasmic area may become saturated at larger concentrations (75 $\mu\text{g/mL}$), which would cause the PLECDs to build up in the cytoplasm and amass around the nucleus. This result is in agreement with our earlier reports on the staining of cytoplasm and nuclei of the B16F10 cell line by carbon dots from stems of *Tinospora cordifolia* (TCSCD) at their higher concentration (Mohapatra et al. 2021).

The obtained IC_{50} values of PLLEE and PLECDs against SiHa cell line were 57.1 $\mu\text{g/mL}$ and 36.95 $\mu\text{g/mL}$, respectively (Fig. 6a & Fig. 6b). The comparative results of percentage SiHa cell viability v/s concentration graph (Fig. 6c) confirmed the high cytotoxic potential of PLECDs compared to PLLEE, which might be due to its nano

structure and better up taking compared to neat PLLEE. The SiHa cell line examined with the PLECDs exhibited dose-dependent bioimaging results (Fig. 6d). Similarly, the calculated IC_{50} values of PLLEE and PLECDs against MDA-MB-231 cell line were 52.66 $\mu\text{g/mL}$ and 45.31 $\mu\text{g/mL}$, respectively (Fig. 7a & Fig. 7b). The comparative percentage cell viability v/s various concentration of PLLEE and PLECDs was shown in Fig. 7c. Figure 7d showed dose-dependent bioimaging results of PLECDs in the MDA-MB-231 cell line. As shown by nuclear counterstaining with DAPI, the PLECDs was successfully internalized in the above-tested cancer cells, predominantly to the cytoplasm. Higher concentrations, however, resulted in complete fluorescence with a cytotoxic effect. Many of the currently used metallic quantum dots and fluorescent dyes are unstable and inadequately water-soluble, rendering them inappropriate for *in-vitro* bio imaging. CDs have been regarded as an appropriate agent for bioimaging in this regard. (Peng et al. 2017). When choosing a sample to be a probe for bioimaging applications, cytotoxicity is a key factor to consider. For *in-vitro* cell imaging, fluorescent probes must be highly fluorescent, water-soluble, chemically inert, biocompatible, and photostable (Mohapatra et al. 2021). At higher concentrations, the PLECDs showed remarkable cytotoxicity, but at lower concentrations, they exhibited minimal cytotoxicity. Therefore, the PLECDs may be used for bioimaging of cancer cells at low concentrations and at higher concentrations, and over a longer incubation time, they will serve as a cytotoxic agent.

3.5. Radical Scavenging

Free radicals generated in the living body are highly reactive, which cause DNA damage, lipid peroxidation, protein oxidation and accelerate the aging process (Ji et al. 2019). Numerous available reports suggest the free radical scavenging potential of CDs (Huang et al. 2017; Li et al. 2018; Pal et al. 2018; Zhang et al. 2018; Jia et al. 2019; Wang et al. 2019; Mohapatra et al. 2021).

3.5.1. DPPH scavenging assay

The DPPH is a free radical containing a lone pair of electrons on the nitrogen atom; upon reaction with an antioxidant, the formation of the complex (DPPH-H) occurs, and the conjugated π system is shrunk, resulting change of color from purple to light yellow and decrease in the absorbance (Li et al. 2018; Pal et al. 2018; Ji et al. 2019). Here, UV-visible spectroscopy was used to assess the scavenging capacity of PLLEE and PLECDs by calculating the decline in DPPH radical absorbance. The PLLEE and PLECDs scavenging responses were dose-dependent, and a progressive alteration of color from a deep purple color to bright yellow was observed (Fig. 8a and Fig. 8b). With PLLEE and PLECDs at various doses, a dose-dependent scavenging response with a reduction in the absorption intensity was observed (Fig. 8c and Fig. 8d). A linear relation between scavenging percentage and concentrations were observed within 0.04 to 0.62 mg/mL and 0.04–0.3 mg/mL concentration range of PLLEE and PLECDs, respectively (Fig. 8e, Fig. 8f, and inset Fig. 8f). The corresponding regression equations were $Y = 92.937X + 6.028$ ($R^2 = 0.989$) for PLLEE and $Y = 217.246X + 3.22$ ($R^2 = 0.994$) for PLECDs. The calculated values of IC_{50} (concentration required for 50% inhibition) for PLLEE and PLECDs were 0.473 mg/mL and 0.216 mg/mL, respectively. When the concentration of vitamin C was gradually increased from 0 to 8 $\mu\text{g/mL}$, it also demonstrated decreasing absorption intensity (Fig. 8g). The linearity was observed within the tested concentration with regression equation $Y = 6.356X + 6.053$ with the correlation coefficient $R^2 = 0.991$ and IC_{50} value of 6.913 $\mu\text{g/mL}$ (Fig. 8h). The antioxidant activity of PLECDs is due to the direct or indirect hydrogen atom transfer (HAT) mechanism assigned to the presence of $-NH_2$ & $-OH$ functional groups (Ji et al. 2019; Jia et al. 2019). The obtained IC_{50} of PLECDs (0.216 mg/mL) is very low than that of the PLLEE (0.473 mg/mL). This may be because PLECDs possess a nanostructure,

unpaired electrons, high surface-to-volume ratio, and surface defects that enable them to scavenge free radicals at low doses. (Sharma et al. 2020).

3.5.2. ABTS.⁺ radical scavenging

The ABTS.⁺ radical is scavenged by absorbing protons or electrons from antioxidants (Shahat et al. 2019). Consequently, the antioxidant substances causes ABTS.⁺ to discolor. The scavenging of ABTS.⁺ was examined by the decrease of its signature peak at 734 nm. In contrast to the DPPH, which is unstable in acidic pH, the ABTS.⁺ assay provides additional versatility and can be employed at various pH levels. Additionally, ABTS.⁺ is suitable for the antioxidant test of hydrophilic and lipophilic candidates since it is soluble in a wide range of aqueous and non-aqueous solvents. In contrast to DPPH, which responds slowly and needs a longer incubation period, samples quickly react with ABTS.⁺ and achieve saturation. Several research teams have previously reported on the ABTS.⁺ scavenging activity of CDs (Rosenkrans et al. 2020; Dong et al. 2021; Roy et al. 2021; Zhao et al. 2021; Mohapatra et al. 2022). With PLLEE and PLECDs, dose-dependent ABTS.⁺ scavenging was noticed, and the blue-green color of the ABTS.⁺ solution gradually discolored (Fig. 9a and Fig. 9b). The decrease in absorbance with a progressive rise in the amounts of PLLEE or PLECDs from 0 mg/mL to 0.56 mg/mL further supported this (Fig. 9c and Fig. 9d). The linear relationship was found between 0.035 to 0.368 mg/mL of PLLEE with the linear equation $Y = 158.973x - 1.05$ and correlation coefficient (R^2) of 0.993 (Fig. 9e & inset Fig. 9e). The calculated IC_{50} value of PLLEE was found to be 0.321 mg/mL. Similarly, the linearity was obtained between the concentration of 0.035 to 0.144 mg/mL for PLECDs with the linear equation $Y = 523.312x + 14.391$, R^2 value of 0.979 and IC_{50} value 0.068 mg/mL (Fig. 9f and Inset Fig. 9f). Trolox as a standard antioxidant showed the gradual decrease in the absorbance of ABTS.⁺ from 1 µg/mL to 4.5 µg/mL (Fig. 9g) with the regression equation $Y = 18.693x - 23.029$ ($R^2 = 0.996$) and IC_{50} value of 3.906 µg/mL (Fig. 9h). The obtained IC_{50} value of PLECDs (0.068 mg/mL) for scavenging of ABTS.⁺ (Inset Fig. 9f) is very minimum than that of PLLEE (0.321 mg/mL) (Inset Fig. 9e), which is in accordance with the scavenging activity of DPPH scavenging results. Collectively the results of DPPH and ABTS.⁺ scavenging assay reflected the free radical scavenging capacity of PLECDs.

3.6. Microbial biocompatibility

Zones of inhibition from the microbial biocompatibility experiment were displayed in Fig. 10 (a-f) & Table 1. The ciprofloxacin, used as a positive control, displayed a wider zone of inhibitions against all pathogenic bacterial strains, demonstrating the antimicrobial susceptibility of strains. In contrast, the tested samples (PLLEE and PLECDs) did not display any zone of inhibition, except for slight inhibition zones against *Aeromonas hydrophila* & *Enterococcus faecalis*. The outcomes demonstrate the biocompatibility of PLLEE and PLECDs against the bacterial strains. The PLECDs can be employed for live bacterial cell imaging because of their fluorescent feature.

Table 1
Disc diffusion assay results in terms of zone of inhibition

Type of microorganism	Microorganism	Zone of Inhibition (mm)			
		PLLEE (140.8 µg/disc)	PLECD (140.8 µg/disc)	Ciprofloxacin (20 µg/disc)	DMSO (10 µL)
Gram negative strains	<i>Aeromonas hydrophila</i>	6.5	6.5	28 ± 0.02	–
	<i>Klebsiella pneumoniae</i>	–	–	45 ± 0.023	–
	<i>Edwardsiella tarda</i>	–	–	24 ± 0.041	–
	<i>Escherichia coli</i>	6.5	–	35 ± 0.013	–
Gram positive strains	<i>Enterococcus faecalis</i>	6.6	6.5	22 ± 0.032	–
	<i>Staphylococcus aureus</i>	–	–	27 ± 0.012	–

3.7. Stability

3.8. Stability of PLECDs

Various stability-indicating parameters were accessed at the end of the stability study. At the end of long-term and accelerated stability (after 3 cycles of the heating-cooling cycle and freeze-thaw cycle), the PLECDs was found to retain their physical properties without any noticeable alterations. No change in color, odor, the appearance of precipitation, agglomeration, sedimentation, pH, percent transmittance, and the refractive index was observed, signifying sufficient physical stability of synthesized PLECDs. The unaltered percent transmittance and refractive index of the PLECDs signify the retention of optical clarity and homogeneity of the PLECDs.

The FTIR spectral data of PLECDs is shown in (Fig. 10g). Consistent spectral results without any shifting or vanishing of the characteristic vibrational peaks at the end of the stability study represent the chemical stability of PLECDs. Similarly, the consistent emission pattern (Fig. 10h) with no substantial alteration of fluorescent intensity, bathochromic or hypsochromic shift was noticed, depicting fluorescence stability of the PLECDs. The retention of physical, chemical, and fluorescence properties in various storage conditions (-20°C, 25°C, 4°C, and 40°C) represents the broad storage conditions for storing PLECDs without any alterations.

A major prerequisite of CDs for bioimaging purposes is photostability. Intentional exposure of the PLECDs to 365 nm was made, and the fluorescence behavior was periodically checked. There was no sign of photobleaching after three hours of continuous exposure. The pink and greenish fluorescence remains intact while observed in the naked eye (Fig. 11a and Fig. 11b) at various time points. The data of absorption (Fig. 11c) and fluorescence (Fig. 11d) obtained at various time points reflected the consistent optical properties with no alteration. As a result, the PLECDs can be employed for bioimaging purposes owing to their excellent photostability.

The emission spectra (Fig. 11e) were acquired by exposing 60µL of PLECDs (14.08mg/mL) to the 3mL aqueous sample of different pH (pH 1 to 13). The maximum fluorescence (1055616.25 ± 5346.773 a.u.) was obtained within the pH range of 3–9, after which the intensity was found to be decreased. The pH-dependent emission alterations

were ascribed to the surface alteration owing to the ionization of surface functional groups (Sachdev et al. 2015; Mohapatra et al. 2021; Mohapatra et al. 2021). The higher fluorescence intensity within the pH value of 3 to 9 covers the physiological pH of body fluid (pH 7.4), representing its possible fluorescence utility in *in-vitro* and *in-vivo* tests. Further, the unaltered intensity within the acidic to the basic region (pH 3–9), represents its pH stability and broad applicability window under varieties of pH conditions.

TGA analysis was used to examine the thermostability of PLECDs. The TGA data showed five steps degradation event up to 800°C (Fig. 11f). The PLECDs showed thermal stability up to 196.75°C with an initial minute loss of weight (2.68%), which is due to the evaporation of water molecules & weakly bonded chemical groups (Mehta et al. 2014). Afterward, the step-wise continuous loss of weight, i.e., 24.69%, 13.01%, 7.98%, and 8.87%, is probably owing to the denaturation of strongly anchored functional groups that are linked to the PLECDs via covalent bonds (Mewada et al. 2013). This demonstrates the availability of various chemical moieties in the PLECDs. At 800°C, the overall weight loss is 57.23%.

4. Conclusions

In the current study, we used a facile, one-pot, and environmentally friendly solvothermal carbonization process to develop dual fluorescent (pink and green) carbon dots from Indian long pepper (*Piper longum*) leaves. The produced carbon dots (PLECDs) are spherical, multifunctional, nanocrystalline materials with good physicochemical stability, excitation-dependent emission property, pH-dependent fluorescence behavior, photostability, and thermal stability. It demonstrated outstanding Cu^{2+} sensitivity and selectivity, making it a useful probe for Cu^{2+} sensing, which might be used for environmental and health aspects. When compared to PLLEE, the PLECDs demonstrated substantially higher cytotoxicity against melanoma (B16F10), cervical cancer (SiHa), and breast cancer (MDA-MB-231) cell lines. In the aforementioned cell lines, it showed concentration-dependent bioimaging outcomes with green fluorescence. In addition, as compared to plain PLLEE, the PLECDs showed outstanding free radical scavenging activity against DPPH and ABTS.⁺, suggesting their potential utility as an antioxidant agent. The absence of any zone of inhibition or very negligible zone of inhibition represents the bacterial biocompatibility of the synthesized PLECDs. The high degree of bacterial biocompatibility against Gram + ve and Gram -ve bacterial strains may be advantageous for *in-vitro* live bacterial cell imaging. The PLECDs offer considerable prospects for their potential uses in Cu^{2+} sensing, cancer cell imaging, and free radical scavenging thanks to their straightforward and environmentally friendly uni-pot fabrication, excellent optical features, biocompatibility, and stability.

Declarations

Ethical Approval: Not applicable

Consent to Participate: Not applicable

Consent to Publish: Not applicable

Authors Contributions:

Debadatta Mohapatra carried out most of the experimental work, collected, processed, analyzed, validated, and interpreted data, and wrote the manuscript. Ravi Pratap, Vivek Pandey, Singh Shreya, and Gaurav Gopal Naik participated in the experiment, reviewed, edited, and revised the manuscript scientifically. Alakh N. Sahu, Avanish S.

Parmar, Pawan K. Dubey, Subhash C. Mandal, and Sunday O. Otimenyin contributed to supervising, evaluating, drawing conclusions from data, providing expert assistance, reviewing, and editing the manuscripts.

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Competing Interests:

The authors declare that they have no conflicts of interest.

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Availability of data and materials:

All the data are encompassed in this manuscript. The scientific data in terms of graphs and figures were generated using MS office package, a 21-day free trial version of Origin Pro 2021 (Microcal Software, Inc., Northampton, USA), GraphPad Prism 5.0 (GraphPad Software Inc.; San Diego, CA, USA), and ImageJ software (National Institutes of Health, Bethesda, MD).

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Figures

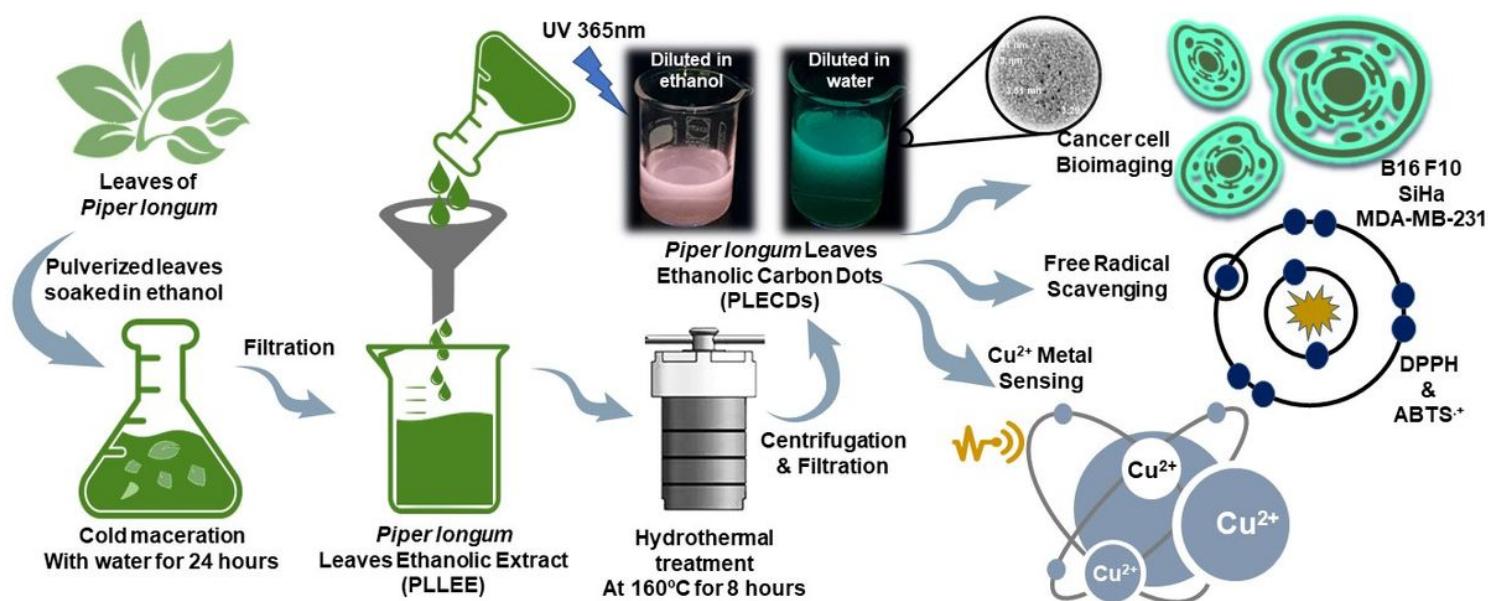


Figure 1

Schematic representation of synthesis and applications of PLECDs from leaves of long pepper

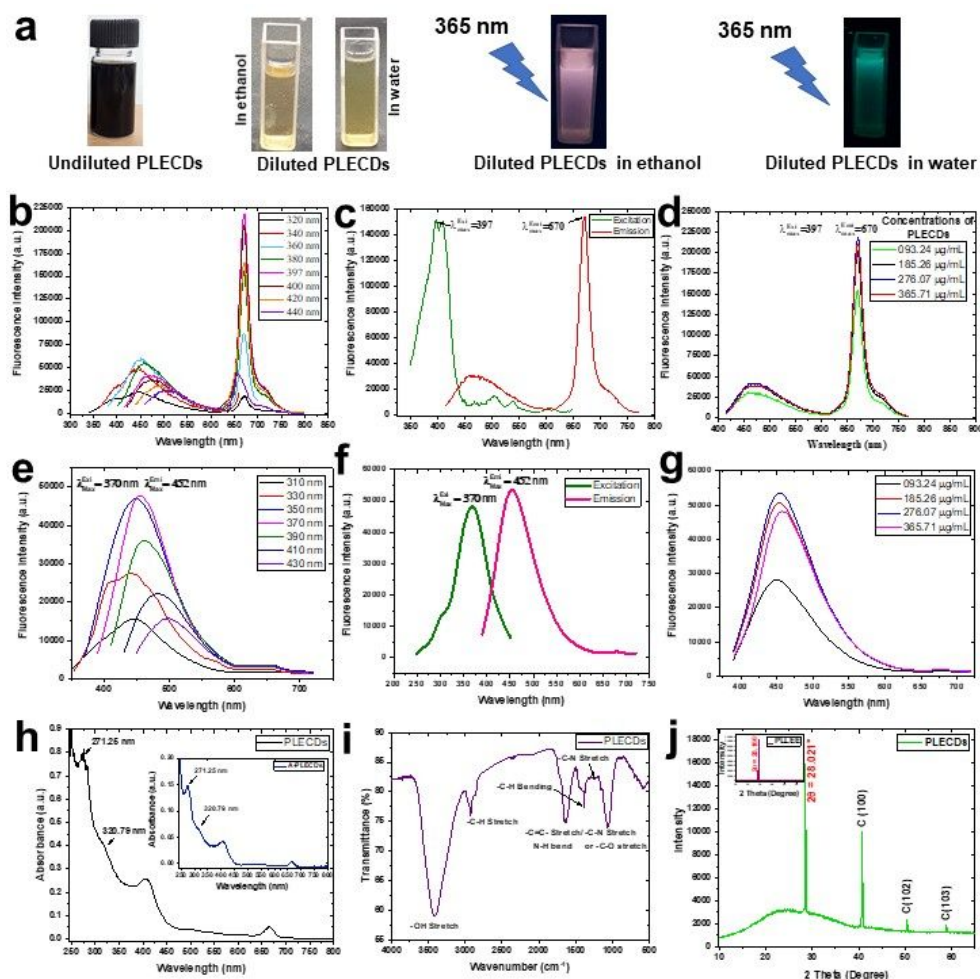


Figure 2

Physicochemical properties of PLECDs (a) visual observations, (b) excitation-dependent emission spectra of PLECDs in ethanol, (c) maximum excitation and emission spectra of the PLECDs in ethanol, (d) concentration-dependent fluorescence spectra of PLECDs in ethanol, (e) excitation dependent emission spectra of PLECDs in water, (f) maximum excitation and emission spectra of the PLECDs in water, (g) concentration-dependent fluorescence spectra of PLECDs in water, (h) UV-vis absorption spectra PLECDs in ethanol with inset image showing the UV-vis spectra of A-PLLECDs, (i) FTIR spectra of PLECDs, and (j) XRD diffractogram of PLECDs with inset figure showing the diffractogram of PLLEE

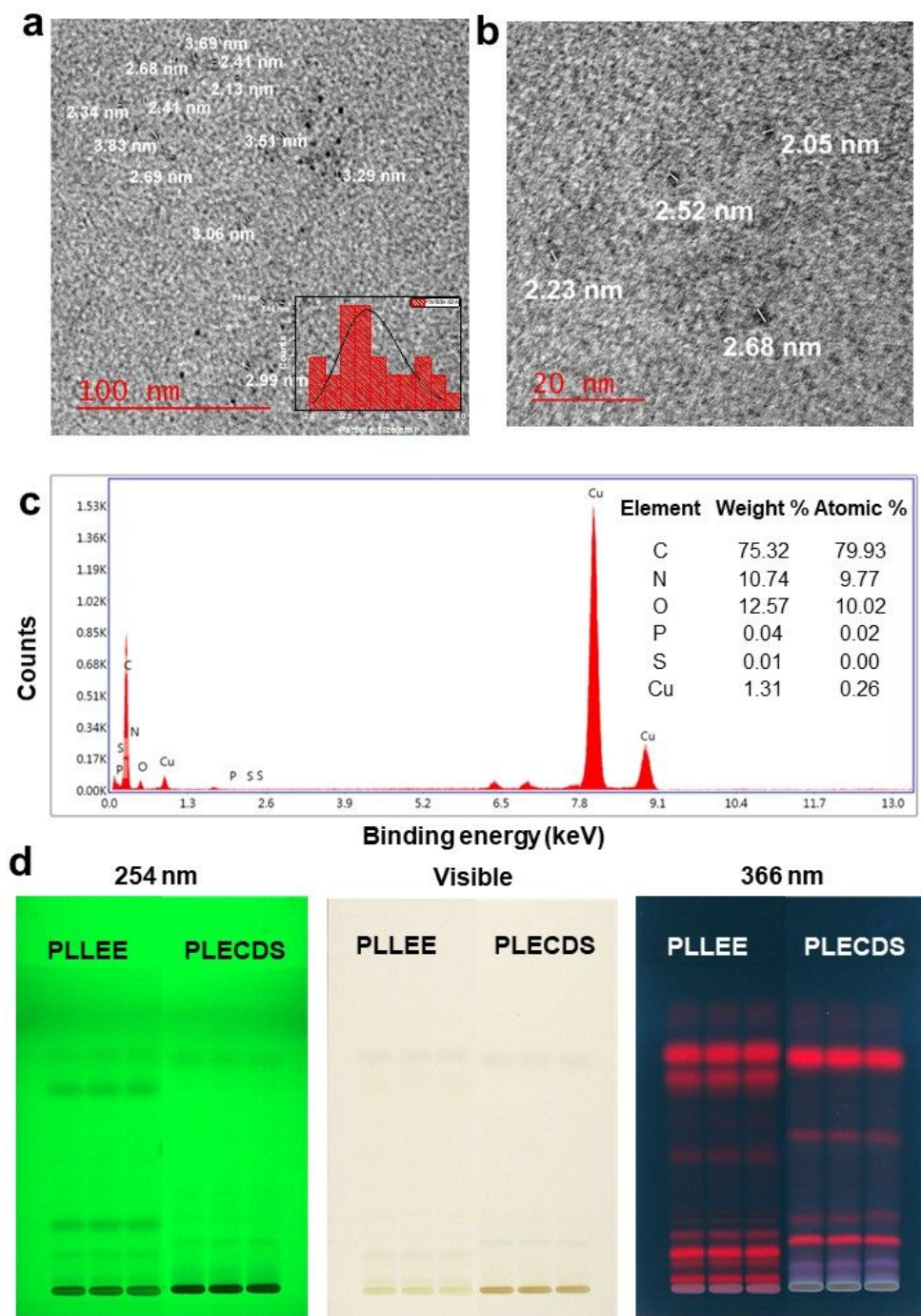


Figure 3

HRTEM, EDAX, and HPTLC results (a) HRTEM photomicrograph of PLECDs at 100 nm scale with inset image showing size distribution analyzed by Image J software, (b) HRTEM photomicrograph of PLECDs at 20 nm scale, (c) EDAX spectra of PLECDs with inset data displaying the elements with their weight & atomic percentage, and (d) HPTLC fingerprint of PLLEE and PLECDs at 254 nm, visible light, and 366 nm

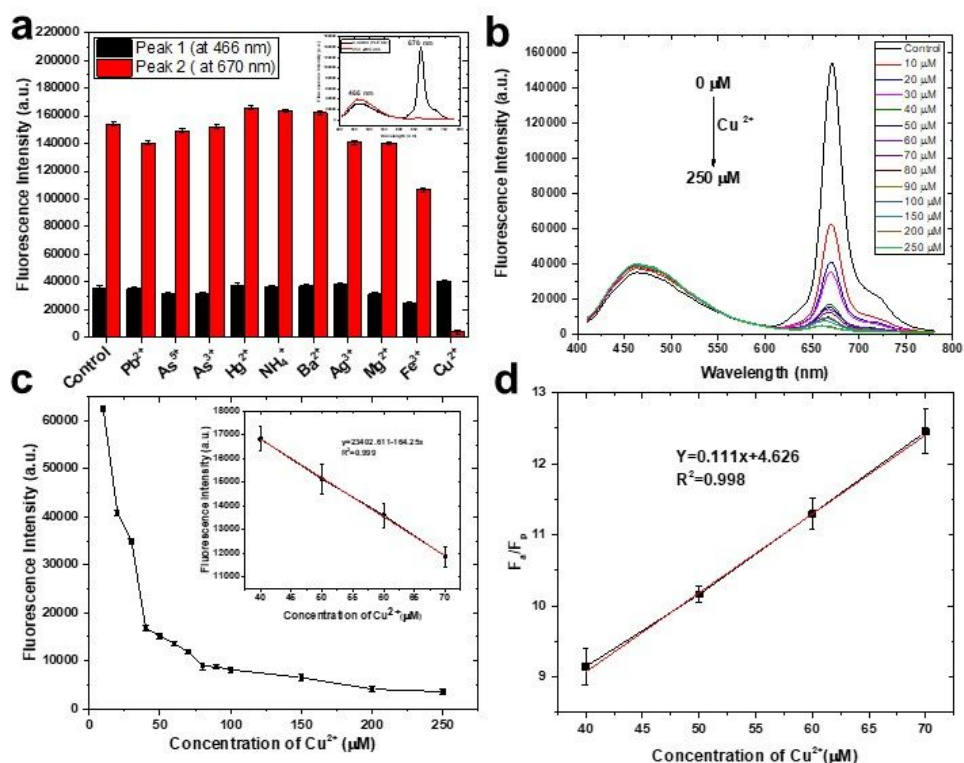


Figure 4

Metal ion sensing of PLECDs (a) fluorescence intensity of PLECDs in the presence of various metal ions (250 μM) with inset figure showing the decrease in the intensity of peak 2 in the presence of Cu^{2+} , (b) fluorescence spectra of PLECDs in the presence of different concentrations of Cu^{2+} (from 0-250 μM), (c) fluorescence intensity of PLECDs v/s concentration of Cu^{2+} from 0 to 250 μM (Inset image showing linearity range), and (d) relative fluorescence intensity (F_a/F_p) versus concentration of Cu^{2+} from 40 to 70 μM

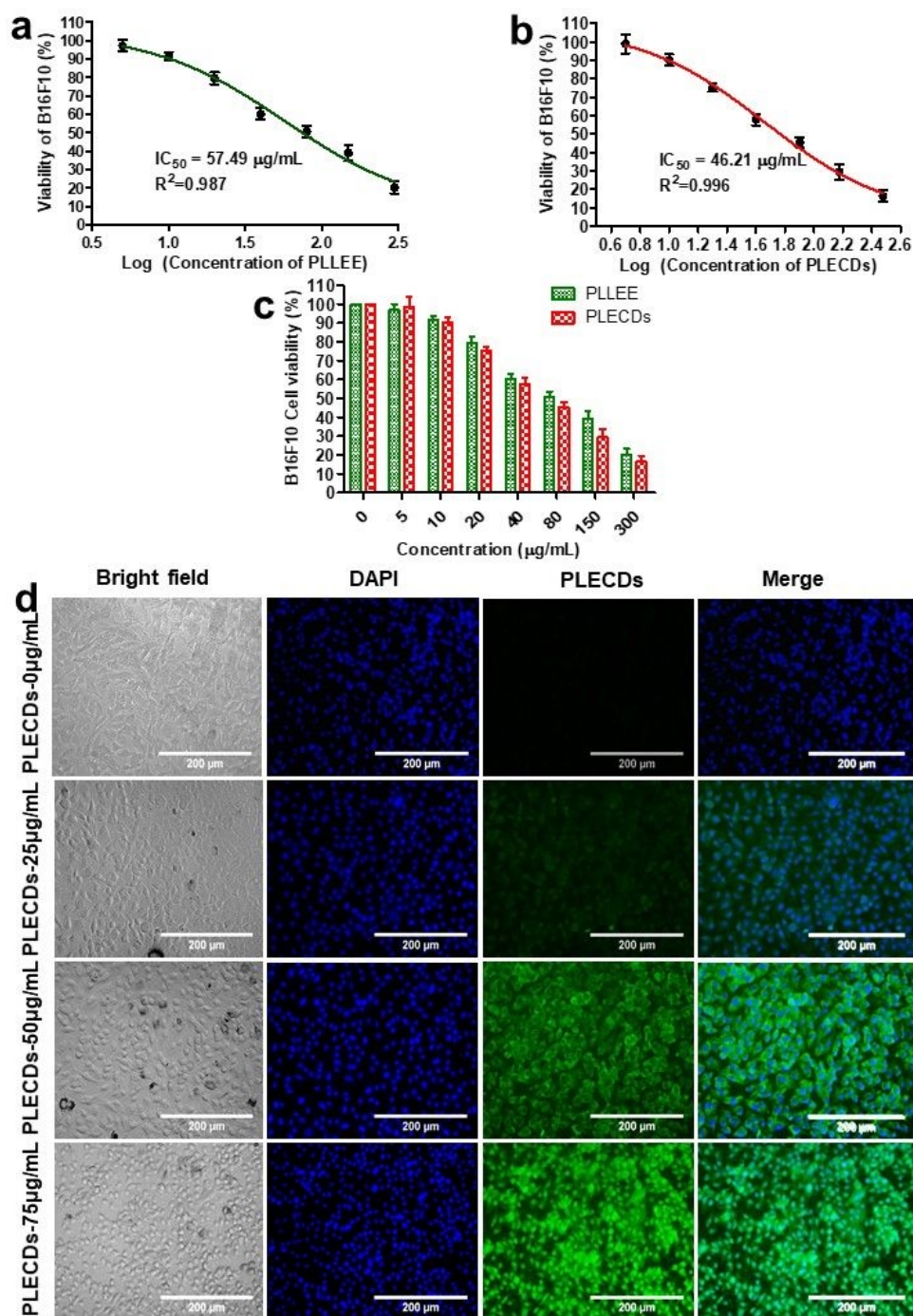


Figure 5

In-vitro cytotoxicity and bioimaging results against B16F10 cell line after 24 hours of incubation (a) percentage cell viability v/s log concentration of PLLEE, (b) percentage cell viability v/s log concentration of PLECDs, (c) comparative percentage cell viability v/s various concentration of PLLEE and PLECDs, and (d) in-vitro bioimaging of B16F10 cell lines under bright field, blue field (DAPI stained), green field (PLECDs treated) and merged micrographs of DAPI and PLECDs after 24 h of incubation

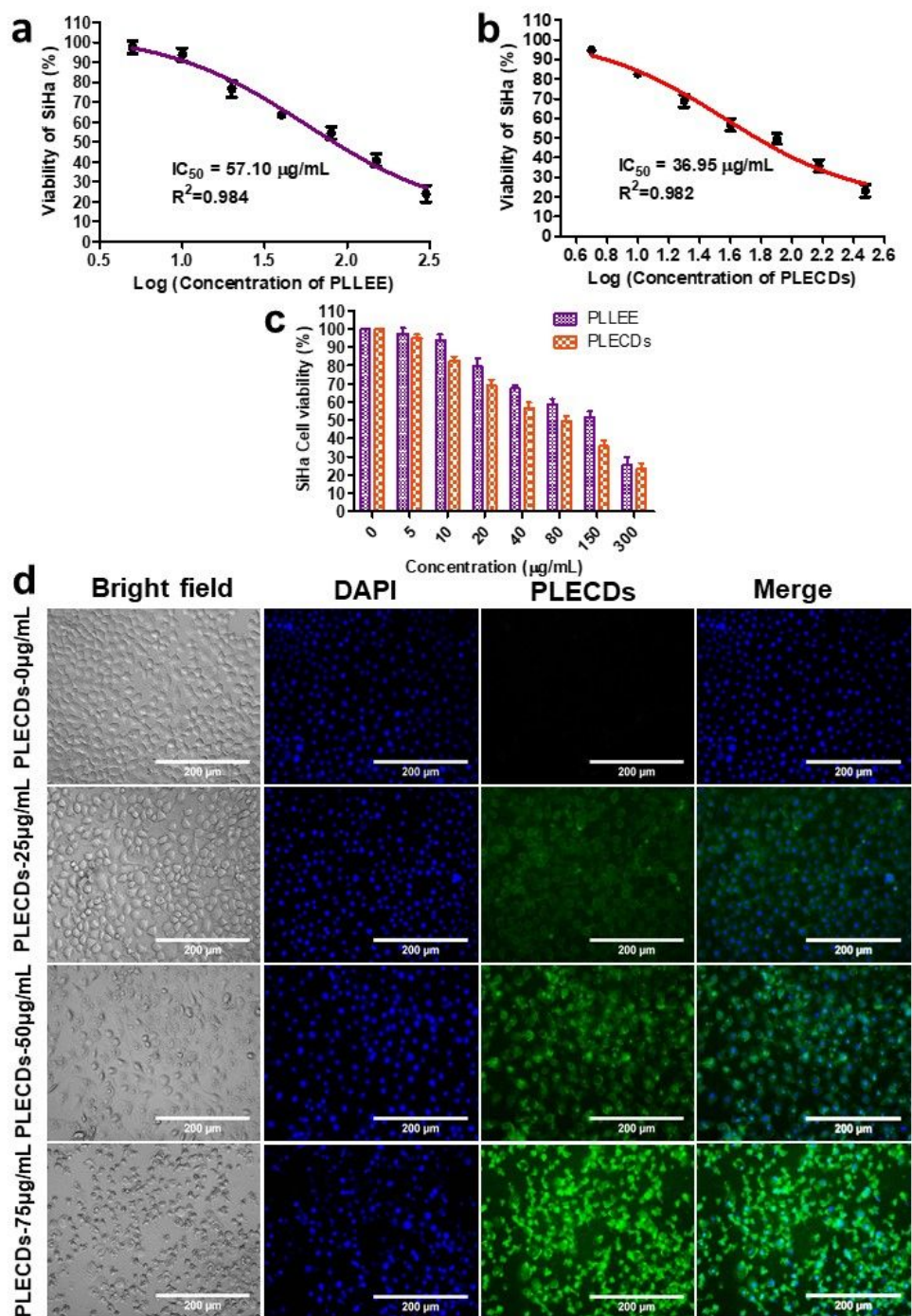


Figure 6

In-vitro cytotoxicity and bioimaging results against SiHa cell line after 24 hours of incubation (a) percentage cell viability v/s log concentration of PLLEE, (b) percentage cell viability v/s log concentration of PLECDs, (c) comparative percentage cell viability v/s various concentration of PLLEE and PLECDs, and (d) in-vitro bioimaging of SiHa cell lines under bright field, blue field (DAPI stained), green field (PLECDs treated) and merged micrographs of DAPI and PLECDs after 24 h of incubation

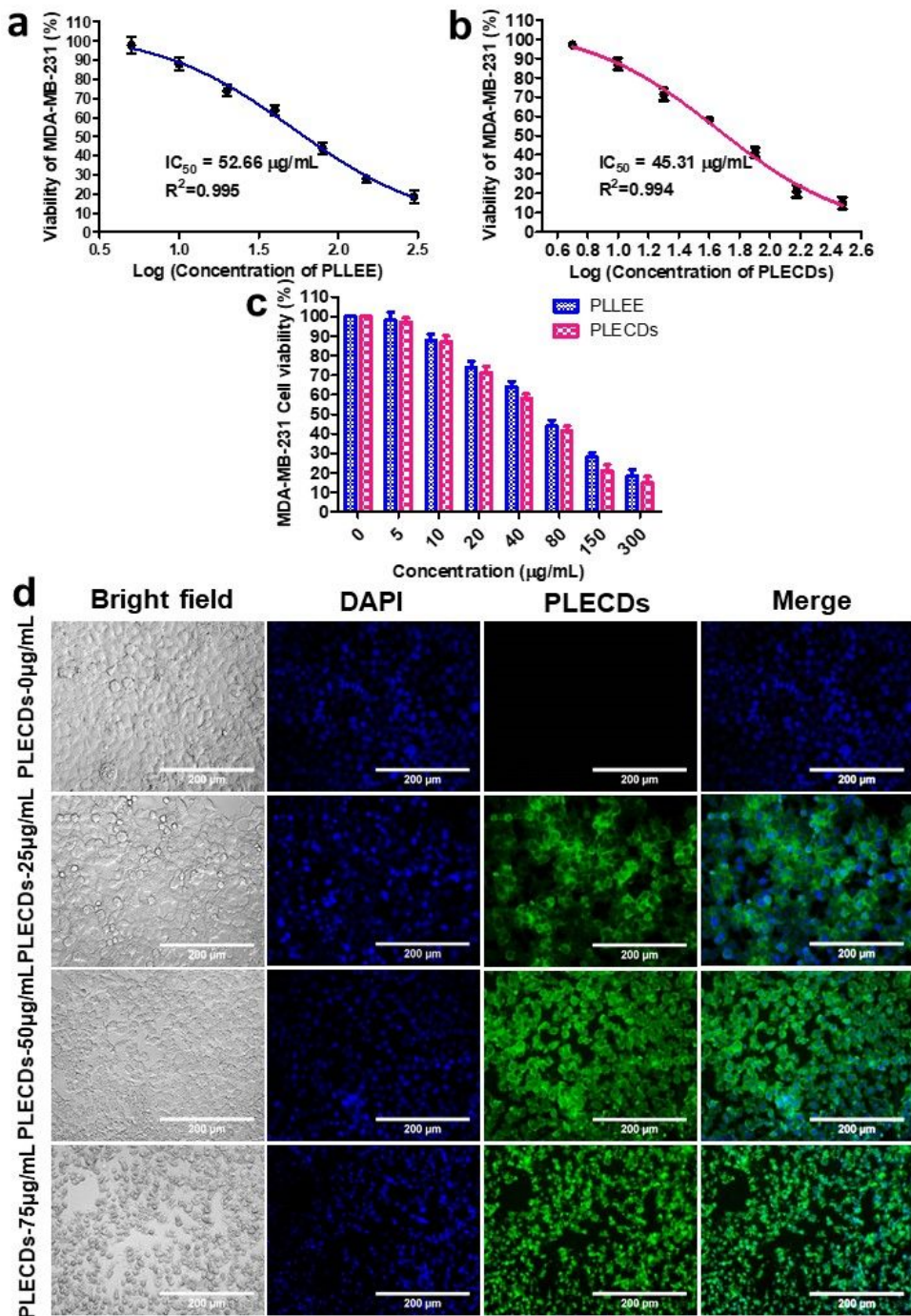


Figure 7

In-vitro cytotoxicity and bioimaging results against MDA-MB-231 cell line after 24 hours of incubation (a) percentage cell viability v/s log concentration of PLLEE, (b) percentage cell viability v/s log concentration of PLECDs, (c) comparative percentage cell viability v/s various concentration of PLLEE and PLECDs, and (d) in-vitro bioimaging of MDA-MB-231 cell lines under bright field, blue field (DAPI stained), green field (PLECDs treated) and merged micrographs of DAPI and PLECDs after 24 h of incubation

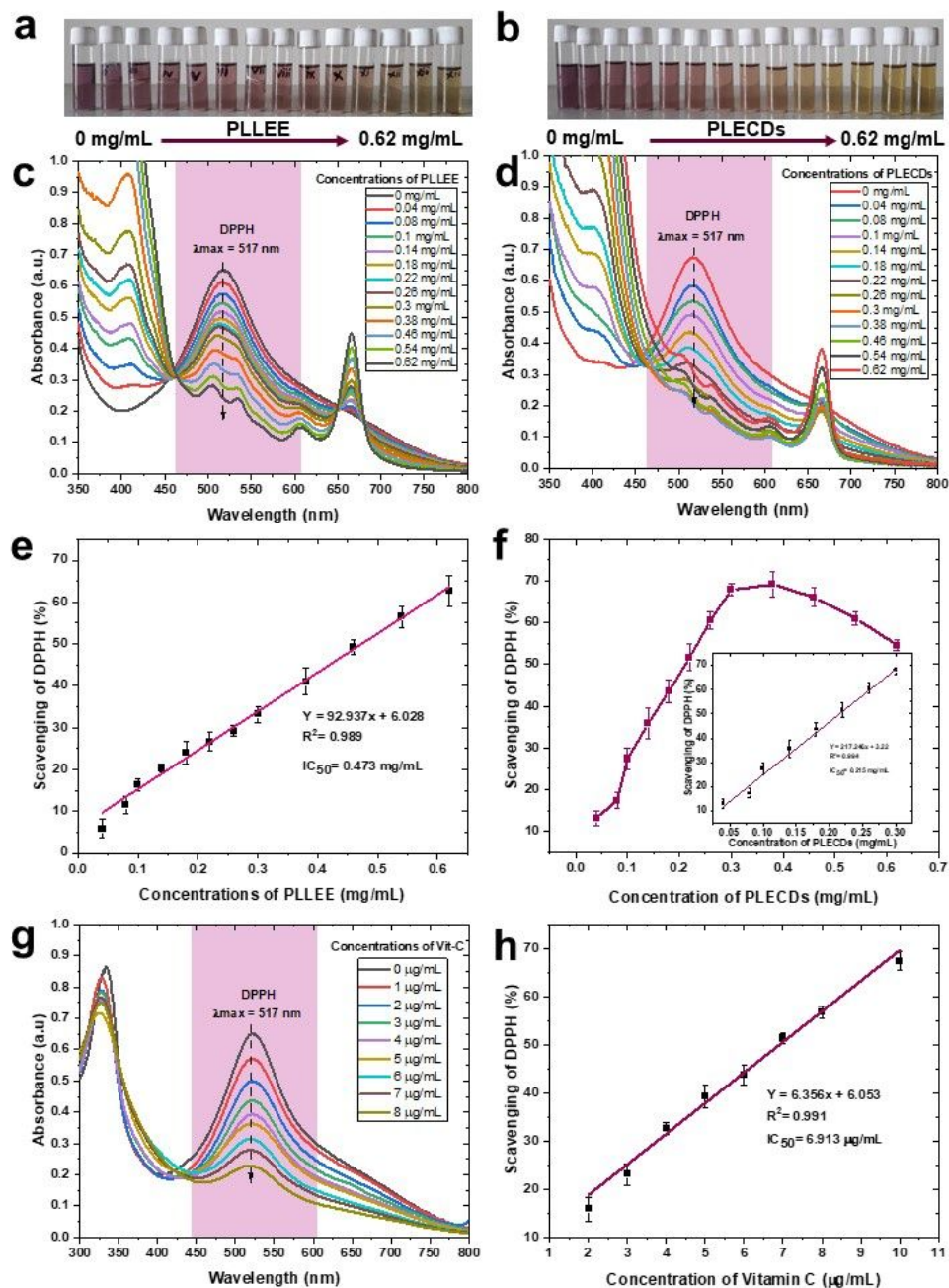


Figure 8

DPPH radical scavenging assay of PLLEE and PLECDs (a) picture of DPPH solution incubated with different concentrations of PLLEE, (b) picture of DPPH solution incubated with different concentrations of PLECDs, (c) UV absorption spectra of DPPH radical with a gradual increase in PLLEE concentration, (d) UV absorption spectra of DPPH radical upon a gradual increase of PLECDs concentration, (e) plot of percentage DPPH scavenging v/s PLLEE concentrations, (f) plot of percentage scavenging of DPPH radicals v/s PLECDs concentrations with inset graph showing the linearity region, (g) UV absorption spectra of DPPH radical with a gradual increase in vitamin C concentration, and (h) calibration curve of percentage scavenging of DPPH radical v/s different concentrations of vitamin C

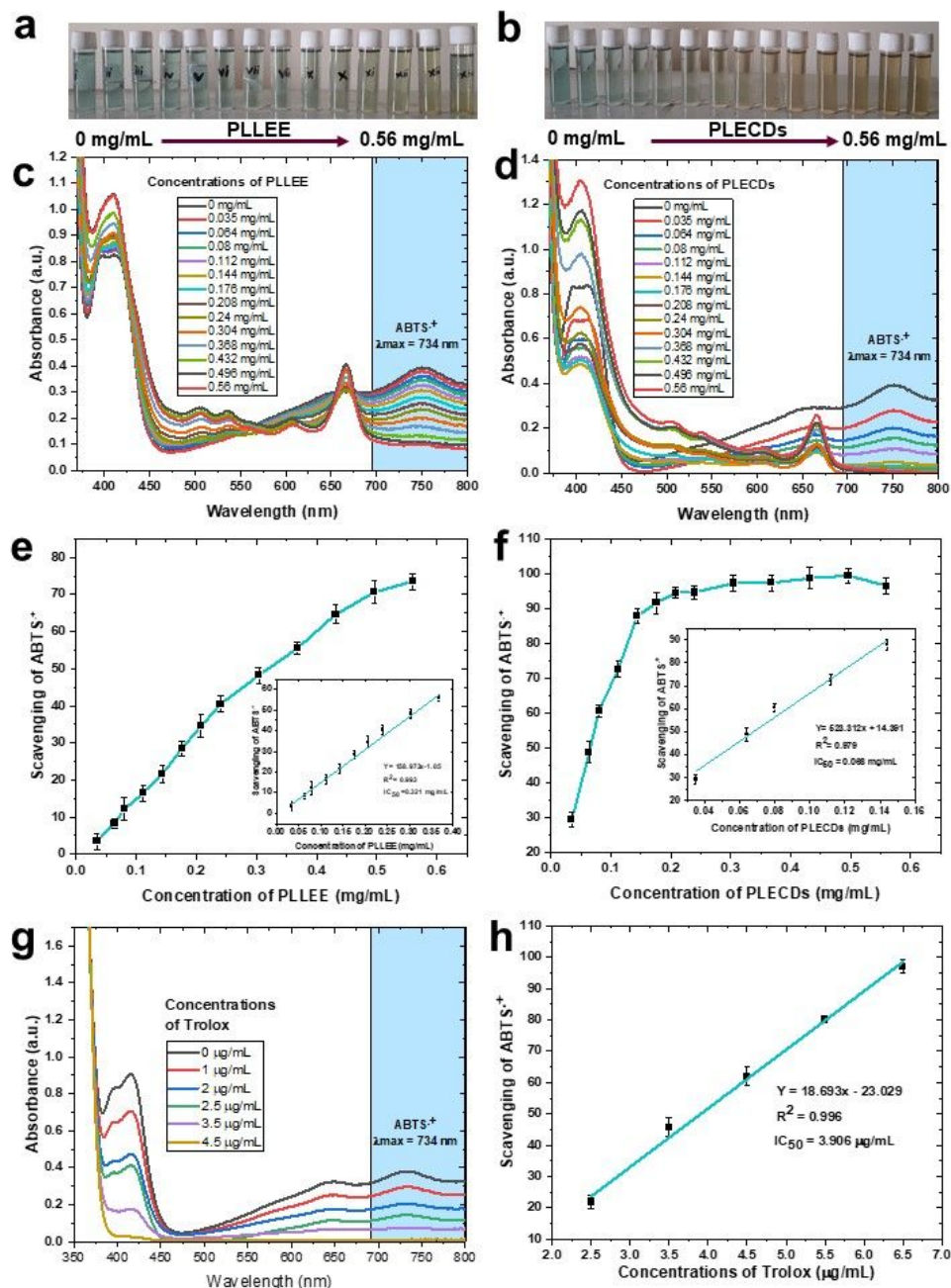


Figure 9

ABTS.+ scavenging assay of PLLEE and PLECDs (a) photograph of ABTS.+ solution exposed to different concentrations of PLLEE, (b) photograph of ABTS.+ solution exposed to different concentrations of PLECDs, (c) UV absorption spectra of ABTS.+ radical upon a gradual increase in the PLLEE concentration, (d) UV absorption spectra of ABTS.+ upon a gradual increase in the PLECDs concentration, (e) calibration curve of percentage scavenging of ABTS.+ radicals v/s PLLEE concentrations with an inset graph representing the linearity region (f) calibration curve of percentage scavenging of ABTS.+ v/s PLECDs concentrations with inset graph representing the linearity region, (g) UV absorption spectra of ABTS.+ upon a gradual increase in the Trolox concentration, and (g) calibration curve of percentage ABTS.+ scavenging v/s Trolox concentrations

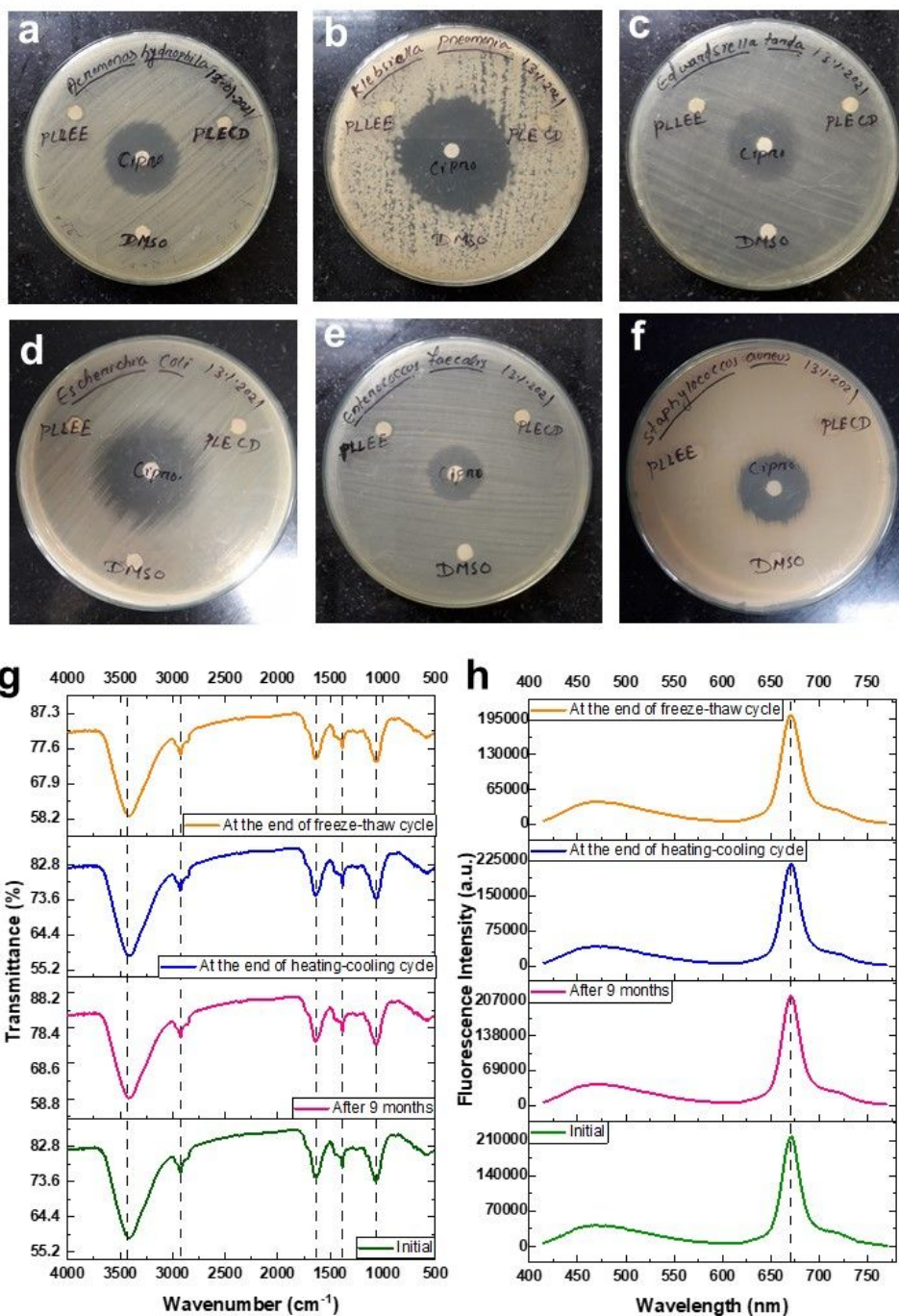


Figure 10

Bacterial biocompatibility study and stability study: (a-f) bacterial biocompatibility against *Aeromonas hydrophila*, *Klebsiella pneumoniae*, *Edwardsiella tarda*, *Escherichia coli*, *Enterococcus faecalis*, and *Staphylococcus aureus*, (g) chemical stability study of PLECDs by FTIR spectroscopy, and (h) fluorescence stability of PLECDs by fluorimetry

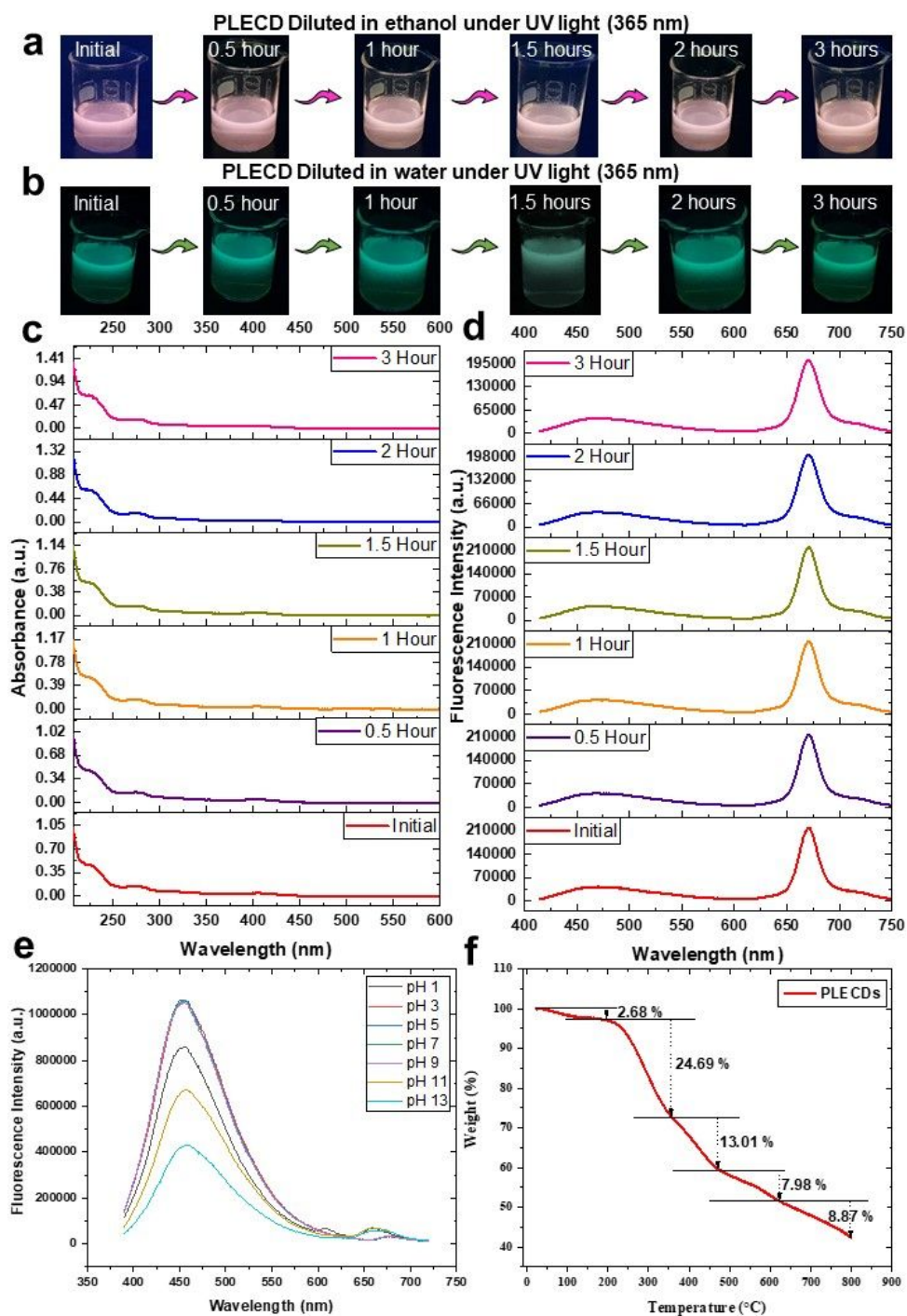


Figure 11

Stability study of PLECDs (a & b) photostability of PLECDs in ethanol and water (A-PLECDs) exposed to UV (365 nm) up to 3 hours, (c & d) UV-absorption spectra and fluorescence emission spectra of PLECDs exposed to UV (365 nm) up to 3 hours at different time intervals, (e) pH-dependent fluorescence stability of PLECDs, and (f) thermal stability of PLECDs via TGA analysis