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Toxicity Profiling of *Marsilea Minuta* Conjugated ZnO Nanoparticles for Therapeutics as Nanomedicine

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Abstract

Nanomedicine is widely explored nowadays for the treatment of life-threatening diseases, yet it comes with various challenges and questions. In this regard, the exploration of the sanative effect of nanoparticles on cell viability for therapeutic applications is going to be a highly developing research field. Nanomedicines should be properly screened before they can be proclaimed as future technology. The present study encapsulates the acute and sub-acute toxicological aspects of the *Marsilea minuta* conjugated ZnO *(*MM-ZnO) nanohybrids synthesized. Biochemical, hematological, and histopathological analyses were performed to assess the toxicity of the nanocomposite. The toxicological profiles of the nanocomposite were studied *in vivo* in an experimental animal model (mice). Sub-chronic studies in Swiss albino mice of either sex showed little or no change in the biochemical and hematological parameters in low and middle doses of MM-ZnO nanohybrids. Histopathological data analysis of the high dosage revealed very modest alterations at the tissue level. Thus, this study concludes that the synthesized nanohybrids are safe and non-toxic and can be used as therapeutic nanomedicine.

Keywords: Toxicity; Nanomedicine; *Marsilea minuta;* MM-ZnO nanohybrids; Mice model; Hematology.

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1. Introduction

Nanomedicines have been acclaimed as the boon of the medicinal world [1-4]. The applications of nanomedicine have been widespread. From the development of diagnostic devices, analytical tools, and physical therapy applications to therapeutic drug delivery and targeted drug delivery [5-8].

In vivo, nanoparticles will be translocated to and entrapped in other tissues or organs along the blood circulation. The small size and large surface area endow them with enhanced activity along with possible intrinsic toxicity. Toxicity refers to the negative consequences caused by nanomaterials' interactions with cells. Even at the cellular level, nanoconjugates can cause detrimental health effects [9]. The hazardous characteristics of nanomaterials must be evaluated before they are used in biomedical science. In practice, the

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evaluation typically includes acute, sub-chronic, and chronic studies [10]. The current status of toxicology due to nanoparticles has been reported by Becker [11].

*Marsilea minuta (*Family: Marsileaceae*)* is a medicinally potent pteridophyte with anti-bacterial, anti-cancerous, antidiabetic, anti-amnesic, and anti-depressant properties [12, 13]. Bioactive constituents present in *M. minuta are* biocompatible and hepatoprotective [13].

During this work, ZnO NP (MM-ZnO) was synthesized via green routes using *M. minuta* leaf extract. The optical properties of the synthesized NPs were measured using UV-visible (UV-VIS) spectroscopy. The morphology of the prepared samples was analyzed by field emission scanning electron microscopy (FESEM). The toxicity of the MM-ZnO nanoconjugates was analyzed using overall health, blood biomarker assay, and hematological and histopathological parameters of the experimented mice. The present work evaluates the toxicity of the prepared samples and ensures their safety upon experimental mice model *in vivo* application.

2. Material and Methods

2.1. *Materials*

The plant material *M. minuta* (Marsileaceae) leaves, were obtained from the Sreegopal Banerjee College campus. Zinc acetate dehydrates, trisodium citrate, and other chemicals utilized in this study were obtained from Merck (India), and they were all in the research grades.

Swiss albino mice of either sex (20-25 days old) weighing 32 ± 5 g were procured from an animal house, authorized by a committee for the purpose of control and supervision of experiments on animals (CPCSEA), Chennai, India (Registration No. 50/CPCSEA/1999). The animals were divided into 4 groups and maintained under standard laboratory conditions (temperature 25 °C ± 2 °C with a day/night circle of 12h/12h). Free access to a dry plate diet (Hindustan Liver, Kolkata) and water *ad libitum* were provided.

The studies were carried out according to the CPCSEA guidelines and authorized by the institutional animal ethics committee (Approval No. AEC/PHARM/1503/ 03/2015 dated 30.11.2015).

2.2. *Synthesis of M. minuta conjugated ZnO nanoparticles (MM-ZnO NPs)*

2.2.1. *Leaf extract preparation*

M. minuta leaf extract was prepared following the protocol of previously published work [14]. Briefly, 1 g of the sterilized air-dried leaves were ground to form a fine powder. This powder was boiled at 50 °C for 15 min in 50 mL of distilled water. The extract was filtered with Whatman filter paper no. 1 and then vacuum filtered with a pore size of 0.2 µm. The finalized filtrate (solution A) was kept in a cold, dry area for future use.

2.2.2. Green synthesis of MM-ZnO

Under stirring conditions, 50 mL of 91 mM of zinc acetate solution was added dropwise in preheated leaf extract (30 mL) [14]. The reaction mixture became light brownish, and a zinc hydroxide precipitated. The reaction mixture was allowed for 30 min to achieve full reduction to ZnO. The precipitate was collected by centrifugation at 16000 rpm for 15 minutes at 4 °C. The precipitate was vacuum-dried at 60 °C and kept for further investigations.

2.3. *Characterization of MM-ZnO nanoconjugates*

MM-ZnO nanoconjugates were characterized using UV-VIS light spectra (λ25 spectrophotometer, Parkin Elmer, Germany), and the size was determined by FESEM (Inspect F50, FEI, Netherland).

2.4. *Toxicity test*

The animals were administered with the appropriate dosages (150 mg kg^{-1} body weight, 300 mg/kg body weight, and 500 mg kg^{-1} body weight) of nanoconjugate once a day for 28 days (Table 1). Body weight measurements were carried out on 0, 7, 14, 21, 27, and 28 days. Feed consumption per cage was assessed over three-day intervals by weighing the feeders. Throughout the dosing procedure, the animals were monitored for clinical indicators of morbidity, mortality, changes in body weight, and changes in food intake. At the completion of the therapy, blood was drawn from the animals' orbital sinuses for clinical pathology study, which included hematological and biochemical parameters.

Table 1. Grouping of mice for experimental setup.

Groups	Treatments	Remarks
GR I	None	Control
GR II	150 mg kg^{-1} body weight of MM-ZnO	Low Dose
GR III	300 mg kg^{-1} body weight of MM-ZnO	Medium Dose
GR IV	500 mg kg^{-1} body weight of MM-ZnO	High Dose

Consequently, the animals were sacrificed by cervical dislocation and necropsied for the gross evaluation of the various organs. The necropsy also included careful and consistent dissection of various target organs like the liver, kidneys, heart, lungs, spleen, and stomach.

We chose the administration of MM-ZnO nanoconjugate by intraperitoneal mode as it is predominantly used for its ease compared to other parental methods during animal testing for the administration of systemic drugs and fluids. Additionally, one can administer a large volume of nanoconjugate suspensions (~1000 μL) to the mice if one chooses the intraperitoneal route compared to the intravenous method. The report of more than this amount via the intravenous route is very rare.

2.5. *Blood biomarker assay*

Blood samples were collected from the retro-orbital sinus. The serum was obtained by centrifugation of the whole blood at 3,000 rpm for 15 min. Liver function was evaluated based on the serum levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), as well as on blood glucose and cholesterol levels. Nephrotoxicity was determined by Blood Urea Nitrogen (BUN), total protein, albumin, and globulin.

2.6. *Haematological parameter determination*

Blood samples were analyzed for routine hematological parameters. Blood samples were taken from the orbital sinus in the morning using heparin as an anticoagulant. Blood cell count was performed using blood smears. Hematological parameters have been examined using a Sysmax-K1000 Cell Counter. Parameters studied were Haemoglobin (Hb), Total Red Blood Corpuscles (RBC), Reticulocyte (Rt), Haematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Platelets, Total White Blood Corpuscles (WHC), Neutrophils (N), Lymphocytes (L), Eosinophils (E) and Monocytes (M).

2.7. *Histopathological examination*

After sacrifice, the heart, liver, and kidneys were removed and placed into buffered formalin. Pathological examinations followed normal laboratory protocols for histopathological testing. Tissues were fixed in paraffin blocks, diced to 5 µm thickness, and mounted on glass slides. After hematoxylin-eosin staining, the slides were examined and photographed using an optical microscope.

2.8. *Statistical analysis*

For statistical analysis, each of the experimental values was compared with its corresponding control. The results are provided as mean ± standard deviation. The means of many groups were compared using the one-way analysis of variance test (ANOVA). The statistical significance level for all tests was set at P 0.05.

3. Results and Discussion

3.1. *Characterization of synthesized MM-ZnO NPs*

UV-VIS spectra of the synthesized nanoparticles were recorded (λ25 spectrophotometer, Perkin Elmer, Germany). A sharp Plasmon Resonance (SPR) band at 379 nm was observed in Fig. 1A, confirming the presence of ZnO. In our previously published work X-ray diffraction (XRD) patterns of the same nanocomposites showed 2θ values at 31.77, 34.41,

36.22, 47.60, 56.58, 62.86, 66.41, 67.93, 69.09, 72.54, and 76.84º corresponds to (100), (002), (101), (102), (110), (103), (200), (112), (201), (004) and (202) planes confirmed the formation of MM-ZnO NPs [14]. The surface morphology of the samples was analyzed using FESEM. FESEM micrograph reveals MM-ZnO NPs were spherical in nature and about 20-50 nm in diameter (Fig. 1B).

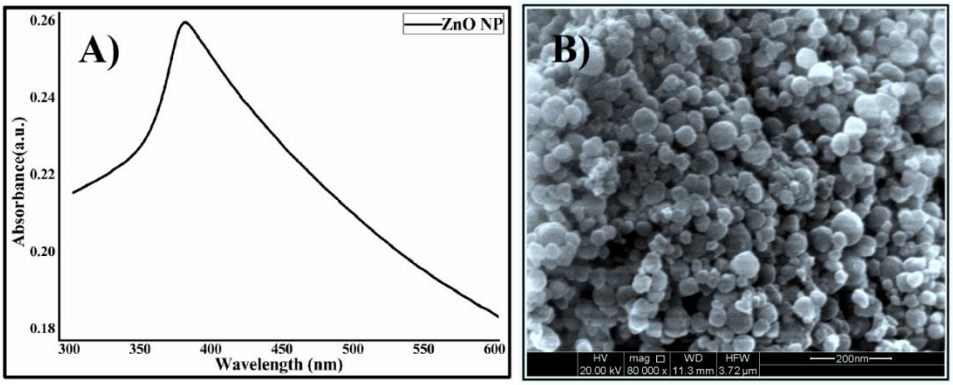

Fig. 1. (A) UV-VIS spectrum of MM-ZnO NPs B) FESEM micrograph of the MM-ZnO NPs.

3.2. *Sub-chronic toxicity analysis of MM-ZnO nanoconjugates*

No animal mortality was recorded in any of the test groups over the whole sub-chronic study period. After long-term dosing, the sub-chronic toxicity results reveal that the nanoparticle formulations had no harmful effects on the animals' overall health. These findings were also supported by our previous findings of Wistar rats [14]. The body weights of male and female animals did not differ considerably. These results are given in Tables 2-4 (either sex). There was no change in the general systemic health of the animals. The organ–body weight indices are shown in Table 5. The organ–body weight indices of the liver and kidney, the major organs of concern, did not show any significant change.

Table 2. Body Weight (g) Changes in male (♂) and female (♀) mice treated with MM-ZnO for 28 days of treatment.

Groups	0 Day		7th Day		14th Day		21th Day		28th Day	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
GR-I	24.1± 2.74	22.3± 1.54	27.5± 2.08	25.7± 1.66	30.1± 0.97	28.1± 1.29	34.7± 2.43	31.8± 1.02	36.2± 3.01	35.2± 0.99
GR-II	24.4± 1.31	23.2± 0.98	25.1± 2.24	23.6± 2.07	27.8± 1.12	24.1± 0.90	30.3± 2.54	25.3± 2.09	35.4± 2.76	28.4± 1.06
GR-III	26.7± 0.89	22.7± 1.77	25.9± 1.33	21.8± 0.87	27.1± 1.64	21.4± 0.78	29.3± 2.35	22.9± 2.11	33.5± 0.94	25.6± 1.44
GR-IV	27.0± 0.86	24.0± 1.12	23.4± 1.17	21.4± 0.95	26.9± 1.16	22.9± 2.04	30.6± 2.45	24.5± 0.78	33.7± 1.15	27.0± 1.17

Table 3. Food Consumption (g day^{-1}) Changes in male and female mice treated with MM-ZnO for 28 days of treatment.

Groups	0 Day		7th Day		14th Day		21th Day		28th Day	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
GR-I	5.0± 0.61	4.3± 0.64	6.5± 0.53	5.2± 0.77	7.8± 0.77	7.1± 0.38	8.5± 0.45	8.5± 0.68	9.2± 0.52	8.6± 0.62
GR-II	4.5± 0.43	3.8± 0.41	4.9± 0.46	4.6± 0.34	6.3± 0.63	5.6± 0.59	7.0± 0.56	6.3± 0.42	8.0± 0.44	8.0± 0.45
GR-III	5.1± 0.74	4.2± 0.35	4.6± 0.52	3.5± 0.21	4.9± 0.54	3.9± 0.37	5.9± 0.23	5.3± 0.55	7.9± 0.37	7.2± 0.32
GR-IV	6.0± 0.71	4.0± 0.44	4.8± 0.34	2.7± 0.76	5.5± 0.44	3.3± 0.54	7.1± 0.36	4.3± 0.83	8.1± 0.41	5.9± 0.42

Table 4. Daily water intake (mL day^{-1}) Changes in male and female mice treated with MM-ZnO for 28 days of treatment.

Groups	0 Day		7th Day		14th Day		21st Day		28th Day	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
GR-I	9.0± 0.71	8.5± 0.43	9.8± 0.42	8.8± 0.35	10.6± 0.82	9.5± 0.34	11.1± 0.28	10.1± 0.33	11.5± 0.40	10.4± 0.37
GR-II	8.8± 0.73	8.0± 0.24	8.9± 0.54	8.9± 0.66	9.5± 0.44	9.7± 0.29	10.4± 0.37	10.2± 0.49	11.1± 0.61	10.7± 0.74
GR-III	9.2± 0.67	8.7± 0.56	10.9± 0.47	9.5± 0.91	11.2± 0.63	10.3± 0.41	11.0± 0.48	11.0± 0.22	11.5± 0.33	10.8± 0.39
GR-IV	9.0± 0.88	9.0± 0.74	12.1± 0.85	10.8± 0.42	11.7± 0.92	11.4± 0.62	11.5± 0.98	10.8± 0.55	11.3± 0.49	10.7± 0.65

Table 5. Absolute organ weight (g) in male and female mice treated with MM-ZnO for 28 days.

Groups	Liver		Stomach		Kidney		Spleen	
	♂	♀	♂	♀	♂	♀	♂	♀
GR-I	1.71± 0.66	1.53± 0.12	0.73± 0.35	0.70± 0.23	0.20± 0.66	0.22± 0.19	0.22± 0.26	0.20± 0.24
GR-II	1.69± 0.53	1.58± 0.22	0.72± 0.71	0.70± 0.31	0.24± 0.24	0.25± 0.35	0.19± 0.23	0.19± 0.34
GR-III	1.79± 0.23	1.61± 0.16	0.80± 0.74	0.69± 0.27	0.29± 0.36	0.30± 0.44	0.24± 0.17	0.22± 0.44
GR-IV	1.85± 0.21	1.72± 0.24	0.81± 0.42	0.79± 0.18	0.35± 0.31	0.33± 0.31	0.23± 0.34	0.26± 0.25

3.3. *Haematological changes*

Changes in hematological parameters were used to find out the physiological and pathological changes in animals. Tables 6 (male) and 7 (female) showed the hematological parameters. There was no significant change in mean corpuscular hemoglobin concentration, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular haemoglobin, reticulocyte, prothrombin time, and activated partial thromboplastin time in either sex.

For acute toxicity, there was a significant increase (P 0.05) in the WBC count, while changes in the RBC and Platelet count were insignificant in the animals [15]. The findings

indicate a disruption in cellular immune function and a suppression of the immunological responses in mice. Lymphocytes circulate in the blood and migrate to injured tissues [11]. This may account for the significant decrease in lymphocytes and increase in neutrophil numbers at the highest dose of treatment. This may be attributed to local reactions at the injection site [16]. There was no significant change in the hematological parameters of the treated group for sub-acute toxicity when compared with the control group in animals. This indicates that the nanoparticle formulations are safe at 150 mg kg^{-1}, 300 mg kg^{-1} body weight, and 500 mg kg^{-1} body weight.

In the sub-chronic toxicity analysis, the biochemical, physiological, and pathological changes due to multiple administrations were analyzed. When the test material induces tissue damage, hematological parameters will alter in a direct way. Overproduction of red blood cells was observed in case of tissue damage [17].

Table 6. Hematological values in male mice treated with MM-ZnO for 28 days.

Parameter	Control	Low Dose	Middle Dose	High Dose
RBC ($\times 10^6$ μg^{-1})	6.79±0.41	6.88±0.38	6.97±0.21	6.95±0.86
Hb (g dL^{-1})	13.1±0.6	12.4±0.6	11.9±0.4	11.7±1.1
HCT (%)	41.8±2.1	40.4±1.9	39.3±1.1	39.1±3.7
MCV (fl)	62.4±1.4	59.9±1.6	57.2±1.1	58.5±2.4
MCH (pg)	19.1±0.4	18.7±0.7	18.6±0.4	18.4±0.6
MCHC (g dl^{-1})	30.4±0.3	29.8±0.5	28.6±0.6	28.1±0.4
RET (%)	4.34±1.16	3.59±1.32	7.22±1.81	5.32±1.26
PLT ($\times 10^3$ μg^{-1})	1640±68	1825±170*	1913±156**	2049±142**
PT (sec)	13.9±1.6	14.7±0.6	13.2±0.3	13.1±0.4
APTT (sec)	16.5±1.8	18.7±1.2	15.2±1.8	15.9±4.1
WBC ($\times 10^3$ μg^{-1})	5.22±1.12	6.41±1.38*	6.77±1.62*	6.95±1.38*
NEU (%)	15.9±2.4	19.5±1.4	21.6±1.6**	24.1±1.3**
LYM (%)	80.0±6.5	76.7±10.2*	73.9±5.4*	71.9±17.1**
MON (%)	02.8±0.5	02.67±0.6	03.43±0.5	03.3±1.1
EOS (%)	01.1±0.3	00.9±0.5	00.8±0.2	0.4±1.4 *
BAS (%)	00.20±0.1	00.23±0.1	00.27±0.1	00.3±0.1

RBC, red blood cells; HB, haemoglobin, HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RET, reticulocyte; PLT, platelet; PT, prothrombin time; APTT, activated partial thromboplastin time; WBC, white blood cells; NEU, neutrophil; LYM, lymphocyte; MON, monocyte; EOS, eosinophil; and BAS, basophil. The values are reported as mean ± SD.

*, **P<0.05, P<0.01 level vs. the vehicle control group

Table 7. Hematological values in female mice treated with MM-ZnO for 28 days.

Parameter	Control	Low Dose	Middle Dose	High Dose
RBC ($\times 10^6$ μg^{-1})	7.02±0.21	7.62±0.55	7.21±0.22	7.15±0.56
Hb (g dL^{-1})	13.7±0.8	14.4±1.1	12.9±0.6	12.4±1.2
HCT (%)	44.5±0.9	45.4±4.2	42.2±1.3	43.3±3.87
MCV (fl)	61.4±1.1	58.1±2.6	58.7±1.1	57.7±1.4
MCH (pg)	19.4±0.4	18.8±0.6	18.8±0.4	18.9±0.7
MCHC (g dL^{-1})	32.1±0.3	31.8±0.7	31.1±0.5	30.7±0.8
RET (%)	3.75±0.46	3.19±0.72	3.22±0.61	3.33±0.64
PLT ($\times 10^3$ μg^{-1})	1843±162	1894±172	1915±157	1905±112
PT (sec)	16.9±1.4	14.8±0.6	14.2±0.3*	13.7±0.4*

APTT (sec)	14.5±1.2	16.7±1.0	15.8±1.7	16.3±3.1
WBC ($\times 10^3$ μg^{-1})	4.85±1.02	5.33±2.11*	5.64±4.1*	5.85±8.14*
NEU (%)	12.9±3.4	24.2±5.4**	27.5±4.1**	31.1±5.3**
LYM (%)	83.0±4.5	71.5±9.9**	68.3±8.5**	65.82±14.1**
MON (%)	02.5±0.4	02.77±0.5	02.84±0.4	03.1±0.6
EOS (%)	01.4±0.3	01.3±0.3	01.1±0.4	0.9±0.6 *
BAS (%)	00.20±0.1	00.23±0.1	00.26±0.1	00.29±0.1

RBC, red blood cells; HB, hemoglobin, HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RET, reticulocyte; PLT, platelet; PT, prothrombin time; APTT, activated partial thromboplastin time; WBC, white blood cells; NEU, neutrophil; LYM, lymphocyte; MON, monocyte; EOS, eosinophil; and BAS, basophil. The values are reported as mean ± SD. *, **P<0.05, P<0.01 level vs. the vehicle control group

3.4. *Biochemical estimation*

Changes in enzyme parameters are due to their leakage from intracellular sites or target tissues due to cellular/tissue injury [18]. The biochemical parameters for animals are given in Figs. 2-4. There were no significant changes in serum cholesterol and lipid profile in experimented mice (Fig. 2). There was a slight change in AST values and ALP values in animals (P 0.05) at a higher dose (Fig. 3). The fact that all the animals survived throughout the investigation period indicated that the liver was not seriously damaged, and neither was the formulation fatal. The ALP levels were only affected at a greater dosage, indicating that metabolism was not disturbed with the usage of MM-ZnO in the system at a low dose. However, in the present study, the raised AST and ALT levels suggested that it was not lethal since all of the mice survived throughout the experiment. The conjugation reduces the toxicity of the nanoparticle. Bioactive constituents of *M. minuta* eliminate the side effects induced by nanoparticles; some proportion of these particles should be excreted by the kidneys [19]. At high doses, nanoconjugate was difficult to remove from the liver and kidney, resulting in elevated AST and ALP. Blood Urea Nitrogen Test (BUN) showed elevated results in male mice (Fig. 4A), while these values were decreased in female mice (Fig. 4B) in comparison to the control group, but the changes in values in different test groups were insignificant. Liver enzymes are present within the liver cells. When the liver cells get damaged, liver enzymes spill into the blood [20-21]. This results in an elevation in enzyme levels. There were no significant changes in total protein plasma proteins (albumin and globulins) between the control and MM-ZnO-treated mice, indicating no abnormalities in the liver, even at the highest dose of nanoparticle treatment. As reported previously, the toxicity associated with ZnO was not found in our study, which may be due to the conjugation formation in MM-ZnO nanoparticles [22]. The results of the present study were supported by toxicity analysis on Wistar rats [14]. Bioactive constituents like polyphenols and flavonoids in *M. minuta* may also be responsible for reduced toxicity over the chemically synthesized ZnO NPs [19].

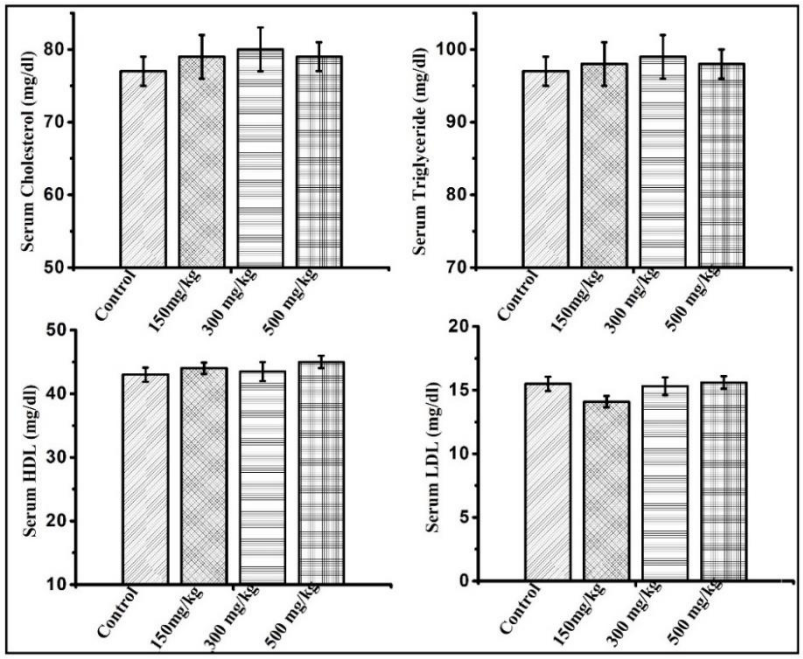

Fig. 2. Serum cholesterol, triglyceride, HDL and LDL profile in experimented mice.

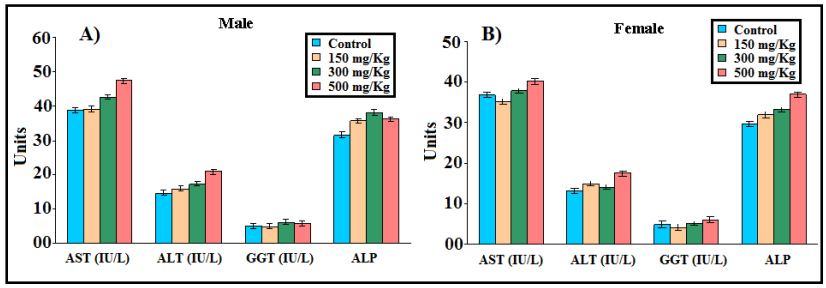

Fig. 3. Liver Function test in A) male and B) female mice.

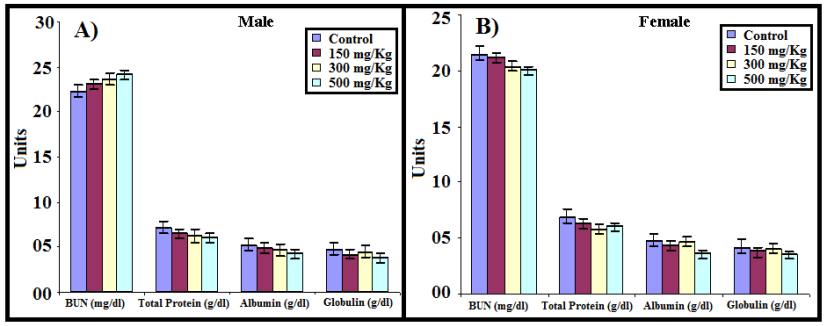

Fig. 4. A) Blood Urea Nitrogen (BUN) and protein profile in male mice, B) BUN and protein profile in female mice.

3.5. *Histopathological analysis*

The histopathological analysis of MM-ZnO conjugates treated mice has been depicted in Fig 5. In the 300 mg kg^{-1} and 500 mg kg^{-1} body weight nanoconjugate groups, there was no change in hepatocytes, portal area, or integrated hepatic side effects as compared to the control set. Similar observations were reflected in the liver and the kidney. They did not reproduce any abnormal pathological changes even at 500 mg kg^{-1} body weight exposure to MM-ZnO nanoconjugates. Mendoza-Milla *et al.* reported that chemically synthesized ZnO nanoparticles caused damage to cardiovascular tissues [23]. In the current study, no such observations were noted. This may be due to non-toxic green routes of ZnO synthesis where secondary metabolites of *M. minuta* reduce the adverse effects of ZnO on cellular toxicity.

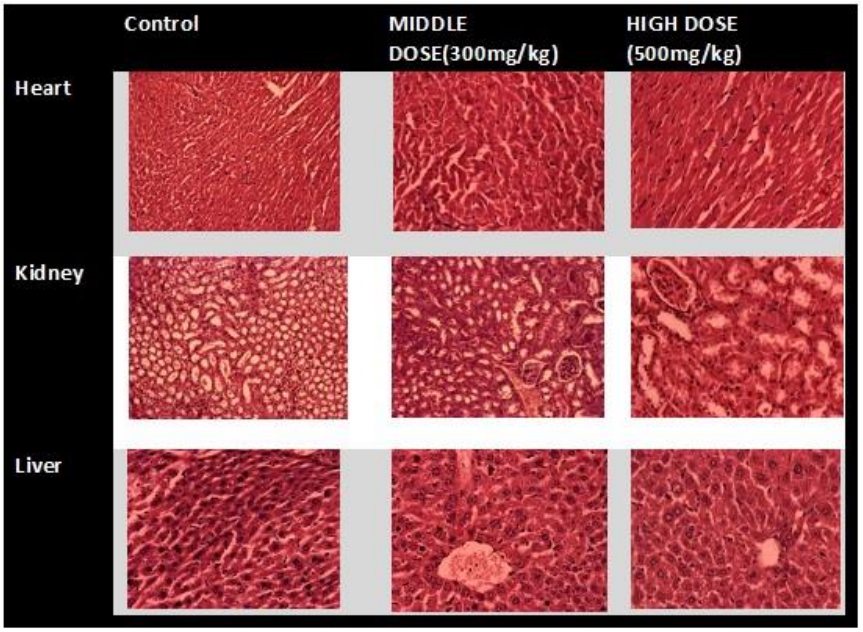

Fig. 5. Histopathological images of heart, kidney and liver of experimented mice.

4. Conclusions

Acute and sub-chronic toxicological analyses of MM-ZnO nanoconjugate confirmed the safety of the developed nanoconjugate. Hematological and biochemical parameters showed no significant changes when compared with control animals. During the study of acute and sub-chronic toxicity, the results showed that there was no toxic effect in either male or female animals. There was no change in the general health of the animals throughout the study. The results indicate that the nanoconjugate did not exhibit any toxicity. These findings may facilitate the development of safe and efficient MM-ZnO nanoconjugates as an effective therapy against many health disorders.

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