Chapter-3

MATERIALS AND METHODS

3.1 Introduction

This chapter describes materials and experimental methodologies for the fabrication of fluorescent carbon nanodots (CNDs), which have been utilized for various pharmaceutical, biomedical and environmental applications. This chapter also deliberates on the various modern characterization techniques used in the present study, such as High resolution- Transmission Electron Microscopy (HR-TEM), Selected Area Diffraction (SAED) pattern, Energy Dispersive X-ray Spectroscopy (EDS), X-Ray Diffraction (XRD), X-ray photoelectron spectroscopy(XPS), Raman spectroscopy, Fourier-transform infrared spectroscopy (FT-IR), UV-visible spectroscopy and Fluorescence spectroscopy. In addition, it covers details about the determination of fluorescent quantum yield (QY), Stern-Volmer plot and detection limit.

3.2 Materials

This study deals with the fabrication of CNDs incorporating ultrapure water as a green solvent and ethanol as a Class 3 solvent as per USP 467 ICH Q3C Guidelines. The fluorescence quantum yield of CNDs has been further improved by surface passivation with biocompatible polymers such as carrageenan, polyethylenimine, and polyethylene glycol. The chemicals and reagents used in this study were of analytical grade and used without further purification (Table **3.1**).

Table 3.1 List of materials and chemicals

S.No	Name	Manufacturer
1	Dulbecco's Modified Eagle's Medium (DMEM/F-12),	Sigma-Aldrich, USA
2	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT),	Sigma-Aldrich, USA
3	dimethyl sulfoxide (DMSO),	Sigma-Aldrich, USA
4	1, 1-diphenyl-2-picrylhydrazyl (DPPH),	Sigma-Aldrich, USA
5	Doxycycline	Sigma-Aldrich, USA
6	4,6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, USA
7	Quinine sulfate	Sigma-Aldrich, USA
8	MCF-7 cells	National Centre for Cell Sciences (NCCS; Pune, India)
9	SiHa cells	NCCS Pune, India
10	MDA-MB-231 cells	NCCS Pune, India
11	HEK-293 cells	NCCS Pune, India
12	K-562 cells	NCCS Pune, India
13	Mueller-Hinton agar (MHA) me-Dia	Himedia (Mumbai, India).
14	Quercetin	Sigma-Aldrich, USA
15	Sodium chloride	Sisco Research Laboratories Pvt. Ltd (Mumbai, India),

16	Potassium chloride	Sisco Research Laboratories Pvt. Ltd (Mumbai, India),
17	Magnesium chloride	Sisco Research Laboratories Pvt. Ltd (Mumbai, India),
18	Calcium chloride	Sisco Research Laboratories Pvt. Ltd (Mumbai, India),
19	Ammonium chloride	Sisco Research Laboratories Pvt. Ltd (Mumbai, India),
20	Copper chloride	Sisco Research Laboratories Pvt. Ltd (Mumbai, India),
21	Mercuric chloride	Fisher Scientific (MA, USA)
22	Barium chloride	Fisher Scientific (MA, USA)
23	Silver nitratc	Merck & Co. (NJ, USA)
24	Ferric chloride	Finar Chemical Ltd (Mumbai, India).
25	Sodium arsenite	SD Fine Chemical Ltd (Mumbai, India).
26	Methanol	Merck & Co. (NJ, USA)
27	HPLC grade water	Merck & Co. (NJ, USA)
29	Ethanol	Merck & Co. (NJ, USA)
29	Acetone	Merck & Co. (NJ, USA)
30	Acetic acid	Merck & Co. (NJ, USA)
31	Thiobarbituric acid	Sigma-Aldrich, USA

32	Formaldehyde	Merck Ltd., Mumbai, India
33	Paraffin wax	Sigma-Aldrich, USA
34	Xylene	SD Fine Chemicals, Mumbai, India
35	Hematoxylin and Eosin (H & E) dye	Hi Media, Mumbai, India
36	Disodium hydrogen phosphate	SD Fine Chemicals, Mumbai, India
37	Potassium dihydrogen phosphate	SD Fine Chemicals, Mumbai, India
38	Sodium dodecyl sulphate (SDS)	Hi Media, Mumbai, India

3.3 **Equipment and software**

The equipment and software used in the study are mentioned in **Table 3.2** and **Table 3.3**, respectively.

Table 3.2: List of equipments

S. No.	Instruments	Model, make & country
1.	Digital electronic balance	AUX220, Shimadzu, Japan
2.	Digital magnetic stirrer	1MLH, REMI, India
3.	Digital pH meter	PC 700, Eutech Instruments, India
4.	Cooling centrifuge	CM-12 Plus, REMI, India
5.	Micro centrifuge	LABQUEST, Borosil, India

6.	Digital microscope	MLX PLus, Magnus, India
7.	Electric oven	Khera Instruments, India
8.	Disposable syringes	Hindustan Syringes, India
9.	Optical plate reader	Bio-Rad Laboratories, California, USA
10.	Water bath sonicator	PCI Analytics Mumbai, India
11.	UV-Visible Spectrophotometer	Cary 60, Shimadzu, Japan
12.	Vortex mixer	REMI Instruments, Mumbai, India
13.	Water bath	DENTPRO, India
14.	Micropipettes	LABQUEST, Borosil, India
15.	Glucometer	One Touch Select, UK
16.	Biochemical analyzer	Chem-5 Plus v2, Erba, Germany
17.	Deep freezer	Thermo Fischer Scientific, USA
18.	Melting point apparatus	PERFIT, India
19.	Refrigerator	SAMSUNG, India
20.	Microtome	Leica, Bensheim, Germany
21.	Distilled water assembly	Saritorius, USA
22.	Rotatory shaker	RS-12 Plus, REMI, India
23.	Western blot assembly	Bio-Rad, USA

24.	Rotatory vacuum evaporator	IKA RV 10, India
25.	Gel-documentation system	Bio-Rad, USA
26.	Electric grinder	Philips, India
27.	Heating mantle	IKON Instruments, India
28	UV-Vis chamber	PERFIT, India

Table 3.3: List of software

S. No.	Software	Source
1.	Magnus MLX	Magvision, Magnus, India
2.	GraphPad prism 7 [Free version]	GraphPad Software, Inc., USA
3.	Image J software	NIH
4	Origin software	OriginPro 2017 , OriginLab Corporation, Northampton, USA

3.4 Methods

Various methods and techniques used in this study have been summarised in this section.

3.4.1 Collection and authentication of plant specimen by DNA fingerprinting

Fresh leaves of *Andrographis paniculata* and roots of *Asparagus racemosus* were collected from IIT (BHU) campus, India, and were authenticated by certified taxonomist

Prof. N.K. Dubey at the Department of Botany, Banaras Hindu University (BHU), Varanasi India (Specimen No. Acantha. 2020/1 & 2021/5). These samples have been submitted to the herbarium for future reference. NucleoSpin® Plant II Kit (Macherey-Nagel) was used to extract 100 mg of plant specimen DNA [White *et al*., 1990]. Using an agarose gel electrophoresis, the integrity of the obtained DNA preparations was examined, and the quantity and purity of the DNA were assessed using a UV transilluminator (Genei, India). Exosap purification process was carried out before sequencing PCR. 1 µL of DNA, 0.25 µL of forward and reverse primers, 5 µL of 2X Phire Master mix, and 4 µL of distilled water were used in each 10.5 µL PCR. The amplification profile consisted of a 98 °C/30 s initial denaturation, followed by 40 cycles of 98 °C/5 s, 45 °C/10 s, and 72 °C/15 s, followed by a 72 °C/1 min extension. Ethanol clean-up was performed after sequencing PCR. Using an ABI 3500 DNA Analyzer, the resultant amplicons were separated electrophoretically (Applied Biosystems, USA). Sequence Scanner Software v1 examined the sequence quality (Applied Biosystems, USA). Geneious Pro v5.1 was used to perform sequence alignment and necessary modification on the acquired sequences.

3.4.2 Fabrication of CNDs

Various plant materials have been utilized as precursors to fabricate CNDs via the one-pot hydrothermal method. Typically, the precursors were uniformly dispersed in suitable solvents to obtain a clear solution devoid of particles and transferred to a teflon-lined hydrothermal autoclave at high temperature up to 8 h. Upon cooling the reaction vessel at room temperature, the obtained dark-brown solutions were centrifuged for 15 min at 12,000 rpm to remove agglomerated and large particles. Then, the obtained

solution was further purified using a membrane syringe filter (0.22 μm). For further characterization and biological evaluations, CNDs were stored at 4°C. The detailed procedures for the fabrication of CNDs using *Andrographis paniculata*, *Asparagus racemosus*, and Quercetin are provided in chapters 3, 4, 5, and 6 under the experimental section.

3.4.3 Instrumentations and characterization of CNDs

The fabricated CNDs were characterized by different techniques described in brief. The absorption behavior of fabricated CNDs was investigated using a UV–vis double beam spectrophotometer (Cary 60 UV vis, USA). The fluorescence attributes of CNDs were studied by employing a Fluorolog-Horiba fluorescence spectrophotometer, USA. Fourier transform infrared spectrometer (Shimadzu FTIR-8400S, JAPAN) examined the surface functional groups in 4000-400 cm^{-1}. A High Resolution-Transmission Electron Microscope (Tecnai G2 20 TWIN, USA) analysis of the CNDs was conducted to study their morphology. After calculating the size of 50 particles with ImageJ software, the average particle size was computed. Elemental analysis was performed using Energy Dispersive X-ray Spectroscopy (EDAX Inc.'s TEAM EDS SYSTEM, USA), and selected area diffraction (SAED) patterns were obtained using Octane Plus SDD Detector. K-Alpha, Thermo Fisher Scientific (USA), was utilized to record X-ray photoelectron spectroscopy. Rigaku Miniflex 600 Desktop X-Ray Diffraction System, USA was utilized to obtain X-ray diffraction (XRD) patterns of nanodots. Horiba's LabRam HR evolution spectrometer (JAPAN) measured Raman spectra. The sample was irradiated with a HeNe laser (633nm, 30 mW), 1800 groves/mm grating, with laser exposure of 20 seconds was

used to acquire a spectrum. A Peltier cooled (-60 °C) CCD detector (model: Syncerity 356399) was employed to collect the Raman scattering signal.

3.4.3.1. High resolution-transmission electron microscopy (HR-TEM)

HR-TEM is a powerful technique that uses an electron beam to focus on the sample, which creates a detailed and highly magnified image of the samples. It enables the characterization of morphology, crystallization, and compositions of the nanoparticles. It operates on a principle similar to that of a light microscope; however, electrons are used in TEM instead of light as in the light microscope. Electrons have higher resolution capability as they have both particle and wave nature, and also, the de Broglie wavelength of electrons is significantly smaller than that of light. This feature facilitates the user to analyze minute details even as small as a single column of atoms. The transmission electron microscope consists of the following parts:

- Electron source
- Electromagnetic lens system
- Sample holder
- Imaging system

In this study, the HR-TEM analysis of the CNDs was carried out by employing Tecnai G2 20 TWIN operating at 200 keV. The samples were prepared by diluting the samples in a 1:10 ratio and then mounting the diluted samples on carbon-coated copper grids. The samples were vacuum dried overnight. Selected Area Diffraction (SAED) pattern for analyzing the amorphous/ crystalline nature and Energy Dispersive X-ray Spectroscopy (EDS) for elemental composition was performed on the same instrument.

3.4.3.2 X-ray photoelectron spectroscopy (XPS)

The widely used surface analysis technique is XPS, as it offers valuable chemical states and quantitative information about the surface of the specimen. It also provides information on the elements present within the samples and other elements bonded to them. Elements are identified based on the binding energy of an electron which is a unique property of each atom. The XPS system consists of the following parts:

- X-rays source: A1 Kα or Mg Kα
- A monochromator is an ultra-high vacuum (UHV) stainless steel chamber with UHV pumps
- Electron collection lens
- Electron energy analyzer
- Electron detection system
- Moderate vacuum sample introduction chamber

In this study, XPS analysis of the sample was carried out employing K-Alpha, Thermo Fisher Scientific XPS system by making the film of CNDs on a 1cm×1cm glass plate.

3.4.3.3 X-ray diffraction (XRD)

The standard technique used to understand the atomic spacing and crystal structures of the samples under investigation is XRD which is based on constructive interference of crystalline samples and monochromatic X-rays. The XRD principle is based on Bragg's law. X-ray diffractometers consist of the following:

- X-ray tube
- Sample holder

- X-ray detector

In this study, we have analyzed all samples using Rigaku Miniflex 600 Desktop X-Ray Diffraction (XRD) System by making the film of CNDs on a 1cm×1cm glass plate.

3.4.3.4. Raman spectroscopy

The most comprehensive technique used to study the rotational, vibrational, and other low-frequency modes present in specimens is Raman spectroscopy which is a non-destructive light scattering spectroscopic technique. It provides valuable information about phase and polymorphism, intrinsic stress or strain, chemical structure, and the presence of impurities in the samples. It is based on the phenomenon of inelastic scattering known as Raman scattering. In this study, Raman spectroscopy was carried out using Horiba's LabRam HR evolution spectrometer. The samples were irradiated with a HeNe laser (633nm, 30 mW) and 1800 groves/mm grating, with laser exposure of 20 seconds to acquire spectra. A Peltier cooled (-60 °C) CCD detector (model: Syncerity 356399) was employed to collect the Raman scattering signal.

3.4.3.5. Fourier transfor- infrared spectroscopy (FT-IR)

The widely employed technique for the identification of functional groups and types of chemical bonds by generating an infrared absorption spectrum is FT-IR. Its working principle is based on the Michelson interferometer, consisting of a beam splitter, a fixed mirror, and a movable mirror that translates back and forth precisely. In this study, the FT-IR analysis of as-prepared CNDs was carried out employing FT-IR (Shimadzu FTIR-8400S) spectrometer. The sample pellets were prepared by mixing the samples with

Kbr in 1:100 and then scanned in the range of 400-4000 cm^{-1} with a scan speed of 0.2cm/sec and spectral resolution of 4.0 cm^{-1}.

3.4.3.6 UV-visible spectroscopy

The interaction of light with chemical compounds is studied by employing UV-visible spectroscopy, and the UV-Visible range of electromagnetic radiation ranges from 200-800 nm. The principle of UV-visible spectroscopy is based on Beer-Lambert's law. Most of the absorptions observed exhibit π-π*, n-π*, and n-σ* transitions as most of the transitions from bonding orbitals are of too high frequency and too short wavelength, which makes their measurements difficult. UV-visible spectrophotometer consists of the following:

- Light sources- deuterium lamp, tungsten lamp
- Monochromator
- Detector

  In the present study, the absorption behavior of CNDs was investigated using a UV–vis double beam spectrophotometer (Cary 60 UV vis, USA) in the wavelength range of 200-800nm with 1cm path length cuvettes.

3.4.3.7. Fluorescence spectroscopy

The most widely used method to study the fluorescence or excitation/emission intensity of the samples is fluorescence spectroscopy which deals with the transition from the excited state to the ground state. Fluorescence occurs due to returning excited molecules to the ground state, emitting a photon of lower energy corresponding to a

longer wavelength than the absorbed photon. The fluorescence spectrophotometer consists of the following:

- Source
- Excitation monochromator
- Emission monochromator
- Detector
- Amplifier
- Recorder

In this study, the fluorescence spectrum of CNDs was recorded on a Fluorolog-Horiba fluorescence spectrophotometer.

3.4.4 Determination of the quantum yield of CNDs

The determination of quantum yield (QY) of CNDs was carried out using quinine sulfate (QY is 54% in 0.1 M H_2SO_4 solution) as a reference sample and calculated by equation **3.1**.

$$Q_{CNDs} = Q_R \times \frac{I_{CNDs}}{I_R} \times \frac{A_R}{A_{CNDs}} \times \frac{{\eta^2}_{CNDs}}{{\eta^2}_R} \tag{3.1}$$

Where η represents the refractive index of the solvent used, A refers to the absorbance measured at an exciting wavelength, Q represents the quantum yield, and I represents the fluorescence intensity. The subscript "R" denotes the reference sample, and CNDs denote carbon nanodots.

3.4.5 Stability studies of CNDs

Colloidal dispersion stability, thermal stability, and photostability of as-prepared CNDs were evaluated using different instruments.

3.4.5.1 Colloidal dispersion stability

The stability of the colloidal dispersions is indicated by Zeta potential (ζ), whose magnitude indicates the degree of electrostatic repulsion between adjacent and similarly charged particles. Higher the zeta potential (positive/negative) better the stability, whereas lower zeta potential makes the colloids coagulate or flocculate. A Malvern Zetasizer Pro was used to assess zeta potential. An average of three measurements (each with 15 runs) was considered for analysis.

3.4.5.2 Thermal stability

It was determined by measuring the loss of material weight as an increase in the temperature, employing Shimadzu (Asia Pacific) TGA-50 Thermogravimetric analyzer.

3.4.5.3 Photostability

UV light at a suitable excitation wavelength was used to test photostability for up to 2 hours at various time intervals. UV–vis double beam spectrophotometer (Cary 60 UV vis) was used for this purpose.

3.5 Applications of CNDs

3.5.1 Sensing of biologically and environmentally relevant metal ions

The sensing ability of relevant metal ions by various CNDs was studied as per earlier reported studies with slight modifications [Edison *et al*., 2016]. The sensing selectivity of

these CNDs towards various metal cations such as Mg^{2+}, Hg^{2+}, Fe^{3+}, Ba^{2+}, Cu^{2+}, Ca^{2+}, K^{+}, Na^{+}, NH_4^{+}, As^{3+}, Ag^{+}, Al^{3+}, Bi^{3+}, Pb^{2+}, etc., was analyzed by adding 2 mL of CNDs solution (300µg/mL) to 2 mL of aqueous metal solutions, with the final concentration of each metal ion 250µM. After incubating for a suitable time, the fluorescence intensity was measured at a suitable excitation wavelength. The metallic ion that dramatically altered the fluorescence intensity of CNDs was noted. To study the sensitivity of CNDs towards selected metal ions, fluorescence titrations were carried out using different concentrations of an aqueous metal cation and measuring the fluorescent intensities at the same excitation wavelength. The Stern-Volmer equation (equation **3.2**) was used to analyze the quenching efficiency of metallic ions.

$$\frac{Fo}{F} = 1 + K\,sv\,[Q] \tag{3.2}$$

Where [Q] represents the concentration of metal ions, Ksv represents the Stern–Volmer quenching constant, and F_0 and F are the fluorescence intensities of CNDs in the absence and presence of metal cation, respectively. The equation **3.3** was used for computing the limit of detection (LOD) of carbon nanodots on a selected metal ion:

$$LOD = 3\sigma/s \tag{3.3}$$

Where s is the slope of the linear line and σ is the standard deviation of F_0/F values.

3.5.2 Cytotoxic potential of CNDs

Cytotoxicity assay was employed to evaluate the cytotoxic potential of CNDs on different cell lines as per the previously reported method with slight modifications [Pandey *et al*., 2020]. In a 96-well culture plate, the cells were plated at a density of 1

$\times 10^6$/well and allowed to grow up to 60–70% confluency. The medium was withdrawn after incubation, and the cells were washed twice with PBS (10 mM, phosphate-buffered saline, pH 7.4) before being incubated for a suitable time duration with various doses of CNDs. Next, the cells were exposed to serum-free media containing 10 µL of MTT (5 mg/ml) and incubated for 3 hours at 37°C. The formazan crystals were dissolved in 150 µL DMSO after incubating for 15 minutes, and the absorbance was measured at 570 nm using an ELISA microplate reader. The detailed procedures for the cytotoxic potential of CNDs using different cell lines are provided in **chapters 4**, **5**, **6**, and **7** under the experimental section.

3.5.3. Free radical scavenging potential

The radical scavenging potential of CNDs was assessed using the DPPH assay as per the previously reported method with slight modifications [Sheng *et al*., 2022]. A fresh DPPH solution (100 µM) was prepared in the dark conditions using methanol. To make 1000 µg/ml stock solutions, CNDs were diluted with double-distilled water. To make immediate working solutions of various concentrations, different quantities of the stock mentioned above were diluted with water up to 1 ml. Then, to obtain varying samples in total volume (i.e., 3 ml), 1 ml of those mentioned above primary working solutions were added to 2 mL of prepared methanolic DPPH solution and incubated for 30 minutes in a dark atmosphere. The sample and standard concentrations that cause 50% inhibition (scavenging) of the DPPH radicals (IC_{50}) were computed. The % radical scavenging activity of CNDs can be calculated from the equation 2:

$$\%\ Scavenging\ activity = \frac{A_o - A_s}{A_o} \times 100 \quad (3.4)$$

Where Ao and As refer to the absorbance of the DPPH at 515 nm without and with CNDs, respectively.

3.5.4. Viability studies using multidrug-resistant bacterial cells

The disk diffusion method with slight modifications [Torkian *et al*., 2022] was employed to investigate the antibacterial activity of CNDs against different clinically isolated multidrug-resistant (MDR) Gram-positive (G+) and Gram-negative (G-) bacterial strains. These organisms were collected from the Department of Microbiology, Institute of Medical Science (BHU). From cultivated bacterial strains grown on Mueller-Hinton agar (MHA) media, bacterial suspensions with an optical density of 0.5 McFarland were made in isotonic sodium chloride solution. Using a sterile cotton bud, Petri plates containing 60mL of solidified sterile MHA medium were inoculated with 100µL of suspension containing 10^8 CFU/ mL (OD_{600}) of bacterial suspension and incubated at 37°C for 30 minutes. Sterile filter paper discs were gently placed on seeded plates after initial incubation. Then, on a pre-placed sterile paper disc, different concentrations of CNDs were supplied. Positive control of 10 µL/disc (1.00 mg/mL) of ciprofloxacin or doxycycline was utilized, while a negative control of one additional sterile blank disc was soaked with sterile water. The widths of the inhibition zones were measured in millimeters using a vernier caliper to observe the anti-bacterial activity. At least three independent experiments were carried out to obtain valid data. The detailed procedures for the antibacterial assay of CNDs are provided in **Chapters 4**, **5**, **6**, and **7** under the experimental section.

3.6. Animal studies

3.6.1 Animal ethics

All ethical principles laid down by the Committee for Control and Supervision of Experimental Animals (CPCSEA), Government of India (No. 2123/G0/Re/S/21/CPCSEA)] were followed while performing studies on animals (female swiss albino mice) which the Institutional Animal Ethical Committee approved (No. IIT (BHU)/IAEC/2022/003).

3.6.2 Animal husbandry

The animal house of BHU, Varanasi, India, supplied the thirty-five female Swiss albino mice, six weeks old, with a body weight of 25 ± 2.0 g. The mice were kept in polypropylene cages in the animal house quarantine facility of the Department of Pharmaceutical Engineering & Technology, IIT (BHU), Varanasi, India. Before the onset of the experiment, they were acclimatized for ten days with standard climatic conditions (humidity: 55% - 60%, room temperature (RT): 22 ± 2˚C, 12/12h light, and dark phases) with *ad libitum* food and water entitlements.

3.6.3 *In-vivo* toxicity evaluation

CNDs are expected not to induce severe adverse effects in animals to be used in therapeutic settings. The animals' overall health and body weight were checked regularly throughout the experiment. CNDs were tested *in-vivo* on mice for 14 days to see if they were biocompatible. Routine blood tests, blood biochemistry assays, and histological

examinations for the control group (PBS group), ARCD, and ARCCD groups were performed to examine the biosafety of the CNDs.

On day 0, the CNDs were dispersed in PBS to make different concentration dispersions, as shown in **Table 3.4.** and administered intraperitoneal injection. Mice in the control group were given PBS. All experimental animals survived the experiment, and their body weight changes were identical to those of the control group. On the 14th day, blood was obtained from the mice, and routine hematological and biochemical assays were performed using kits.

Table 3.4: Animals grouping for *in-vivo* toxicity studies of CNDs.

S.No	**Group Name**	**Group Particulars**	**Dose, if any (mg/kg)**	**Total No. of animals used**
1	Group I	ARCD	1 mg/kg, 2 mg/kg,	10 (5*2)
2	Group II	ARCCD	4 mg/kg, 8 mg/kg,	10 (5*2)
	Group III	AAPCD	2 mg/kg	5
3	Group IV	EAPCD	0.7 mg/kg	5
4	Group IV	Control	Saline	5
TOTAL				35

3.6.4. Evaluation of hematological and biochemical parameters in CNDs-treated mice

Blood biochemistry study was performed to investigate the potential toxicity of the CNDs components *in-vivo*. Various hematological and biochemical markers were assessed after the treatments ended. Briefly, 100 mL of blood was taken through heart puncture of mice and placed in a 1.5 mL centrifuge tube containing 10 μL of EDTA-Na_2 and hematological parameters such as hemoglobin (Hb), red blood cells (RBC), white blood cells, and others were measured. Blood samples were allowed to coagulate before the serum was separated for 10 minutes at 4 °C, and the samples were calculated using an automated biochemical analyzer (Erba EM360, Japan). Alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate transaminase (AST), total bilirubin (TB), blood urea nitrogen (BUN), creatinine (CRE), cholesterol, creatine phosphokinase (CPK), and other biochemical markers were examined.

3.6.5. Histological analysis of organs in treated mice

The major organs such as kidney, liver, lung, spleen, heart, brain were collected from sacrificed animals and then 10% paraformaldehyde (PBS buffered) was used to fix overnight. After embedding small part of fixed organs in wax blocks, about 4-5 μm thick section slides were subjected to hematoxylin and eosin (H & E) staining for visualization of changes in histological organs. The images of histopathological examination were captured using a Leica microscope.

3.7 Statistical analysis of data

GraphPad Prism-5 software, USA was used for statistically analyzing the results. Obtained values are demonstrated as Mean± Standard Deviation. One-way ANOVA gave differences among group means. For finding statistical significance in the mean among different experimental groups, Tukey's multiple comparison post hoc test was applied unless otherwise stated.