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TOXICITY EVALUATION OF NANOCONSTRUCT OF CHITOSAN AND TRIPOLYPHOSPHATE (TPP)

Mehendra Kumar Dewangan*, Durgeshnandani Sinha, Trilochan Satapathy, Vijay Kumar Singh and Amit Roy

Columbia Institute of Pharmacy, Tekari, Raipur (C.G.), India, Pin Code-493111.

***Corresponding Author: Mehendra Kumar Dewangan**
Columbia Institute of Pharmacy, Tekari, Raipur (C.G.), India, Pin Code-493111.

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1. INTRODUCTIN

The prefix "nano" has found in last decade an ever-increasing application to different fields of the knowledge.[1] Nanoscience, nanotechnology, nanomaterials or nanochemistry are only a few of the new nano-containing terms that occur frequently in scientific reports, in popular books as well as in newspapers and that have become familiar to a wide public, even of non-experts.[2] The prefix comes from the ancient Greek *νᾶνος* through the Latin *nanus* meaning literally *dwarf* and, by extension, *very small*. Within the convention of International System of Units (SI) it is used to indicate a reduction factor of 109 times.[3] So, the nanosized world is typically measured in nanometers (1nm corresponding to 10^{-9} m) and it encompasses systems whose size is above molecular dimensions and below macroscopic ones (generally > 1 nm and < 100 nm).[4]

Nanoparticles (NPs) represent a specific type of matter (from about 1 to 100 nm in size).[5] They are intermediate in size between bulk materials and atomic/molecular structures, and possess unique physical and chemical properties.[6] These distinctive properties, related to a high surface area to volume ratio and/or quantum effects, have spawned notable interest from engineers, biologists, chemists, and physicists. In the past decade, there has been an exponential growth in the synthesis of NPs, commonly termed as engineered nanoparticles (ENPs), due to their extensive use in emerging technologies and in consumer products such as electronic devices and other products used for personal care, biomedicine, agriculture, water/soil treatment, and renewable energy.[7-9] An array of ENPs have been manufactured which include mainly metals, non-metals, metal oxides, lipids, and polymers as well as various nanocomposites.[10,11]

Nanoparticle drug delivery systems are nanometeric carriers used to deliver drugs or biomolecules.[12] Generally, nanometeric carriers also comprise sub-micro particles with size below 100 nm and with various morphologies, including nanospheres, nanocapsules, nanomicelles, nanoliposomes, and nanodrugs, etc. Nanoparticle drug delivery systems have outstanding advantages:[13-16]

(1) They can pass through the smallest capillary vessels because of their ultra-tiny volume and avoid rapid clearance by phagocytes so that their duration in blood stream is greatly prolonged;
(2) They can penetrate cells and tissue gap to arrive at target organs such as liver, spleen, lung, spinal cord and lymph;
(3) They could show controlled release properties due to the biodegradability, pH, ion and/or temperature sensibility of materials;
(4) They can improve the utility of drugs and reduce toxic side effects; etc.

Chitosan is a non-toxic (In case of low amount non-toxic but in high amount in show toxic) biodegradable poly-cationic polymer with low immunogenicity.[17-20] Chitosan is a linear polysaccharide composed of randomly distributed-(1-4)-linked d-glucosamine and N-acetyl-d-glucosamine.[21-23] Due to the advantageous biological properties of chitosan, such as relative non-toxicity, biocompatibility, biodegradability, cationic properties, bio-adhesive characteristics and permeability-enhancing properties, chitosan-based particles have been extensively studied for delivery of anti-cancer agents, therapeutic proteins, genes, antigens.[24,25]

Chitosan (CS), the N-deacetylation form of chitin mostly found in the exoskeleton of crustacean, insects, and fungi, is a natural polysaccharide.[26-28] CS is not only non-toxic and biodegradable with low immunogenicity, but also possesses a high density of positive charge in an acid solution attributed to the glucosamine group on its backbone.[29,30] Because of these beneficial characteristics, increasing attention has been drawn to the applications of CS-based micro and nanoparticles in

the pharmaceutical and nutraceutical field.[31,32] Ionic gelation is the most studied and widely used method for fabricating CS nanoparticles, in which cationic CS and multivalent polyanions interact to form CS nanoparticles under simple and mild conditions. Among various polyanions, Tripolyphosphate (TPP) is the most investigated due to its quick gelling capability and non-toxic property.[33-36]

As drug delivery system, nanoparticles can entrap drugs or biomolecules into their interior structures and/or absorb drugs or biomolecules onto their exterior surfaces. Presently, nanoparticles have been widely used to deliver drugs, polypeptides, proteins, vaccines, nucleic acids, genes and so on.[37,38] Over the years, nanoparticle drug delivery systems have shown huge potential in biological, medical and pharmaceutical applications Chitosan has attracted considerable interest because of their unique combination of properties, such as biocompatibility, biodegradability, metal complexation and antibacterial activity.[39] Therefore, chitosan has a variety of current and potential applications in various fields, for example, biotechnology, pharmaceutics, wastewater treatment.[40]

The antibacterial activity of chitosan has been widely explored. A number of chitosan derivates with different modifications have been prepared to improve its antibacterial activity.[41] The metal-chelating property of chitosan has been mainly used in wastewater treatment. Recently, different metal chitosan complexes have been prepared to improve its antimicrobial activity.[42-44]

Toxicology traditionally addresses adverse poisoning effects of chemicals to humans, animals and the environment.[45,46] Historically, toxicology is often associated with Paracelsus and the concept of dose and dose response. He is attributed with having coined the phrase "the dose makes the poison", implying a linear relationship. However toxicological dose responses can be complex and decidedly non-linear especially in the low and high dose range.[47-54]

2. MATERIALS AND METHODS

2.1 Materials

Low molecular weight (LMW) water-soluble chitosan, Sodium Tripolyphosphate (TPP), glacial acetic acid, sodium hydroxide and all other chemicals were analytical grade, ultrapure water was used throughout this study.[39]

2.2 Animal used

Male Wistar rat of weight 180-250 gm were taken. Experimental protocols used in the experiment were approved by the Institutional Animal Ethic Committee (IAEC reg. no. CIP/IAEC/2015-16/063) of Columbia Institute of Pharmacy Tekari, Raipur (C.G.). The animals were housed in polycarbonate cages in a room with a 12 h day-night cycle, temperature of 22±2 ◦C, and humidity of 45-64%. During the whole experimental period, animals were fed with balanced commercial pellet diet (Ashirwad Industries, Mohali, India) and water *ad libitum* and normal control, Test Control, Reference group each containing six animals for In-vivo Nanoconstruct Toxicity activity.[56,57]

2.3 Preparation of Chitosan/TPP Nanoconstruct

2.3.1 Ionic gelation method

Chitosan was dissolved in an aqueous solution of acetic acid to form a 0.5 mg/mL chitosan solution. The concentration of acetic acid was 0.4 times (0.2 mg/mL) that of chitosan. The chitosan solution was stirred overnight at room temperature using a magnet stirrer. The pH of the resulting solution was around 3.6 and this was adjusted to 4.7-4.8 using 20 wt% aqueous sodium hydroxide solution. The chitosan solution was then passed through a syringe filter (pore size 0.45 µm, Millipore, USA) to remove residues of insoluble particles. TPP was dissolved in ultrapure water at a concentration of 0.5 mg/mL and also passed through a syringe filter (pore size 0.22 µm, Millipore, USA). To prepare chitosan nanoparticles, a magnetic stirrer was placed in a chest freezer, in which the ambient temperature was controlled at 2-4◦C, temperature fluctuations and flow of cold air should be avoided as much as possible. Ten millilitres of chitosan solution in a 25 mL round-bottom flask was preheated in a water bath at 60◦C For 10 min, the flask was then placed on the magnetic stirrer stirring at 700 rpm, 3.0 ml of 2-4◦C TPP solution was quickly added to the chitosan solution with a plastic Pasteur pipette. The reaction was carried out for 10 min and the resulting Suspension was subjected to further analysis.[39]

2.4 Characterization and morphology of Chitosan/TPP Nanoconstruct

2.4.1 Transmission electron microscope (TEM)

The size of the nanoparticles was examined using a high resolution Transmission Electron Microscope (TEM) machine (JOEL 2100F).

2.4.2 Particle size and zeta potential

The sizes and zeta potential of the CNP were measured with a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, U.K.). The particle size distribution of the nanoparticles is reported as a polydispersity index (PDI). All measurements were performed in triplicates. 3 ml of sample was taken in a cuvette and was analyzed at 25 _C with at an angle of 90.

2.5 In-Vivo experimental method

2.5.1 Acute oral toxicity studies

The animals used in the toxicity studies were approved by the Institute Animal Ethics Committee (IAEC), India. Wistar male rats weighing 180-250 gm were used. The acute toxicity study was carried out on male Wistar rats by administering Nanoconstruct orally at one of the levels (0.5, 1.0, 1.5, 2.0 and 2.5 ml/kg) once only. Mortality was not found up to 2.5 ml/kg dose. So, as per

OECD guideline 423 which was half of the maximum dose was considered for therapeutic exploration.[56]

2.5.2 Repeated dose 28 days oral toxicity studies:

The animals used in the toxicity studies were approved by the Institute Animal Ethics Committee (IAEC), India. Wistar male rats weighing 180-250 gm were used. The acute toxicity study was carried out on male Wistar rats by administering Nanoconstruct orally at repeated 28 days of the levels (0.5, 1.0, 1.5, 2.0 and 2.5 ml/kg) single dose daily only. So, as per OECD guideline 407.[57]

2.5.2.1 Haematology

Blood was collected on the initial day, after 7, 14, 21 and 28 days by Retro orbital plexus from the overnight fasted animals. Investigation of whole blood for following was done: Red blood cells (RBCs), White blood cells (WBCs), Haemoglobin (Hb), Platelet count, Haematocrit (HTC), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), RDW-SD, RDW-CV, PDW, MPV, P-LCR, PCT, Neutrophil (N), Lymphocytes (L), Monocytes (M), Eosinophil's (E), Basophils (B).[57]

2.5.2.2 Clinical Biochemistry

Blood was collected on the initial day, after 7, 14, 21 and 28 days from retro orbital plexus of overnight fasting rats and different parameters in blood like determination in serum or plasma should include: Sodium, potassium, glucose, total cholesterol, urea, blood urea nitrogen, creatinine, total protein and albumin, Alkaline Phosphatase (ALP), Alanine aminotransferase, Aspartate aminotransferase, Gamma glutamyl transpeptidase, Sorbitol dehydrogenase, Bile acids.[57]

2.5.2.3 Histopathology

All animals were sacrificed and organs Liver, Kidney were fixed immediately in 10% formalin for routine Histopathological examination. The tissues were embedded in paraffin and then sectioned, stained with Haematoxylin and Eosin and were examined under light microscope.[57]

2.5.2.4 Statistical analysis

All studies were performed in triplicate and the values were expressed in mean ± SD. The data was analysed by one way analysis of variance (ANOVA) Graph Pad Prism Instat Software (version 6.00, Graph Pad Software), using one way ANOVA followed by student test. ANOVA was done to show that the work done is statistically significant a value of $P < 0.05$ was considered to be statistically insignificant and significant.

3. RESULTS AND DISCUSSION

3.1 Morphological characterization

3.1.1 Transmission Electron Microscopy (TEM)

TEM is an important parameter for the surface morphology characterization of Nanoconstruct. The surface morphology of the prepared Nanoconstruct was characterized by Transmission Electron Microscope (TEM) studies. The results of TEM pictures reveal that Nanoconstruct were almost spherical in shape. TEM images of the formulation are shown in fig. 3.1.

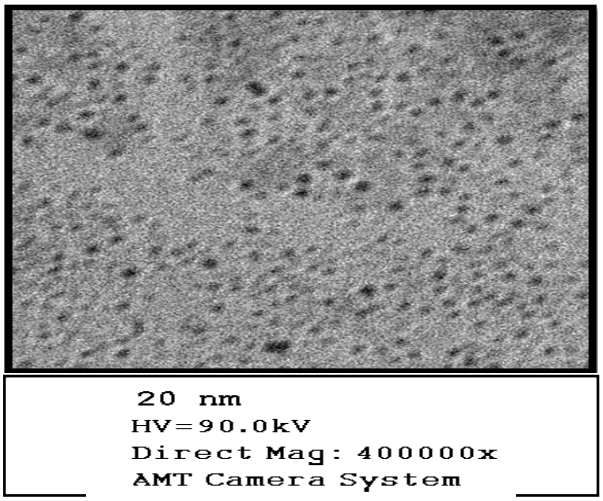
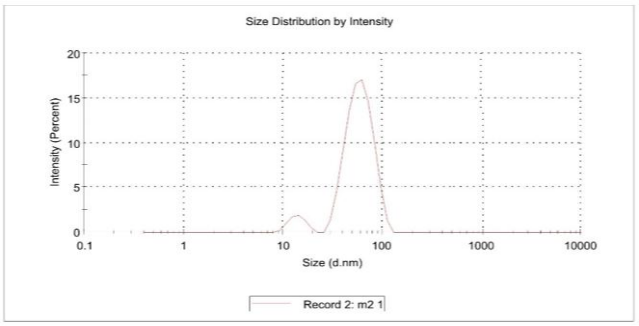


Fig. 3.1: Transmission Electron Microscope (TEM) of the Nanoconstruct Optimized Respectively Size, Range – 20 nm taken at 400000 X Magnification.

3.2 Zetasizer

The Zeta potential of formulations were analysed by using Malvern Zetasizer. Zeta potential is the electric potential of a particle in a formulation. This parameter is useful for the assessment of physical stability of the colloidal dispersion. Zeta potential and particle size of Nanoconstruct were found in Zetasizer and shown in fig.3.2.



Results

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 66.9	Peak 1:	61.5	93.3	74.40
PdI: 0.90	Peak 2:	8.20	0.7	5.35
Intercept: 0.286	Peak 3:	0.000	0.0	0.000

Result quality : Good

Fig. 3.2: Zetasizer analysis graph of the nanoconstruct optimized respectively size range is 66.9.

3.3 Acute oral toxicity studies

There was no mortality or morbidity observed in animals through the 3-days period following single oral administration at all selected dose levels of the Nanoconstructs.

3.4 Repeated dose 28 days oral toxicity study

3.4.1 Body weight (gm)

Table 3.1: Effect of Nanoconstruct on Body weight in Albino Wistar Rats.

Duration	Control	0.5 ml/kg	1.0 ml/kg	1.5 ml/kg	2.0 ml/kg	2.5 ml/kg
0 Day	170.21±1.03	171.97±0.71	173.53±1.05	173.50±0.84	172.62±0.87	171.72±0.43
7 Days	180.66±0.52	181.86±0.48	183.83±0.80	183.09±0.60	182.61±0.75	181.23±0.45
14 Days	190.83±0.58	192.36±0.52	194.87±0.50	192.47±0.63	193.29±0.82	191.01±0.46
21 Days	200.83±0.45	201.27±0.33	205.37±0.83	202.16±0.72	201.76±0.55	200.12±1.41
28 Days	212.25±0.73	211.92±0.70	214.10±0.79	211.90±0.49	209.23±0.34	206.58±1.35

Value are Mean±SEM. (n=6).

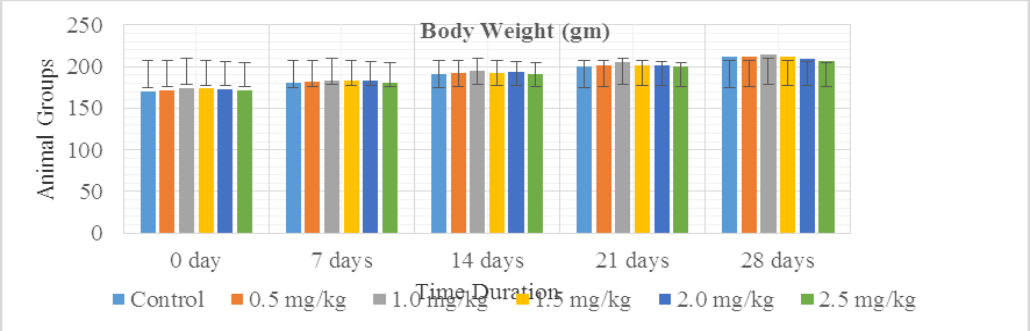


Fig. 3.3: Animals mean body weight during pre-dosing, Number of animals per group=6. All value are reported as Mean±SEM.

3.4.2 Food consumption (gm)

Table 3.2: Effect of Nanoconstruct on Food consumption in Albino Wistar Rats.

Duration	Control	0.5 ml/kg	1.0 ml/kg	1.5 ml/kg	2.0 ml/kg	2.5 ml/kg
0 Day	83.16±0.28	83.33±0.30	82.17±0.28	83.50±0.39	82.50±0.45	83.17±0.36
7 Days	83.50±0.20	84.00±0.40	85.50±0.39	84.67±0.56	84.67±0.38	85.50±0.39
14 Days	87.66±0.38	87.16±0.28	88.00±0.33	87.83±0.43	87.67±0.45	87.83±0.43
21 Days	95.16±0.28	95.67±0.30	94.00±0.33	93.50±0.39	94.33±0.45	94.17±0.28
28 Days	106.16±0.43	106.00±0.33	105.83±0.43	104.33±0.65	106.00±0.33	105.83±0.49

Value are Mean±SEM. (n=6).

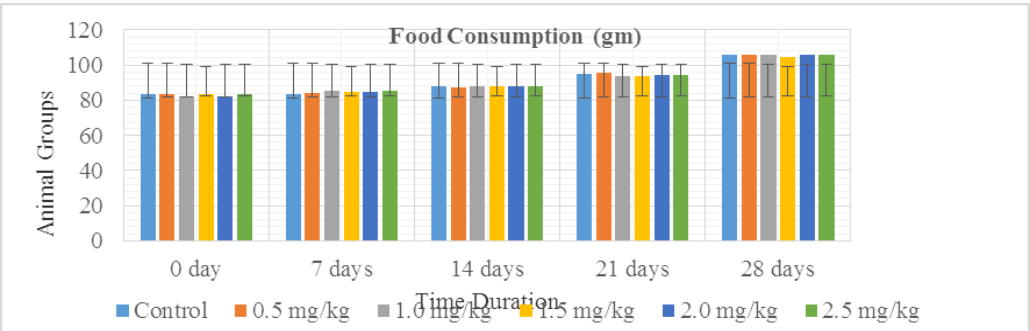


Fig. 3.4: Animals mean food consumption during dosing, Number of animals per group=6. All value are reported as Mean±SEM.

3.4.3 Water consumption (ml)

Table 3.3: Effect of Nanoconstruct on Water consumption in Albino Wistar Rats.

Duration	Control	0.5 ml/kg	1.0 ml/kg	1.5 ml/kg	2.0 ml/kg	2.5 ml/kg
0 Day	80.17±0.28	81.17±0.28	80.67±0.30	81.00±0.33	81.83±0.43	81.83±0.43
7 Days	82.00±0.33	82.00±0.52	82.33±0.38	83.50±0.39	84.67±0.30	82.500.54
14 Days	89.83±0.64	90.17±0.49	90.17±0.54	88.50±0.31	89.17±0.64	89.67±0.50
21 Days	94.00±0.78	94.83±0.43	93.67±0.45	92.50±0.56	93.67±0.93	93.33±0.76
28 Days	106.33±0.30	105.67±0.73	105.67±0.73	105.67±0.38	105.67±0.83	106.00±0.47

Value are Mean±SEM. (n=6).

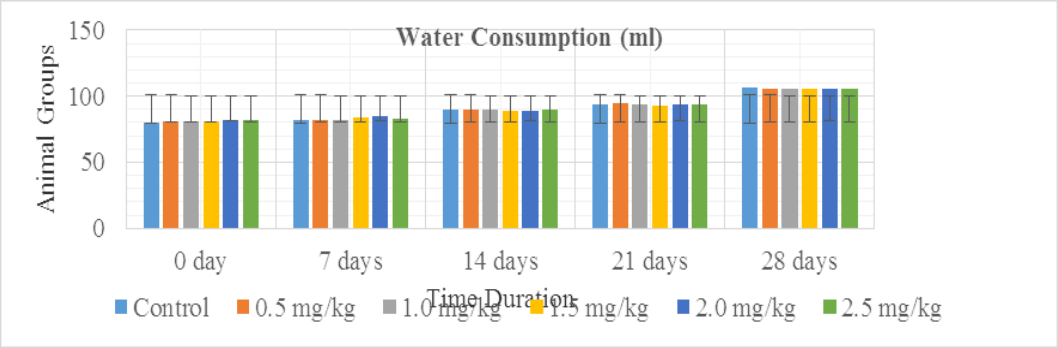


Fig. 3.5: Animals mean water consumption during dosing, Number of animals per group=6. All value are reported as Mean±SEM.

3.4.4 Organ weight (gm)

Table 3.4: Effect of Nanoconstruct on Organ weight in Albino Wistar Rats.

Animal Organ	Control	0.5 ml/kg	1.0 ml/kg	1.5 ml/kg	2.0 ml/kg	2.5 ml/kg
Liver	5.195±0.013	5.175±0.013	5.171±0.013	5.181±0.007	5.230±0.005	5.242±0.002
Kidney	0.573±0.003	0.482±0.004	0.516±0.001	0.542±0.004	0.544±0.003	0.551±0.002
Heart	0.853±0.001	0.835±0.002	0.815±0.002	0.850±0.008	0.849±0.002	0.858±0.002
Pancreas	0.244±0.004	0.241±0.004	0.221±0.002	0.234±0.001	0.238±0.002	0.245±0.004

Value are Mean±SEM (n=6).

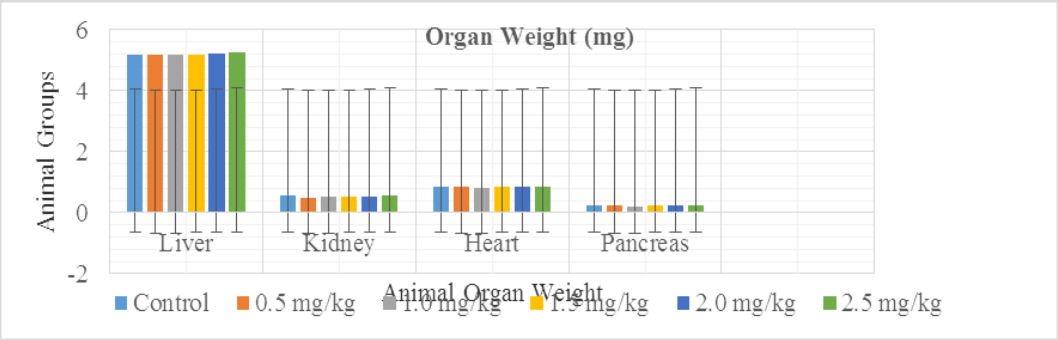


Fig. 3.6: Animals mean Organ weight after during dosing, Number of animals per group=6. All value are reported as Mean±SEM.

3.4.5 Haematological parameters

3.4.5.1 Comparison of First day Haematological parameters (Complete Blood Count, Liver Function Test, Lipid Profile) test reports with Mean±SEM.

Table 3.5: Effect of Nanoconstruct on Haematological parameters (Complete Blood Count) in Albino Wistar Rats.

Parameters	Control	0.5 ml/kg	1.0 ml/kg	1.5 ml/kg	2.0 ml/kg	2.5 ml/kg
Complete Blood Count						
Haemoglobin	15.32±0.142	15.53±0.030	13.53±0.030	14.11±0.028	14.55±0.031	15.63±0.038
WBC Count (10^3/uL)	10.13±0.123	10.16±0.030	9.13±0.019	10.35±0.031	10.13±0.019	10.46±0.019
Neutrophils	41.16±0.597	42.33±0.384	41.5±0.311	44.16±0.495	43.33±0.192	43.33±0.962
Lymphocytes	51.66±0.384	50.66±0.384	48.16±0.495	42.16±0.495	41.33±0.192	41.66±0.962
Eosinophil	5.83±0.152	5.66±0.192	5.66±0.192	4.5±0.311	5.66±0.192	5.16±0.152
Monocytes	1±0	1.33±0.192	2±0.235	1.33±0.192	4.16±0.152	6.16±0.495
Basophils	0.1±0	0.2±0	0.13±0.019	0.13±0.019	0.13±0.019	0.16±0.019
RBC Count (10^6/uL)	6.10±0.012	6.11±0.002	6.14±0.034	6.14±0.009	6.16±0.003	6.25±0.101
Platelet Count	2.9±0	3.00±0.003	2.91±0.008	2.81±0.001	2.91±0.001	2.62±0.004
Mean Platelet Value	10.5±0	9.21±0.003	9.71±0.036	9.78±0.059	10.86±0.031	8.92±0.007
Packed Cell Volume	42.96±0.161	42.25±0.065	40.15±0.031	42.13±0.019	40.18±0.059	42.13±0.019
Mean Corp. Vol.	70.52±0.072	70.15±0.003	68.14±0.003	69.17±0.006	79.18±0.006	80.14±0.002
Mean Corpuscular	24.56±0.013	24.33±0.007	26.34±0.004	26.73±0.005	25.72±0.007	23.23±0.009
Mean Corp. Hb Con.	34.65±0.087	32.66±0.069	31.73±0.030	33.78±0.036	32.2±0.074	33.13±0.019

Red Cells Destribution Width	14.5±0.108	12.6±0.047	11.63±0.038	13.65±0.031	19.18±0.049	19.43±0.019
Biochemistry (Liver Function Test)						
Bilirubin-Total	0.88±0.003	0.67±0.005	0.65±0.002	0.61±0.001	0.75±0.001	0.77±0.005
Bilirubin-Direct	0.20±0.003	1.1±0.003	0.21±0.007	0.24±0.003	0.14±0.001	0.16±0.003
Bilirubin-Indirect	0.67±0.010	2.89±0.003	0.47±0.004	0.46±0.003	0.41±0.001	0.45±0.001
Total Protein	6.6±0.047	6.85±0.020	6.56±0.045	5.45±0.031	6.65±0.031	6.73±0.096
Albumin	3.31±0.047	3.21±0.028	3.13±0.030	3.61±0.049	3.83±0.038	3.43±0.038
Globulin	2.81±0.039	2.63±0.038	2.21±0.036	2.48±0.036	3.13±0.019	3.25±0.031
A/G Ratio	1.17±0.005	1.12±0.003	1.32±0.004	1.11±0.003	1.22±0.003	1.27±0.005
SGOT	80.28±0.042	82.41±0.059	83.53±0.019	78.5±0.023	79.36±0.038	84.33±0.019
SGPT	16.81±0.028	17.68±0.059	18.83±0.019	19.13±0.019	20.13±0.019	22.43±0.019
Alkaline Phosphatase	43.26±0.054	44.16±0.030	40.15±0.031	45.13±0.019	48.86±0.019	58.8±0.057
Biochemistry (Lipid Profile)						
Blood Glucose (Random)	82.86±0.215	87.18±0.028	81.2±0.023	89.55±0.031	89.56±0.019	95.36±0.038
Serum CREATININE	0.71±0.023	0.78±0.028	0.76±0.019	0.75±0.031	0.76±0.030	0.63±0.038
S. Cholesterol	124.5±0.865	136.1±0.028	126.2±0.023	116.6±0.038	136.6±0.040	126.4±0.019
S. Triglycerides	70.0541.16±	81.5±0.031	79.13±0.090	84.05±0.031	89.16±0.019	89.13±0.019
HDL Cholesterol	19.23±0.005	18.24±0.003	19.23±0.005	17.13±0.003	19.13±0.009	19.21±0.003
LDL Cholesterol	62.74±0.006	61.73±0.006	58.74±0.004	68.75±0.002	66.72±0.002	76.74±0.005
VLDL Cholesterol	14.28±0.052	15.23±0.038	16.16±0.019	17.2±0.023	17.36±0.038	19.33±0.019
LDLC/HDLC Ratio	2.44±0.005	2.43±0.010	2.44±0.003	2.46±0.004	2.41±0.001	2.12±0.007
TC/HDLC Ratio	3.67±0.004	3.56±0.003	3.34±0.005	3.31±0.002	3.43±0.005	3.85±0.012
S. Cholesterol/HDLC Ratio	5±0	5±0	5±0	5±0	5±0	5±0

Value are Mean±SEM. (n=6).

3.4.5.2 Comparison of After 7 day Haematological parameters (Complete Blood Count, Liver Function Test, Lipid Profile) test reports with Mean±SEM.

Table 3.6: Effect of Nanoconstruct on Haematological parameters (Complete Blood Count) in Albino Wistar Rats.

Parameters	**Control**	**0.5 ml/kg**	**1.0 ml/kg**	**1.5 ml/kg**	**2.0 ml/kg**	**2.5 ml/kg**
Complete Blood Count						
Haemoglobin	15.65±0.085	15.08±0.036	15.5±0.031	16.13±0.019	15.06±0.038	16.51±0.028
WBC Count (10^3/uL)	10.11±0.064	9.41±0.043	9.48±0.059	9.73±0.045	9.16±0.019	9.58±0.026
Neutrophils	43.5±0.515	45.16±0.435	41.66±0.192	45.5±0.311	42±0.471	41.33±0.384
Lymphocytes	50.5±0.392	54.33±0.384	51.5±0.311	51±0.745	51.33±0.192	54.33±0.192
Eosinophil	6±0	5.33±0.192	5±0.233	5.16±0.152	5.16±0.152	5.66±0.192
Monocytes	1±0	2.33±0.192	2±0.235	2±0.235	1.83±0.152	1.66±0.192
Basophils	0.2±0	0.11±0.015	0.13±0.019	0.13±0.019	0.23±0.050	0.16±0.019
RBC Count (10^6/uL)	6.12±0.002	6.37±0.004	6.36±0.006	6.32±0.004	6.37±0.005	6.42±0.010
Platelet Count	2.91±0.005	3.84±0.002	3.82±0.006	3.74±0.001	3.84±0.001	3.84±0.005
Mean Platelet Value	10.5±0	8.24±0.003	9.24±0.001	9.25±0.001	9.21±0.001	9.23±0.009
Packed Cell Volume	43.13±0.019	39.45±0.031	39.55±0.031	38.55±0.031	39.48±0.015	39.38±0.054
Mean Corp. Vol.	70.61±0.003	63.10±0.003	68.14±0.003	68.10±0.004	68.15±0.001	68.12±0.010
Mean Corpuscular	24.51±0.004	24.74±0.003	25.75±0.003	25.74±0.001	25.79±0.003	25.62±0.010
Mean Corp. Hb Con.	38.75±0.031	42.42±0.005	42.42±0.002	42.46±0.001	42.41±0.009	42.41±0.009
Red Cells Destribution Width	14.18±0.025	14.43±0.019	14.43±0.019	15.4±0.023	14.23±0.078	14.43±0.019
Biochemistry (Liver Function Test)						
Bilirubin-Total	0.81±0.003	0.74±0.003	0.73±0.001	0.74±0.001	0.62±0.001	0.79±0.001
Bilirubin-Direct	0.21±0.004	0.23±0.003	0.21±0.007	0.25±0.002	0.24±0.004	0.23±0.009
Bilirubin-Indirect	0.68±0.005	0.56±0.007	0.57±0.001	0.57±0.004	0.46±0.003	0.52±0.007
Total Protein	6.63±0.035	6.15±0.031	6.2±0.062	6.13±0.019	6.11±0.015	6.56±0.019
Albumin	3.45±0.054	3.21±0.036	3.2±0.023	3.25±0.031	3.21±0.015	3.16±0.019
Globulin	2.35±0.055	3.15±0.031	3.23±0.065	3.13±0.019	3.15±0.031	3.63±0.038
A/G Ratio	1.16±0.003	1.06±0.003	1.07±0.005	1.03±0.006	1.03±0.006	1.07±0.005

SGOT	81.31±0.055	152.2±0.023	152.2±0.059	152.4±0.065	152.5±0.019	132.4±0.061
SGPT	16.76±0.038	31.45±0.031	31.23±0.080	31.13±0.019	31.11±0.015	31.2±0.057
Alkaline Phosphatase	43.16±0.038	101.35±0.03	101.3±0.051	101.8±0.019	101.8±0.045	102.2±0.020
Biochemistry (Lipid Profile)						
Blood Glucose (Random)	81.26±0.054	80.33±0.019	80.23±0.050	82.15±0.020	80.16±0.096	82.36±0.038
Serum CREATININE	0.7±0	0.58±0.015	0.55±0.031	0.56±0.019	0.54±0.152	0.56±0.019
S. Cholesterol	126±0.101	50.15±0.318	50.15±0.031	50.13±0.019	50.13±0.019	50.36±0.038
S. Triglycerides	71.06±0.037	104.8±0.047	104.6±0.038	104.8±0.049	104.8±0.076	104.8±0.038
HDL Cholesterol	19.13±0.003	11.36±0.006	11.37±0.003	11.37±0.004	11.36±0.009	11.36±0.006
LDL Cholesterol	62.55±0.002	24.20±0.004	24.20±0.003	24.25±0.001	24.21±0.009	24.21±0.009
VLDL Cholesterol	14.23±0.052	23.24±0.001	23.24±0.002	23.21±0.003	23.24±0.001	23.54±0.001
LDLC/HDLC Ratio	2.45±0.004	2.41±0.003	2.42±0.002	2.40±0.003	2.41±0.001	2.45±0.002
TC/HDLC Ratio	3.68±0.003	9.54±0.005	9.5±0.007	9.51±0.003	9.74±0.001	9.53±0.005
S. Cholesterol/HDLC Ratio	5±0	5±0	5±0	5±0	5±0	5±0

Value are Mean±SEM. (n=6).

3.4.5.3 Comparison of After 14 days Haematological parameters (Complete Blood Count, Liver Function Test, Lipid Profile) test reports with Mean±SEM.

Table 3.7: Effect of Nanoconstruct on Haematological parameters (Complete Blood Count) in Albino Wistar Rats.

Parameters	Control	0.5 ml/kg	1.0 ml/kg	1.5 ml/kg	2.0 ml/kg	2.5 ml/kg
Complete Blood Count						
Haemoglobin	15.41±0.065	15.05±0.031	15.1±0.074	15.05±0.031	13.6±0.038	15.25±0.155
WBC Count (10^3/uL)	10.28±0.154	7.46±0.019	7.43±0.019	7.2±0.023	7.38±0.036	7.53±0.019
Neutrophils	44.66±0.385	40.33±0.192	42±0.235	41.5±0.204	42.16±0.597	43±0.577
Lymphocytes	51.16±0.282	51.33±0.192	51.66±0.451	51.83±0.597	51.83±0.597	50.66±0.192
Eosinophil	5.66±0.190	4.66±0.192	5±0.235	4.5±0.311	5.33±0.192	5.33±0.192
Monocytes	1.66±0.190	2.16±0.152	2±0.235	1.66±0.192	1.83±0.152	1.66±0.192
Basophils	0.11±0.015	0.13±0.019	0.13±0.019	0.21±0.015	0.16±0.038	0.13±0.019
RBC Count (10^6/uL)	6.41±0.006	6.37±0.005	6.35±0.004	6.16±0.005	6.37±0.005	6.37±0.005
Platelet Count	2.13±0.004	.3.84±0.005	3.43±0.005	3.42±0.006	3.84±0.003	3.84±0.001
Mean Platelet Value	10.23±0.038	9.21±0.003	9.23±0.008	9.14±0.001	9.23±0.009	9.23±0.009
Packed Cell Volume	41.33±0.038	39.45±0.031	39.5±0.023	39.25±0.031	39.23±0.050	39.46±0.019
Mean Corp. Vol.	73.22±0.006	63.15±0.001	68.14±0.002	68.36±0.001	68.11±0.009	68.12±0.010
Mean Corpuscular	21.43±0.006	25.74±0.003	25.63±0.045	25.74±0.001	25.41±0.003	25.72±0.010
Mean Corp. Hb Con.	35.14±0.005	43.70±0.002	42.73±0.006	42.71±0.001	42.23±0.008	42.41±0.009
Red Cells Destribution Width	14.36±0.045	15.38±0.036	14.31±0.068	14.43±0.019	14.43±0.019	14.6±0.147
Biochemistry (Liver Function Test)						
Bilirubin-Total	0.81±0.001	0.75±0.003	0.73±0.003	0.70±0.003	0.71±0.001	0.75±0.017
Bilirubin-Direct	0.25±0.003	0.26±0.003	0.26±0.004	0.21±0.001	0.26±0.001	0.24±0.002
Bilirubin-Indirect	0.72±0.006	0.55±0.006	0.56±0.004	0.56±0.003	0.55±0.010	0.56±0.003
Total Protein	6.55±0.039	6.46±0.019	6.38±0.008	6.46±0.050	6.46±0.019	6.15±0.020
Albumin	3.15±0.020	3.2±0.023	3.26±0.045	3.13±0.019	3.15±0.031	3.16±0.019
Globulin	2.23±0.019	3.13±0.019	3.13±0.019	3.28±0.068	3.2±0.023	3.15±0.020
A/G Ratio	155±0.003	1.07±0.004	1.07±0.003	1.06±0.003	1.07±0.005	1.07±0.001
SGOT	83.40±0.028	159.4±0.028	142.4±0.068	150.4±0.045	150.5±0.019	152.3±0.061
SGPT	12.21±0.028	31.15±0.031	31.28±0.111	31.13±0.019	31.13±0.019	31.21±0.068
Alkaline Phosphatase	38.78±0.028	102.6±0.031	101.6±0.049	100.1±0.019	103.7±0.038	101.3±0.057
Biochemistry (Lipid Profile)						
Blood Glucose (Random)	82.18±0.028	81.2±0.023	81.31±0.068	79.28±0.049	80.33±0.019	80.3±0.057
Serum CREATININE	0.56±0.019	0.55±0.031	0.56±0.019	0.51±0.059	0.55±0.020	0.55±0.020
S. Cholesterol	140.15±0.02	50.4±0.023	50.45±0.051	50.35±0.031	50.33±0.038	50.13±0.019
S. Triglycerides	52.06±0.030	104.8±0.031	104.4±0.031	104.9±0.031	104.9±0.019	104.8±0.076
HDL Cholesterol	21.20±0.003	11.37±0.003	10.0051.27±	11.31±0.003	11.97±0.001	11.38±0.006

LDL Cholesterol	65.52±0.007	24.18±0.002	24.17±0.003	24.17±0.003	24.17±0.004	24.20±0.002
VLDL Cholesterol	25.21±0.028	23.24±0.001	23.24±0.009	23.23±0.008	23.14±0.006	23.24±0.002
LDLC/HDLC Ratio	2.44±0.003	2.41±0.002	2.42±0.004	2.42±0.005	2.42±0.011	2.43±0.006
TC/HDLC Ratio	3.57±0.002	9.55±0.001	9.52±0.006	9.52±0.009	9.51±0.003	9.51±0.001
S. Cholesterol/HDLC Ratio	5±0	5±0	5±0	5±0	5±0	5±0

Value are Mean±SEM. (n=6).

3.4.5.4 Comparison of After 21 days Haematological parameters (Complete Blood Count, Liver Function Test, Lipid Profile) test reports with Mean±SEM.

Table 3.8: Effect of Nanoconstruct on Haematological parameters (Complete Blood Count) in Albino Wistar Rats.

Parameters	**Control**	**0.5 ml/kg**	**1.0 ml/kg**	**1.5 ml/kg**	**2.0 ml/kg**	**2.5 ml/kg**
Complete Blood Count						
Haemoglobin	13.58±0.028	15.05±0.031	15.1±0.062	15.06±0.038	15.1±0.040	16.16±0.096
WBC Count (10^3/uL)	10.51±0.028	5.5±0.023	5.6±0.023	5.36±0.050	5.35±0.045	5.66±0.019
Neutrophils	44.5±0.311	42±0.235	41.83±0.366	41.83±0.597	42.33±0.509	47±0.577
Lymphocytes	51.16±0.280	51.5±0.204	53.83±0.683	51.33±0.192	51.16±0.366	51±0.235
Eosinophil	5.66±0.192	5±0.235	6.5±0.311	4.83±0.152	4.66±0.502	4.83±0.152
Monocytes	2±0.235	1.66±0.192	2±0.235	1.66±0.192	1.66±0.192	1.83±0.152
Basophils	0.15±0.020	0.13±0.019	0.18±0.028	0.13±0.019	0.16±0.038	0.83±0.152
RBC Count (10^6/uL)	6.41±0.003	36.37±0.003	6.46±0.012	6.37±0.001	6.77±0.001	6.37±0.005
Platelet Count	2.14±0.001	3.83±0.003	3.84±0.009	3.84±0.001	3.84±0.001	3.84±0.001
Mean Platelet Value	10.21±0.028	9.25±0.002	9.25±0.002	9.24±0.001	9.22±0.009	9.25±0.001
Packed Cell Volume	41.4±0.047	39.48±0.036	31.53±0.019	39.4±0.057	39.25±0.087	39.4±0.057
Mean Corp. Vol.	73.41±0.002	68.15±0.001	62.15±0.003	68.14±0.001	68.14±0.008	69.21±0.052
Mean Corpuscular	21.54±0.003	25.74±0.003	25.73±0.008	25.73±0.007	25.64±0.011	22.73±0.009
Mean Corp. Hb Con.	35.15±0.003	42.71±0.007	40.71±0.008	42.31±0.009	42.73±0.009	42.70±0.001
Red Cells Destribution Width	14.21±0.028	14.45±0.031	14.43±0.019	14.51±0.015	14.43±0.019	15.43±0.019
Biochemistry (Liver Function Test)						
Bilirubin-Total	0.84±0.001	0.73±0.004	0.75±0.003	0.72±0.001	0.73±0.003	0.73±0.003
Bilirubin-Direct	0.25±0.002	0.26±0.003	0.27±0.002	0.26±0.003	0.26±0.003	0.25±0.010
Bilirubin-Indirect	0.71±0.003	0.56±0.001	0.56±0.003	0.55±0.007	0.58±0.006	0.56±0.003
Total Protein	6.50±0.019	6.36±0.065	6.41±0.036	6.38±0.068	6.51±0.083	6.4±0.057
Albumin	3.25±0.028	3.16±0.019	3.26±0.038	3.21±0.015	3.18±0.015	3.2±0.023
Globulin	2.13±0.019	3.2±0.047	3.1±0.023	3.11±0.015	3.11±0.015	3.13±0.019
A/G Ratio	1.55±0.003	1.07±0.004	1.06±0.005	1.08±0.002	1.07±0.003	1.07±0.005
SGOT	81.41±0.028	151.3±0.065	105.4±0.057	142.5±0.019	153.4±0.049	132.5±0.019
SGPT	12.16±0.019	31.15±0.031	31.23±0.076	31.13±0.019	31.16±0.030	31.13±0.019
Alkaline Phosphatase	31.78±0.028	100.5±0.050	107.5±0.069	108.7±0.031	102.6±0.038	101.6±0.040
Biochemistry (Lipid Profile)						
Blood Glucose (Random)	80.15±0.020	80.2±0.023	80.25±0.031	82.18±0.015	82.4±0.091	80.3±0.057
Serum CREATININE	0.53±0.019	0.56±0.019	0.51±0.059	0.58±0.015	0.55±0.031	0.56±0.019
S. Cholesterol	140.15±0.02	50.36±0.050	52.31±0.049	50.45±0.031	51.43±0.019	50.43±0.019
S. Triglycerides	52.06±0.030	104.8±0.031	114.8±0.049	104.9±0.019	104.9±0.019	104.8±0.076
HDL Cholesterol	21.21±0.005	11.37±0.005	11.37±0.005	11.37±0.005	11.37±0.003	13.36±0.006
LDL Cholesterol	65.55±0.003	24.17±0.003	24.15±0.011	24.23±0.003	23.17±0.005	24.14±0.015
VLDL Cholesterol	25.25±0.020	23.24±0.001	23.23±0.007	23.23±0.003	23.23±0.001	23.24±0.001
LDLC/HDLC Ratio	2.40±0.001	2.41±0.001	2.41±0.003	2.41±0.002	2.42±0.008	2.31±0.002
TC/HDLC Ratio	3.57±0.004	9.56±0.001	9.42±0.002	9.52±0.007	9.57±0.011	9.53±0.006
S. Cholesterol/HDLC Ratio	5±0	5±0	5±0	5±0	5±0	5±0

Value are Mean±SEM. (n=6).

3.4.5.5 Comparison of After 28 days Haematological parameters (Complete Blood Count, Liver Function Test, Lipid Profile) test reports with Mean±SEM.

Table 3.9: Effect of Nanoconstruct on Haematological parameters (Complete Blood Count) in Albino Wistar Rats.

Parameters	Control	0.5 ml/kg	1.0 ml/kg	1.5 ml/kg	2.0 ml/kg	2.5 ml/kg
Complete Blood Count						
Haemoglobin	12.26±0.140	15.05±0.031	14.5±0.031	16.48±0.106	16.06±0.038	16.16±0.096
WBC Count (10^3/uL)	10.28±0.151	3.61±0.049	3.25±0.031	3.33±0.038	3.7±0.357	3.56±0.019
Neutrophils	41.5±0.390	42±0.235	38.8±0.311	38.66±0.192	44±0.235	40.33±0.192
Lymphocytes	51.83±0.280	51.5±0.311	51.5±0.311	54.33±0.192	53±0.577	54.33±0.192
Eosinophil	5.33±0.304	5±0.235	4.16±0.597	4.83±0.152	5.16±0.152	5.16±0.152
Monocytes	2±0.235	2±0.235	2.66±0.192	1.66±0.608	1.33±0.192	1.33±0.192
Basophils	0.13±0.019	0.2±0.023	0.13±0.019	0.13±0.030	0.23±0.019	0.2±0.023
RBC Count (10^6/uL)	6.49±0.057	6.36±0.006	6.17±0.003	5.26±0.009	6.17±0.005	6.27±0.005
Platelet Count	2.12±0.007	3.94±0.003	3.66±0.003	3.54±0.001	3.42±0.007	3.64±0.003
Mean Platelet Value	10.46±0.030	10.25±0.002	9.04±0.001	10.04±0.003	10.24±0.005	10.21±0.096
Packed Cell Volume	41.16±0.030	39.46±0.019	37.13±0.019	39.13±0.019	39.23±0.076	39.23±0.076
Mean Corp. Vol.	73.42±0.003	62.15±0.001	60.25±0.004	61.25±0.002	62.23±0.009	62.25±0.001
Mean Corpuscular	21.49±0.032	25.75±0.002	24.46±0.010	24.47±0.004	23.46±0.006	25.47±0.005
Mean Corp. Hb Con.	35.17±0.008	40.70±0.003	42.92±0.005	40.80±0.003	41.92±0.001	40.93±0.005
Red Cells Destribution Width	14.46±0.030	13.4±0.023	12.56±0.019	13.6±0.023	14.55±0.020	13.56±0.019
Biochemistry (Liver Function Test)						
Bilirubin-Total	0.85±0.003	0.71±0.001	0.74±0.003	0.70±0.003	0.72±0.001	0.73±0.003
Bilirubin-Direct	0.21±0.003	0.23±0.003	0.25±0.001	0.11±0.007	0.24±0.005	0.21±0.004
Bilirubin-Indirect	0.74±0.003	0.57±0.002	0.50±0.003	0.51±0.006	0.55±0.001	0.53±0.003
Total Protein	6.53±0.030	6.4±0.062	6.41±0.049	6.41±0.149	6.56±0.019	6.56±0.019
Albumin	3.36±0.038	3.16±0.019	3.15±0.031	3.35±0.031	3.33±0.038	3.43±0.019
Globulin	2.21±0.054	3.15±0.031	3.16±0.030	3.3±0.057	3.15±0.020	3.23±0.019
A/G Ratio	1.14±0.004	1.07±0.003	1.05±0.003	1.05±0.001	1.05±0.001	1.05±0.001
SGOT	81.16±0.030	132.4±0.031	139.4±0.068	127.4±0.076	157.6±0.031	137.4±0.080
SGPT	12.46±0.030	31.18±0.059	33.2±0.047	30.16±0.038	30.13±0.019	30.13±0.019
Alkaline Phosphatase	31.16±0.030	104.6±0.049	102.6±0.038	103.6±0.090	102.7±0.019	101.6±0.038
Biochemistry (Lipid Profile)						
Blood Glucose (Random)	81.35±0.020	81.2±0.023	80.23±0.019	83.4±0.115	81.5±0.115	83.35±0.093
Serum CREATININE	0.51±0.043	0.56±0.019	0.46±0.019	0.58±0.015	0.56±0.019	0.56±0.019
S. Cholesterol	130.16±0.03	56.45±0.031	57.41±0.015	68.33±0.038	59.33±0.038	58.43±0.019
S. Triglycerides	72.06±0.030	104.2±0.023	101.2±0.023	106.2±0.023	106.2±0.019	106.3±0.057
HDL Cholesterol	21.22±0.004	11.37±0.003	11.63±0.003	12.67±0.005	11.68±0.001	11.67±0.004
LDL Cholesterol	64.55±0.002	24.16±0.006	25.43±0.007	25.47±0.003	25.07±0.005	25.45±0.013
VLDL Cholesterol	25.22±0.028	23.23±0.004	21.22±0.014	24.25±0.012	21.22±0.007	21.24±0.001
LDLC/HDLC Ratio	2.43±0.004	2.47±0.004	2.11±0.004	2.13±0.004	2.37±0.005	2.18±0.003
TC/HDLC Ratio	3.54±0.003	9.58±0.004	9.33±0.019	9.01±0.002	10.24±0.089	9.09±0.001
S. Cholesterol/HDLC Ratio	5±0	5±0	5±0	5±0	5±0	5±0

Value are Mean±SEM. (n=6).

3.4.5 Histopathology:

3.4.6.1 Kidney: Animal organ (Kidney) Histopathology report was shown.

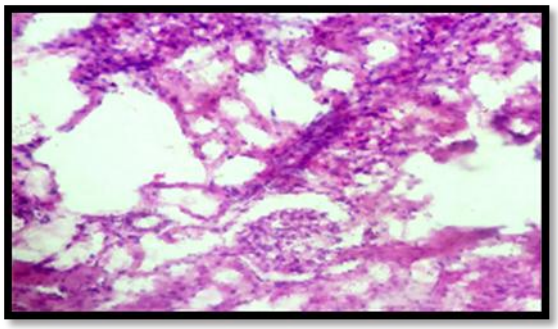


Fig. 6.14: Normal group.

Microscopic features of Test group-I is normal. Hand E 40x10

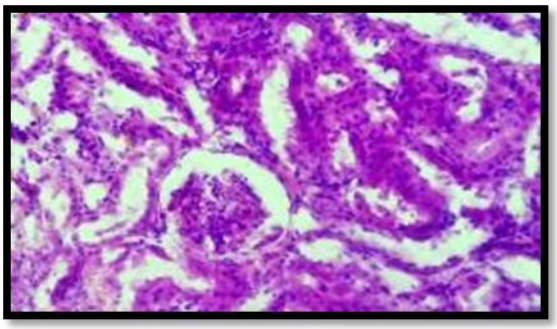


Fig. 6.15: Test group-I.

Microscopic features of Normal group is normal. Hand E 40x10

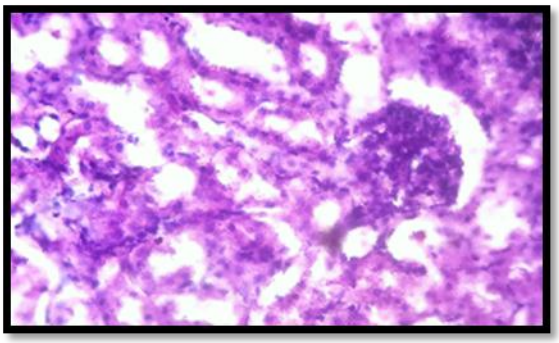


Fig. 6.16: Test group-II.

Microscopic features of Test group-II is showing atrophy of Bowman's capsule and glomeruli. Slight degeneration of renal tubules. Hand E 40x10

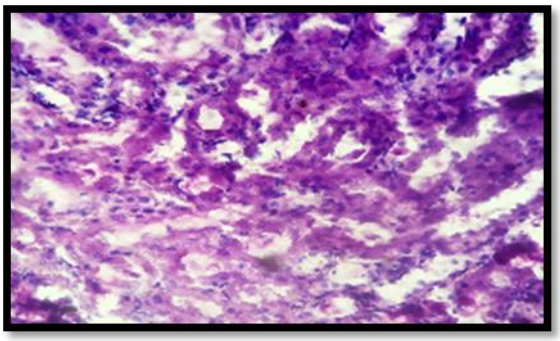


Fig. 6.17: Test group-III.

Microscopic features of Test group-III is showing degeneration of Bowman's capsule, glomeruli and Slight degeneration of renal tubules. Hand E 40x10

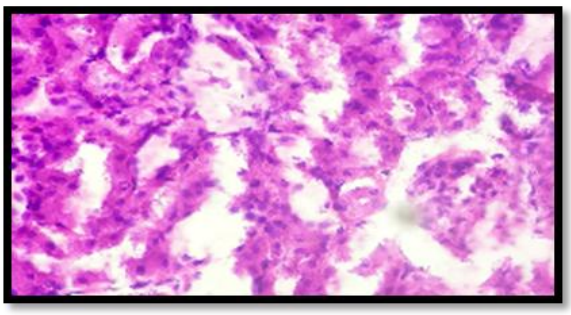


Fig. 6.18: Test group-IV.

Microscopic features of Test group-IV is showing degeneration of Bowman's capsule, glomeruli and moderate degeneration of renal tubules. Hand E 40x10

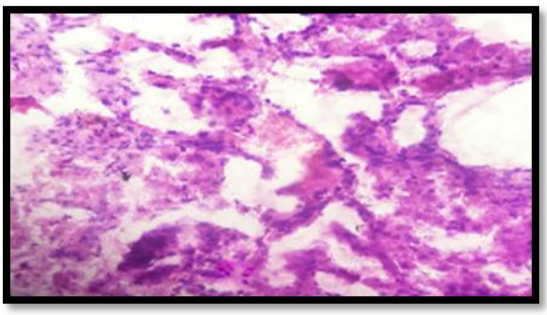


Fig. 6.19: Test group-V.

Microscopic features of Test group-V is showing degeneration of Bowman's capsule, glomeruli and severe degeneration of renal tubules along with Hemorrhage. Hand E

3.4.6.2 Liver: Animal organ (Liver) Histopathology report was shown.

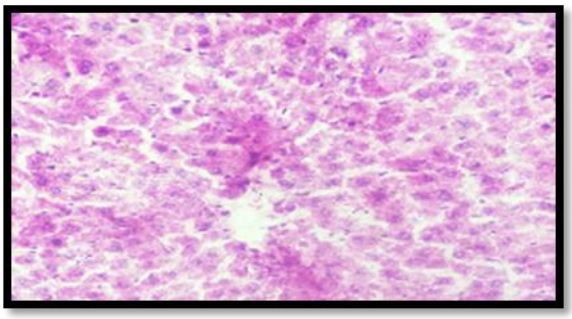


Fig. 6.20: Normal group.

Microscopic features of Normal group is normal. Hand E 40x10.

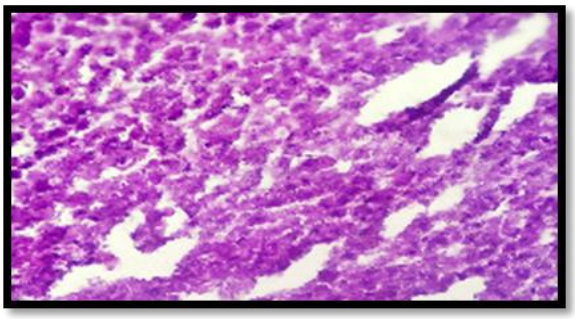


Fig. 6.21: Test group-I.

Microscopic features of Test group-I is normal. Hand E 40x10.

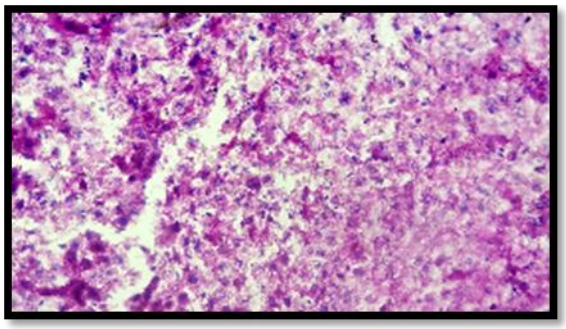


Fig. 6.22: Test group II.

Microscopic features of Test group-II is showing degeneration and necrosis of hepatic cells. Hand E 40x10.

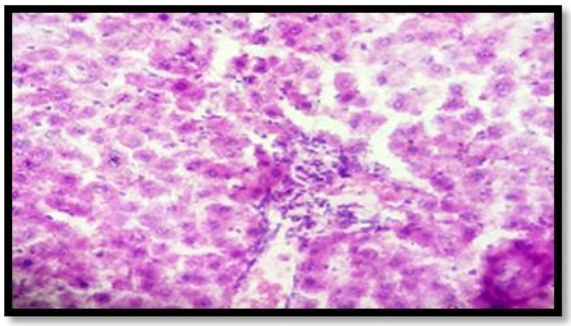


Fig. 6.23: Test group III.

Microscopic features of Test group-III is showing degeneration, necrosis, infiltration of leukocytes and red blood cells in sinusoidal space. Hand E 40x10.

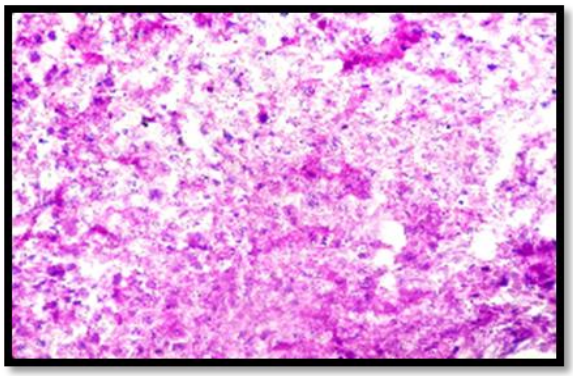


Fig. 6.24: Test group-IV.

Microscopic features of Test group-IV is showing degeneration, necrosis, of hepatic cell and hepatic nuclei. Fatty degeneration are also observed. Hand E 40x10.

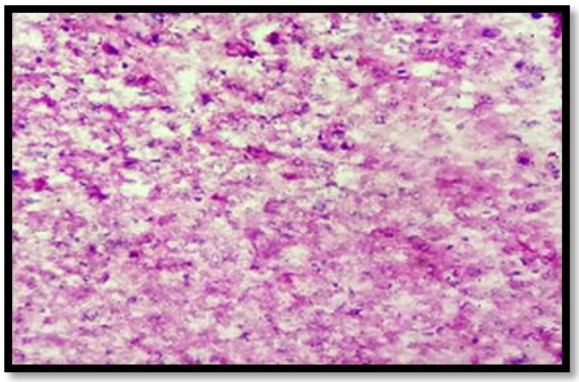


Fig. 6.25: Test group-V.

Microscopic features of Test group-V is showing degeneration, necrosis of hepatic cell and hepatic nuclei. Nucleus become disintegrated and hydropic degeneration are also observed. Hand E 40x10.

4. CONCLUSION

In conclusion, the developed formulations Nanoconstructs were found to be safer in less amount and mortality activity shown in more amount shown in Haematological and Histopathological reports. Toxicity of a formulation is a primary concern for its future applications and the developed formulations may prove their capabilities *In-vivo* due to toxicity. A part from toxicity the formulation must be stable enough to withstand various environmental conditions to give its highest performance whenever used during its self-life.

The prepared formulations were stable enough in normal storage conditions e.g. at room temperature, assuring its easy storage and transportation, if so, in future.

The given Nanoconstruct was found safe in less amount but more amount in mortality shown, toxicity studies in Albino Wistar Rats. The toxicity data of Nanoconstructs may be useful for further development in pre-clinical and clinical studies.

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