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Green synthesis of ZnO nanoparticles by using Syzygium jambolanum seed extract and their toxicity study

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ABSTRACT

Nanomedicines should be properly screened before they can be hailed as the pharmacological boon of the future. The present study encapsulates the acute and subacute toxicological aspects of the ZnO-Siziyumjambolanum (SJ-ZnO) nanocomposites synthesized. The individual components of the formulation,vizZnO in surface modified nano form and at low dose , And Siziyumjambolanumare both biocompatible materials.For toxicity evaluation of the nanocomposite, Biochemical, Hematological and Histopathological analysis were conducted along with Behavioural assays to ascertain any behavioural side effects. For acute toxicity studies according to OCED(Organization for Economic Cooperation Development) guidelines Female Wistar rats(6-7 weeks) were used and were given single intraperitoneal dose of 2000 and 5000 mg/kg body weight and were obsereved for mortality and other side effects for 14 days and for subacute toxicity studies, swiss albino mice of either sex (8-10weeks) were randomly divided into 4 groups (n=6) where in group I as vehicle treated control. Group II and Group III and Group IV received 150,300,500 mg/kg body weight of SJ-ZnO for a period of 28 days. The animals were sacrificed on the 29th day to evaluate the toxicological implications of the nanocomposite. Low and middle dose showed little or no change in the biochemical and haematological parameters. Very slight changes were observed in the tissue level from histopathological data analysis of the high dose. It is thus concluded from this study that the nanocomposite synthesized is safe and nontoxic and can be used as a therapeutic nanomedicine.

Keywords: Acute and. Subacute toxicity, Nanomedicine, S jambolanumsensitizedZnO nanoparticles, Mice model.

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I. INTRODUCTION

Nanomedicines have been acclaimed as the boon of the medicinal world .The applications of nanomedicine has been far and widespread (1,2). From the development of diagnostic devices (3), analytical tools (5), physical therapy applications (3, 4) to therapeutic drug delivery (4, 5, 6), and targeted drug delivery. (4)

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 alongwith possible intrinsic toxicity. Toxicity indicates the adverse effects due to the interaction between nanomaterials and cells. Even at a very small cellular level, they may pose potential long-term health hazards (7,9). The evaluation of toxic properties of nanomaterials is crucial when considering them for use in biomedical science. In practice, the evaluation typically includes acute, subchronic, and chronic studies (10).The status of toxicology due to nanoparticles has been reported by Becker (11).

During our work, we have synthesized ZnO NP via green routes using S. jambolanumseedextract. Optical properties of the synthesized NPs were measured using UV-visible (UV-VIS) spectroscopy. Morphology of the prepared samples was analyzed by field emission scanning electron microscopy (FESEM).

The present work evaluates the acute and sub-acute toxicity profile of the prepared samples and ensures their safety upon experimental mice model and also analyzes their behavioural changes if any upon their in vivo application.

II. MATERIALS AND METHODS

Chemicals and Reagents

Siztyumjambolanumfruitwas collected from University campus and seeds were taken out. Zinc

acetate dehydrate, tri-sodium citrate and other chemicals used in this experiment were purchased from Merck (India)and all are of research grade.

III. EXPERIMENTAL PROCEDURE:

Synthesis ofS.jambolanumfunctionalizedZnO nanoparticles (NPs):

A. Seed extract preparation:

1gram ofS.jambolanumseeds were washed thoroughly with plenty of distilled water and were sterilized using alcohol by gently rubbing. The seeds were then then grinded to form fine powder. This powder was heated for 15 min in 50 ml of distilled water at 50 °C. Then the extract was filtrated with Whatman filter paper no 1 and further filtered using vacuum filter with pore size of 0.2 µ m. The final filtrate (solution A) was stored in cool dry place for further use.

B. Green synthesis of silvernanoparticles:

According to our previous published paper green route of synthesis(12) was chosen, 30 ml of the seed extract was heated at 50 °C for 10 min and 50ml of 91 mM of zinc acetate solution (1 gm of zinc acetate was dissolved in 50 ml of distilled water) was added drop wise to it under stirring. The reaction mixturebecamechoco brownish and light chocolate coloured precipitate of zinc hydroxide was formed. The reaction mixture was left for 30 min for complete reduction to zinc hydroxide. Then the precipitate was collected by centrifugation at 16000 rpm for 15 min at 4 °C. The precipitate was vacuum dried at 60 °C and stored for further studies

Characterizationof the synthesized nanoparticles

UV-VIS light spectra of the synthesized nanoparticles were recorded (λ25 spectrophotometer, Perkin ElmerGermany). Same amount of different samples were considered for UV-VIS data analysis

Surface morphology and elemental analysis of the samples were analyzed by FESEM using INSPECT F50 (FEI, Netherland). For FESEM study, dry powder samples were sprayed over the carbon tape and coated with gold. ZnO-NPs particles were spherical in nature and each particle was aggregation of many smaller particles. FTIR study was conducted as per standard protocol.

Animals

8 Female Wistar rats (6-7 weeks) and 32 Swiss albino mice of either sex (20-25 days old) weighing 32 ± 5 g were obtained from animal house, approved by committee for the purpose of control and supervision of experiments on animal (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 day/night circle of 12h/12h). Free access of dry plate diet (Hindusthan Liver, Kolkata) and water ad libitum were provided. The experiments were carried out according to the guideline of CPCSEA and approved by the institutional animal ethics committee (Approval No.AEC/PHARM/1503/03/2015 dated 30.11.15).

Acute and subacute toxicity profiling

a. Acute toxicity analysis:

Acute toxicity studies of thenanoconjugate was carried out in female rats by using Organisation for Economic Co-operation and Development (OECD) guideline 425. Before administration of a single dose of the test samples, the rats were deprived of food for 3 h. Doses of 2000 and 5000 mg/kg of the test samples were given to Group I and Group II respectively. The respective doses were suspended in water and were administered intraperitoneally because as it's a nanomedicine this route requires less quantity. Once daily cage side observations included changes in skin, fur, eyes, mucous membrane (nasal), autonomic (salivation, lacrimation, perspiration, piloerection, urinary incontinence, and defecation) and central nervous system (drowsiness, gait, tremors and convulsion) changes. Mortality, if any, was determined over a period of 2 weeks. After completion of the treatment, the animals were sacrificed by cervical dislocation and necropsied to facilitate gross pathological examination of organs.

b. Subacute toxicity test

The animals were administered(i.p) with the respective doses (150mg/kg body weight, 300mg/kg body weight, and 500 mg/kg body weight) ofnanocomposite once daily, for a period of 28 days. Body weights were recorded on days 0, 7, 14, 21, 27, and 28. Feed consumption was measured per cage over successive periods of 3 days by weighing the feeders. Throughout the dosing, the animals were examined for any clinical signs of morbidity, mortality, changes in body weight, and changes in food consumption. At the end of the treatment, blood was collected from the animals from the orbital sinus for clinical pathology assessment, which included analysis of various hematology parameters and biochemical parameters..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 liver, kidneys, heart,lungs, spleen, and stomach.

We chose the administration of SJ-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/rats 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.

Behavioural analysis

Open field locomotor activity

The exploratory and anxiety-related behavior of the experimental animals upon the influence of the nanocomposite was assessed in the open field test after the sub-acute studies. Control and SjZnO-NPs-treated mice were tested in the open field for one daily session of 5 min for three consecutive days, with an intertribal interval of 24 h. The device is a dull black circular enclosure (100 cm in diameter and 60 cm high). It is divided into a central circle and a peripheral part which is further divided into 6 parts of equal area (0.135 m2). Three identical objects (length 15 cm; width 10cm; height 5cm) were placed in the same positions of the peripheral part during al trials (13-15). At the beginning of the test, themice was placed in a peripheral part of the open field which then with time on its own reached the central zone. The following behavioral components were measured to ascertain whether the synthesized nanoconjugate has any behavioural side effects: locomotion in the peripheral and central part, total immobility time, number and duration of contact with an object. The whole setup was cleaned immediately with a 20% alcoholic solution so that no behavioral pheromone of the previous subjects affects the next one.

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 minutes. Liver function was evaluated based on theserum levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Nephrotoxicity was determined by BUN, total protein, albumin, and globulin.

Hematological parameter determination

Blood samples were analyzed for routine hematological parameters. Blood samples were collected from orbital sinus following morning using heparin as anticoagulant. Blood cell count was done using blood smears. Hematological parameters were studied using Sysmax-K1000 Cell Counter. Parameters studied were Hemoglobin (Hb), Total Red Blood Corpuscles (RBC), Reticulocyte (Rt), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Platelets, Total White Blood Corpuscles (WHC), Neutrophils (N), Lymphocytes (L), Eosinophils (E) and Monocytes (M).

Statistical analysis

For statistical analysis, each of the experimental values was compared with its corresponding control. The results were expressed as mean ± standard deviation. Multi-group comparisons of the means were carried out by one-way analysis of variance test. Statistical significance for all tests was set at P, 0.05.

IV. RESULTS AND DISCUSSION

Characterization of synthesized nanoparticles

Green synthesis of ZnO nanoparticle using S. jambolanumseeds extract was presented in Figure 1A. UV-VIS light spectra of the synthesized nanoparticles were recorded SharpPlasmon resonance (SPR) bands at 377nm confirm the presence of ZnONP's. FESEM analysis showed a grape like accumulation or agglomeration of the nanoconjugates and further studies revealed the particle diameter of around 20-50nm in diameter (Figure 1B). FTIR analysis showed typical ZnO nanoparticle's feature (Figure 1C)

Acute toxicity analysis of nanoconjugate:

No mortality was observed in the animals in any test group or in the control group. The animals did not show any abnormal behavior throughout the 14 days.The nanoparticle-composite was found to be safe for a single limit dose equivalent to2000 and 5000 mg/kg of SJ-ZnO. All clinical symptoms were evaluated by comparing treated animals with controls (n=6). The toxicity of the nanoparticle formulation due to a single dose for less than 14 days was determined by acute toxicity analysis.The present study showed that intraperitoneal treatment of mice with SJ-ZnO did not induce mortality or significant clinical symptoms of toxicity. In the acute toxicity study, no morbidity or mortality were observed in any mice, which survive throughout the 14 days of observation. According to Organisation for Economic Cooperation and development (OECD) guidelines for IP toxicity, a LD_{50} dose of 2000 mg/kg and above is unclassified and the drug is found to be safe. As the nanocompositeupto the dose level of 5000mg/kg body weight did not exhibit any mortality or toxic implications hence it was designated as safe for further long term assay.

Subacute toxicity analysis of nanoconjugate:

No animal mortality was observed in any of the test groups for the entire period of subacute study for nanoparticles. The subacute toxicity result shows that the nanoparticle formulations did not exhibit any adverse effect with long-term administration on the general health of the animals. The body weights of both male and female animals did not vary significantly. These results are given in Table 1, 2. There was no change in the general systemic health of the animals. The organ–body weight indices are shown in table 4. The organ–body weight indices of liver and kidney the major organs of concern did not show any significant change.

Behavioural changes of the animals treated with the nanocomposite

The spontaneous activity, exploratory behavior, and habituation to novelty were examined in the open field test (Figure 2). All the parameters did not vary significantly between control and ZnO-NPs exposed-mice proving that the synthesized nanoconjugate is safe and symptomatic (Table 5).

Haematological changes

Changes in hematological parameters are used to find out the physiological and pathological changes in animals and humans. Figure 3 shows the hematological parameters. There was no significant change in platelets or mean corpuscular hemoglobin concentration in either sex.

For acute toxicity there was a significant decrease ($P<0.05$) in the WBC count and Platelets count in the animals. This indicates a disturbance of cellular immune function and an inhibition of the immune response of the animals. Lymphocytes circulate in the blood and migrate to injured tissues.[11]This may account for the decrease in lymphocyte 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 forsub acute toxicity when compared with the control group in animals. This indicates that the nanoparticle formulations are safe at 50 mg/kg, 100mg/kg body weight and 200 mg/kg body weight (Table 6).

In the subacute toxicity analysis, the biochemical, physiological, and/or pathological changes due to multiple administrations were analyzed. There will be a direct change in the hematological parameters when the test compound causes tissue injury. When there is damage to tissue, overproduction of red blood cells occurs.

Biochemical estimation

Changes in enzyme parameters are due to their leakage from intracellular sites or target tissues due to cellular/tissue injury. The biochemical parameters for animals are given in Figure 3 & 4. There were no significant changes in blood glucose and cholesterol in animals. There was a slight change in AST values and ALP values in animals (P,0.05) at a higher dose (500 mg/kg body weight). The fact that all the animals survived throughout the investigation period indicated that the liver was not seriously damaged and neither the formulation was fatal. The ALP levels were altered only at a higher dose, indicating that bone metabolism was not disturbed with the usage of ZnO in the targeted drug delivery system at a low dose. However, in our case, the elevated AST and ALT levels indicated that it is not fatal, and all the animals survived through the whole experiment. The conjugation reduces the toxicity of the nanoparticle. The ALP levels are altered only at a higher dose. This shows that the metabolism was not disturbed with the usage of ZnO at a low dose.

The serum creatinine values are given in Figure 4. There was no increase in the serum BUN values. The globulin levels and albumin-to-globulin ratio are given in Figure 7 (17,18). [13]Liver eliminates the side effects induced by nanoparticles; some proportion of these particles should be excreted by the kidneys. At high concentrations, it is difficult for nanoparticles to clear liver and kidney, and this would result in increased AST, ALP. Liver enzymes are present within the liver cells. When the liver cells get damaged, they get spilled into blood. This causes an increase in the enzyme level. The toxicity associated with ZnO as reported previously(19-21) was not found in our study, which may be due to the surface modification.

V. CONCLUSION

Acute and subacute toxicology analyses of SJ-ZnOnanoconjugate confirmed the safety of the developed nanocomposite. Hematological and biochemical parameters showed no significant changes when compared with control animals. During the study of acute and subacute 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 nanoparticles did not exhibit any toxicity. These findings may facilitate the development of safe and efficient SJ-ZnOnanocojuagate as a effective therapy against glucose mediated health disorders.

Conflict of Interest:
The authors declare that there are no conflicts of interest.

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Table 1: Body Weight (g) Changes in female and male mice treated with nanoparticals for 28 days treatment

Female mice	**0 Day**	**7thDay**	**14th Day**	**21th Day**	**28th Day**
Control	22.3±1.54	25.7±1.66	28.1±1.29	31.8±1.02	35.2±0.99
150 mg/kg body wt	23.2±0.98	23.6±2.07	24.1±0.90	25.3±2.09	28.4±1.06
300mg/kg body wt.	22.7±1.77	21.8±0.87	21.4±0.78	22.9±2.11	25.6±1.44
500mg/kg body wt.	24.0±1.12	21.4±0.95	22.9±2.04	24.5±0.78	27.0±1.17
Male mice					
Control	24.1±2.74	27.5±2.08	30.1±0.97	34.7±2.43	36.2±3.01
150 mg/kg body wt	24.4±1.31	25.1±2.24	27.8±1.12	30.3±2.54	35.4±2.76
300mg/kg body wt.	26.7±0.89	25.9±1.33	27.1±1.64	29.3±2.35	33.5±0.94
500mg/kg body wt.	24.1±2.74	27.5±2.08	30.1±0.97	34.7±2.43	36.2±3.01

Values are presented as mean±SD.

Table 2: Food Consumption (g/day) Changes in female and male mice treated with nanoparticals for 28 days treatment

Female mice	**0 Day**	**7thDay**	**14th Day**	**21th Day**	**28th Day**
Control	5.0±0.61	6.5±0.53	7.8±0.77	8.5±0.45	9.2±0.52
150 mg/kg body wt	4.5±0.43	4.9±0.46	6.3±0.63	7.0±0.56	8.0±0.44
300mg/kg body wt.	5.1±0.74	4.6±0.52	4.9±0.54	5.9±0.23	7.9±0.37
500mg/kg body wt.	6.0±0.71	4.8±0.34	5.5±0.44	7.1±0.36	8.1±0.41
Male mice					
Control	4.3±0.64	5.2±0.77	7.1±0.38	8.5±0.68	8.6±0.62
150 mg/kg body wt	3.8±0.41	4.6±0.34	5.6±0.59	6.3±0.42	8.0±0.45
300mg/kg body wt.	4.2±0.35	3.5±0.21	3.9±0.37	5.3±0.55	7.2±0.32
500mg/kg body wt.	4.0±0.44	2.7±0.76	3.3±0.54	4.3±0.83	5.9±0.42

Table 3: Daily water intake (ml/day) Changes in female and male mice treated with nanoparticals for 28 days treatment

Female mice	**0 Day**	**7thDay**	**14th Day**	**21th Day**	**28th Day**
Control	9.0±0.71	9.8±0.42	10.6±0.82	11.1±0.28	11.5±0.40
150 mg/kg body wt	8.8 ±0.73	8.9±0.54	9.5±0.44	10.4±0.37	11.1±0.61
300mg/kg body wt.	9.2±0.67	10.9±0.47	11.2±0.63	11.0±0.48	11.5±0.33
500mg/kg body wt.	9.0±0.88	12.1±0.85	11.7±0.92	11.5±0.98	11.3±0.49
Male mice					
Control	8.5±0.43	8.8±0.35	9.5±0.34	10.1±0.33	10.4±0.37
150 mg/kg body wt	8.0±0.24	8.9±0.66	9.7±0.29	10.2±0.49	10.7±0.74
300mg/kg body wt.	8.7±0.56	9.5±0.91	10.3±0.41	11.0±0.22	10.8±0.39
500mg/kg body wt.	9.0±0.74	10.8±0.42	11.4±0.62	10.8±0.55	10.7±0.65

Table 4: Absolute organ weight (g) in female and male mice treated with nanoparticals for 28 days

Female mice	**Liver**	**Stomach**	**Kidney**	**Spleen**
Control	1.53±0.12	0.70±0.23	0.22±0.19	0.20±0.24
150 mg/kg body wt	1.58±0.22	0.70±0.31	0.25±0.35	0.19.±0.34
300mg/kg body wt.	1.61±0.16	0.69±0.27	0.30±0.44	0.22±0.44
500mg/kg body wt.	1.72±0.24	0.79±0.18	0.33±0.31	0.26±0.25
Male mice				
Control	1.71±0.66	0.73±0.35	0.20±0.66	0.22±0.26
150 mg/kg body wt	1.69±0.53	0.72±0.71	0.24±0.24	0.19±0.23

300mg/kg body wt.	1.79±0.23	0.80±0.74	0.29±0.36	0.24±0.17
500mg/kg body wt.	1.85±0.21	0.81±0.42	0.35±0.31	0.23±0.34

Table 5: Effects of NPs treatment on the open field parameters.

Variables	**Session 1**		**Session 2**		**Session 3**	
	Control	NPs	Control	NPs	Control	NPs
Locomotion in the peripheral part (s)	174.18±11.2	192.46±15.2	106.1±8.7	112.3±4.3	76.8±6.4	114.8±6.9
Locomotion in the central part (s)	2.89±1.5	5.06±2.7	0.85±0.4	2.36±1.03	0.61±0.5	2.54±0.6
Immobility time (s)	104.3 ± 12.03	83.4 ± 13.54	171.5 ± 8.7	174.1 ± 11.14	211.16 ± 5.6	163 ± 24.6
Time of contact with objects (s)	19.8±3.1	18.6 ±2.9	18.66±4.3	12.9±5.2	15.21±3.09	8.72±4.29
Number of contact with objects	10.42±3.87	11.39±2.12	9.06±2.09	7.42±4.11	7.29±2.11	9.46±1.28

Data represent the means ± SEM of 6 animals per group

Table 6. Hematological values in mice treated with ZnO coupled with S. jambolanumfor 28 days

Parameter	Control	Low Dose	Middle Dose	High Dose
RBC (×106/μg)	6.79±0.41[a]	6.88±0.38	6.97±0.21	6.95±0.86
HB (g/dL)	13.1±0.6	12.4±0.6	11.5±0.4 **	11.1±1.1 **
HCT (%)	41.8±2.1	40.4±1.9	38.3±1.1 *	37.3±3.7 **
MCV (fl)	62.4±1.4	58.9±1.6 **	54.2±1.1 **	53.5±2.4 **
MCH (pg)	19.1±0.4	18.7±0.7 **	162±0.4 **	15.5±0.6 **
MCHC (g/dL)	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 (×103/μg)	1640±68	1765±170	2083±252	2419±842
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 (×103/μg)	5.22±1.12	11.41±2.38	12.47±2.62	14.17±8.38
NEU (%)	15.9±6.4	26.5±11.4	33.5±4.6	46.1±17.3
LYM (%)	81.2±6.5	68.7±10.2	65.4±5.4	52.4±17.1
MON (%)	02.1±0.5	03.2±0.6	03.4±0.5	03.3±1.1
EOS (%)	01.1±0.3	00.9±0.5	00.8±0.2	0.4±1.4 *
BAS (%)	00.2±0.1	00.3±0.1	00.2±0.0	00.3±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.

[a]Values are presented as mean±SD.

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

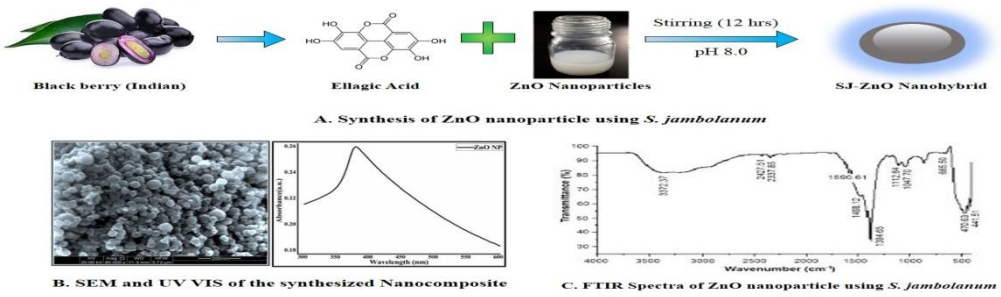

Figure 1: Synthesis and characterization of ZnO nanoparticle using S. jambolanum. A. Synthesis of ZnO nanoparticle using S. jambolanum;B. SEM and UV VIS of ZnO nanoparticle; C. FTIR study of ZnO nanoparticle.

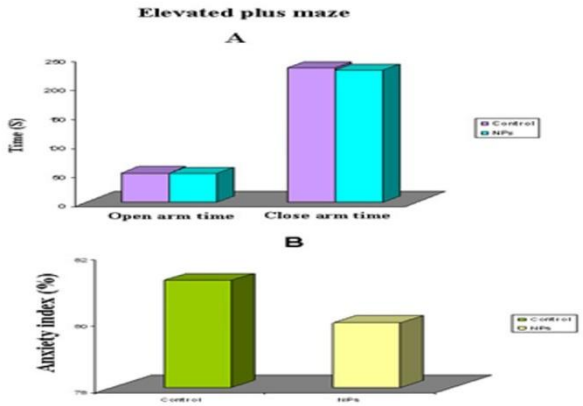

Figure 2: Elevated plus maze test of ZnO nanoparticle using S. jambolanum. A. Open and close arm test; B. Anxiety test.

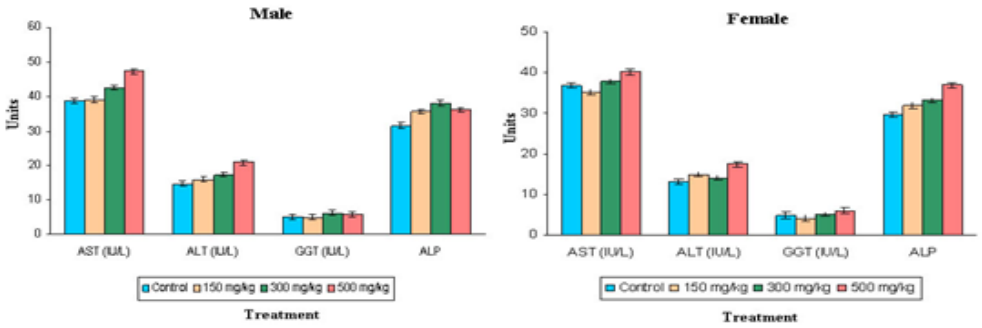

Figure 3: Liver function test of ZnO nanoparticle using S. jambolanum

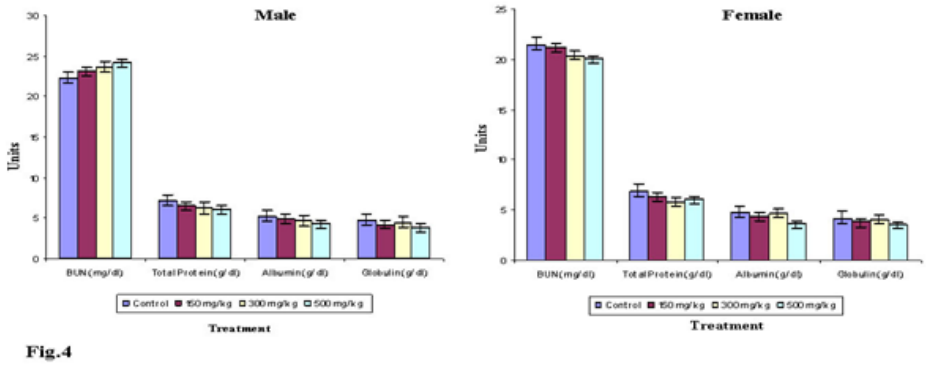

Figure 4: serum biochemical test of ZnO nanoparticle using S. jambolanum