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Research Article

ACUTE & AND CHRONIC TOXICOLOGICAL STUDIES OF THE BIOSYNTHESIZED GOLD NANOPARTICLES ON WISTAR RATS

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ABSTRACT

In the present study, gold nanoparticles were biosynthesized using green extracts of medicinal plants. The synthesized nanoparticles were screened for *in vitro* anti HIV, anticancer and anti-tuberculosis activity. All the three studies demonstrated potential agonistic effects of the synthesized gold nanoparticles against HIV, cervical cancer and tuberculosis. Simultaneously, the *in vitro* cytotoxicity was checked in epithelial cell lineage such as Vero cell line. The results of *in vitro* cytotoxicity showed that the synthesized nanoparticles were non-toxic to the normal cells. The results of the *in vivo* toxicity will help in the effective formulation of the gold nanoparticles towards drug development for HIV, cervical cancer and tuberculosis.

The results of acute toxicity and chronic toxicity implied that the animals did not suffer from any adverse effects following administration of gold nanoparticles. The animals survived both single dose and consecutive dosage for 28 days. The animals were observed to be normal without any abnormal behavioral changes. The blood parameters showed no significant difference between the control and treated animals on various parameters under consideration. Hematology reports suggested that the metabolic parameters of the treated group do not vary significantly with the control group. All the hematology reports implied that the biosynthesized gold nanocompounds did not elicit adverse effects on the animal model system. In the histopathology investigation, the major organs, such asthe brain, the heart, the kidney, the liver and the spleen were excised from the control and treated animals. The tissue samples were sectioned, stained with eosin red and viewed under microscope. The microscopic examination of all the tissues represented normal cellular organization without any distinctive tissue degeneration and organ damage.

The present study has been successfully carried out to check the *in vivo* toxicological effects of the biosynthesized gold nanocompounds adapting Wistar rats as animal model system. The outcome of the study emphasized that the biosynthesized AuNPs were non-toxic to the treated animal model system. Further the study will focus on other toxicological implications such as immunotoxicity, neurotoxicity, reproductive toxicity and genotoxicity. The biosynthesized gold nanocompounds have a promising role as versatile nanomaterials towards multiple applications.

Keywords: Gold nanoparticles, Toxicity, Acute, *In vivo*, chronic, Wistar rats.

1. INTRODUCTION

Rats and mice are the major class of model animals used for experimental animal studies. These animals are smaller, simpler to handle with higher reproduction rate. Moreover rats have nearly 95% resemblance to human DNA and hence the results achieved through experiments could be correlated to humans in most of the cases. The experimental animal models were adapted for various preclinical findings in toxicology, safety and efficacy testing, reproduction and development, behavior, nutrition and pharmacological studies [1].

The impact of nanomaterials on human health, safety and nanotoxicity is one of the major concerns in biomedical applications of nanoparticles. The size and surface area of the nanoparticles have a prominent role in influencing the fate of the nanomaterials within the human system. Various studies have reported on the pharmacokinetics, pharmacodynamics, biodistribution, absorption, retention, elimination and accumulation of nanomaterials in the body and vital organs. In most of the reports, the results indicated size and shape dependent toxicity of nanomaterials [2].

In nanotechnology, gold nanoparticles (AuNPs) are indeed the most versatile nanomaterials of choice with a wide range of applications. There are certain peculiar properties of AuNPs, facilitating their wider applicability such as increased surface-to-volume ratio, optical properties, ease to synthesis, stability and surface functionalization [3].

Among all the metallic nanoparticles, gold nanoparticles (AuNPs) have emerged as a favorable carrier for the site-specific targeted delivery. In a recent study, AuNPs have been used as a multivalent system for targeted delivery of anticancer drugs in combination with their own theranostic properties [4].

Development of highly efficient multifunctional nanoparticles (NPs) is a potential strategy in the treatment of various diseases and disorders. The multifunctional nanoparticles possess several promising features such as increased surface to volume ratio, a unique capacity in adsorbing Aβ monomers and interference with the fibrillation process [5].

The efficiency of the nanoparticles is proportional to their size; smaller sizednanoparticles are more efficient compared to bigger nanoparticles. In order to penetrate the tumor in cancer therapy, the nanoparticles must initially cross the barriers across the cell surface. The smaller sizednanoparticles easily overcome the barriers effectively. Different sized nanoparticles demonstrated different levels of toxicity, clearance, heat generation efficiency, blood circulation time and intratumoral penetration ability [6].

The histological investigation was carried out on the vital organs such as the lung, the kidney, and the liver following administration of AuNPs for 10 days. No subclinical toxicity was observed following the administration of the AuNPs. AuNPs were reported to be excellent anti oxidative and anti-hypoglycemic agents [7].

In general, the dimensions of NPs play a crucial role in enhancing internalization. Cobalt nanoparticles were reported to induce the transformation of cells whereas cobalt ions did not exhibit such response. AuNPs are one of the best exploited nanomaterials due to their versatility in particle size, surface modification and biocompatibility. Several studies have reported that AuNPs induce apoptosis via caspase-dependent mechanisms and also increase cell susceptibility to apoptosis induced by other agents [8].

Stability of AuNPs is one of the major factors in consideration for various applications. Protective agents such as capping molecules help to improve the stability of the nanomaterials. In case of poorly stable systems, PEG can be employed as the capping agent to prevent the aggregation of AuNPs [9].

In the present study, the biosynthesized gold nanoparticles were subjected to *in vivo* acute and chronic toxicological effects on Wistar rats as animal model system. The novel nanoformulations were developed through biosynthetic method. Gold nanoparticles were synthesized using plant extracts as reducing and stabilizing agents. The synthesized nanoparticles were characterized by spectroscopic analysis, SEM, HRSEM, TEM and other techniques which demonstrated the morphology, dimensions and stability of the nanoparticles. The nanoparticles demonstrated high stability without any additional stabilizers or capping agents. The *in vitro* studies showed that the synthesized nanoparticles have potential agonistic effects against HIV, cervical cancer and Tuberculosis at very low EC_{50} and IC_{50} concentrations. It was also observed that the gold nanoparticles were non-toxic to the normal cell line with high cell viability at all concentrations. Further, the success of the preclinical study can be utilizedfor effective formulation of the nanoparticles as drugs and drug delivery molecules.

2. METHODOLOGY

2.1. Animal Profile of the animals under study:

The albino animal model Wistar rats were selected for the study. The animals were Male/Female, around 8-10 weeks old and weighted about 200±30g. The animals (21 animals) were purchased from TANUVAS, Madhavaram and were transported to C.L. Baid Metha College of Pharmacy, Thoraipakkam, where the study was carried out. All the animals were maintained in the Animal House in the College of Pharmacy, Thoraipakkam. The animals were maintained at a constant temperature with a 12-hour light/dark cycle and fed with clean water and food. All the experimental procedures have been adopted as per OECD guidelines and approved by the Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) with reference number (05/321/PO/Re/S/01CPCSEA/dated 30/03/2019).

2.2. Biosynthesized gold nanoparticles

The gold nanoparticles (AuNPs) were biosynthesized (nanoparticles formed or synthesized through biological materials like plants) using green extracts of plants as discussed previously in our other reports [10]. The

characterization was carried out to get an insight of the morphology and dimensions of the AuNPs [11] [12]. The effect of the underlying parameters such as temperature and pH were optimized, which play a crucial role in the synthesis [13]. The applications of gold nanoparticles [14], *in vitro* cytotoxicity [15], anticancer assay [15-20] and anti HIV assay [21] were carried out in different cell lines. The results demonstrated non-toxicity of the AuNPs to normal cell line.

2.3. Characterization of the AuNPs

The initial confirmation of the AuNPs was carried out by UV-vis spectrophotometer which showed Surface Plasmon Resonance (SPR) peaks corresponding to gold nanoparticles. The AuNPs were characterized through High Resolution Scanning Electron Microscopy (HRSEM) which revealed the size and morphology of the nanoparticles. The stability of the AuNPs was checked using zeta potential analyzer.

2.4. Experimental setup

The animals (Wistar rats) were divided into 5 groups. Group A comprising 3 Males around 8-10 weeks old and weighted about 210±10g, Group B comprising 3 Females around 8-10 weeks old and weighted about 240±20g, Group C comprising 6 animals with 3 Males and 3 Females, Group D comprising 6 animals with 3 males and 3 Females, Group E comprising 3 animals with 2 Male and 1 Female. First two groups (A, B) were assigned for initial level of acute toxicity. 3 Male and 3 Female were randomly chosen and marked. This study was monitored for 14 days. Based on the observation, the chronic study was further commenced.

Next two groups (C, D) were followed for the chronic toxicity. This study was monitored for 28 days. One group (E) was maintained as control group.

2.5. Acute toxicity

Animals from the Groups A and B were treated with single dose of 1.3 µg/ml AuNPs. The dosage was administered following oral route. The animals were weighed every day. The treated animals were observed for 14 different parameters like body weight, assessments of posture, signs of convulsion, body tone, lacrimation, salivation, changes in skin color, piloerection, defecation, sensitivity response, locomotion, muscle gripness, rearing and urination. Additionally, other parameters such as any changes in behavior and depression were assessedbased on the animals' behavior and daily routine activities. After the end of 14th day, the animals were examined. The chronic toxicity study was further commenced.

Table 1: Experimental setup of the study

Groups	No. of animals	Description of the group
Acute Group A (3 animals)	Male (3)	Treated with synthesized Gold nanoparticles (AuNPs)
Acute Group B (3 animals)	Female (3)	Treated with synthesized Gold nanoparticles (AuNPs)
Chronic Group C (6 animals)	Male(3) Female(3)	Treated with biosynthesized Gold nanoparticles (AuNPs) (high dose) Treated with biosynthesized Gold nanoparticles (AuNPs) (low dose)
Chronic Group D (6 animals)	Male(3) Female (3)	Treated with biosynthesized Gold nanoparticles (AuNPs) (low dose) Treated with biosynthesized Gold nanoparticles (AuNPs) (high dose)
Group E (3 animals)	3 (random)	Control group

2.6. Chronic toxicity

The animals from Groups 3 and 4 were administered with different oral doses of AuNPs every day for 28 days. The high dose was fixed at 1.3µg/ml of AuNPs and the low dose was fixed at 0.13µg/ml of AuNPs. After dosage administration, the animals were observed for 14 different parameters like body weight, assessments of posture, signs of convulsion, body tone, lacrimation, salivation, changes in skin color, piloerection, defecation, sensitivity response, locomotion, muscle gripness, rearing and urination. Additionally, other parameters such as any change in behavior and depression were assessed based on the animals' behavior and daily routine activities. At the 15th day of study, about 0.5ml of blood was collected from the treated (high dose) and control group and compared for various blood parameters. Similar observation was followed at the 28th day of study. After

the end of 28 days, the animals were sacrificed to follow with histopathological analysis. In the tissues samples, the major organs, such as the brain, the liver, the kidney, the spleen, and the heart were excised, stained, and examined. From the outcome of the study, the toxicological implications of the biosynthesized AuNPs were elucidated.

2.7. Histopathological analysis

At the end of the 28 days study, the rats were anesthetized with Chloroform and sacrificed. The major vital organs, such as the heart, the brain, the liver, the kidney and the spleen were excised. All the excised organs were immersed in 10% neutral buffered formalin (NBF) which hardened the tissues that helped in the survival for the preparatory steps. The Formalin-fixed Paraffin embedded (FFPE) sections were prepared through infiltration by wax. The tissues were dehydrated with different concentrations of ethanol. Further the tissues were embedded into IHC-grade paraffin for fixed time periods. The vital organs were sectioned and stained with eosin red from the control & treated group. The stained tissue sections were observed under a light microscope. The prepared FFPE tissue blocks and tissue slides were stored at room temperature [22].

2.8. Determination of gold nanoparticles concentration in the tissue samples: ICP-OES

The FFPE tissue blocks were analyzed to determine the concentration of gold nanoparticles present/retained in the tissues. The tissue was digested completely in nitric acid solution. The solution was made up to 10 ml with double distilled water [23]. The solution was quantitatively analyzed by ICP-OES to determine the concentration of gold nanoparticles in the tissue sample at Centralized instrumentation SAIF, IIT Madras, Chennai.

3. RESULTS

3.1. Biosynthesized gold nanoparticles (AuNPs)

The biosynthesis of AuNPs was carried out using the precursor solution, Chloroauric acid. Chloroauric acid was pale yellow in color in the higher metallic dimensions. When reduced to nano dimensions, the biosynthesized AuNPs were observed to be deep wine red in color due to the shift in the absorption spectrum.

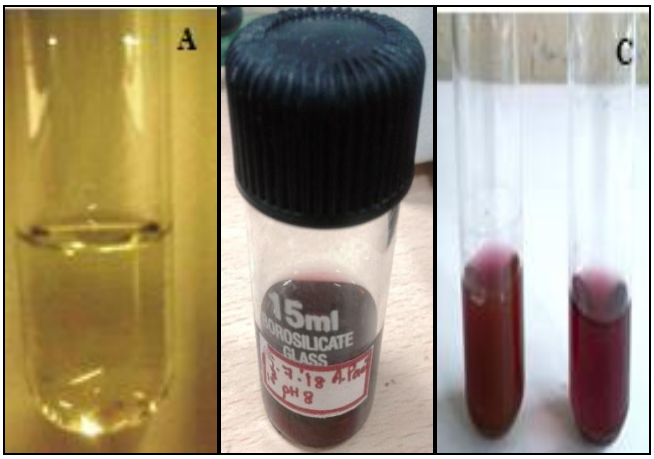


Fig. 1: a) Chloroauric acid, b) & c) Synthesized AuNPs

3.2. UV Visible Spectrophotometry

The UV absorption peak was the corresponding wavelength at which the absorption spectrum of metallic nanoparticles was measured by the surface plasmon resonance (SPR). There was a notable correlation existing between the wavelength and the particle size of the NPs, as the particle size increased, the wavelength of the absorption peak shifted to longer spectrum.

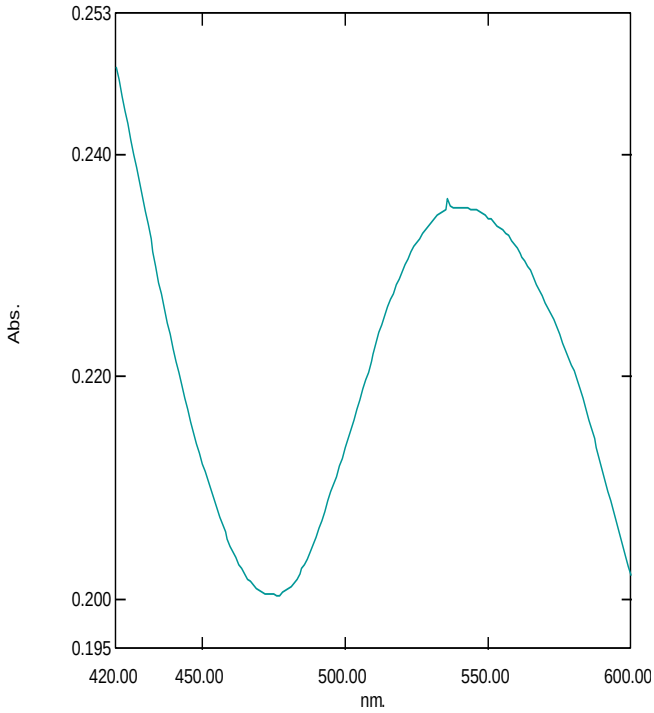


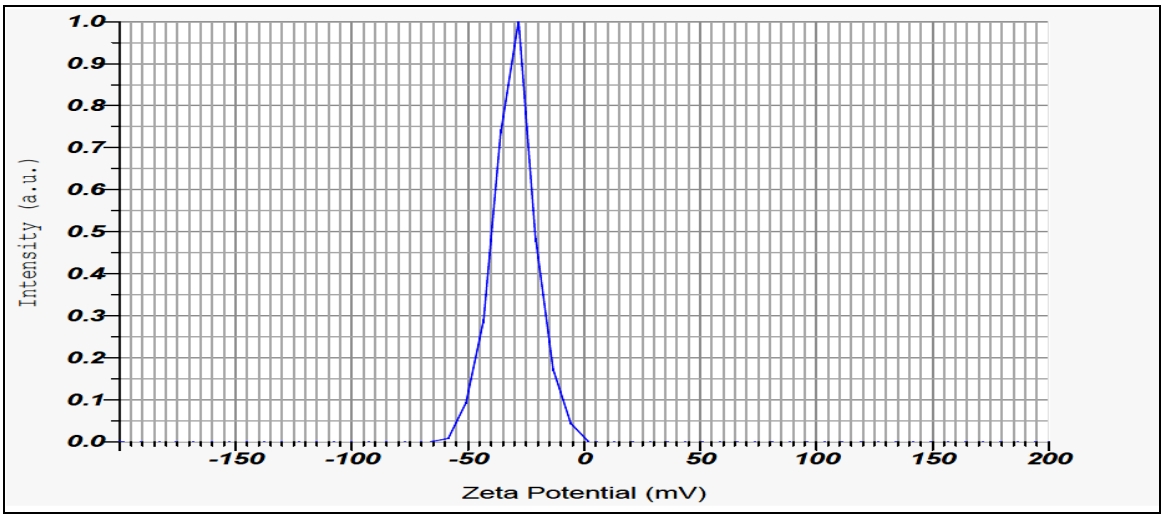
Fig. 2: UV spectrum of synthesized AuNPs

The UV absorbance spectrum of biosynthesized AuNPs was observed with SPR peak at 536nm which

confirmed the formation of gold nanoparticles. The preliminary confirmation of the synthesized nanoparticles was carried out through UV visible absorption spectrum, which showed the characteristic peak for nanoparticles. The peak confirmed the formation of gold nanoparticles. Another research has reported on the similar absorption spectrum of biosynthesized gold nanoparticles [24].

3.3. Zeta Potential

The stability of the synthesized AuNPs was analyzed through Zeta potential measurement. The Zeta potential measurement corresponded to the charges present on the surface of the NPs. The positive or negative charge was responsible for the stability of the AuNPs. The charge enabledthe particles to be evenly dispersed and impeded any aggregations of AuNPs. When the Zeta potential value was <-30mV, the particles were held together with strong electrostatic forces. These forces rendered high stability to the nanoparticles with meager chances of accumulation [25]. The observed zeta potential values were in concordance to similar results stated by previous report.



Zeta potential (Mean): -34.9mV

Fig 3: Zeta potential analysis

3.4. High Resolution Scanning Electron Microscopy (HRSEM) Imaging

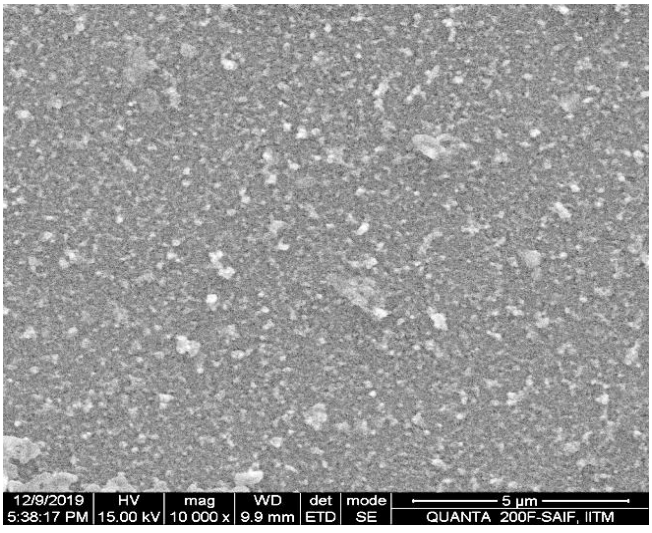


Fig. 4a: HRSEM images of the nanoparticles

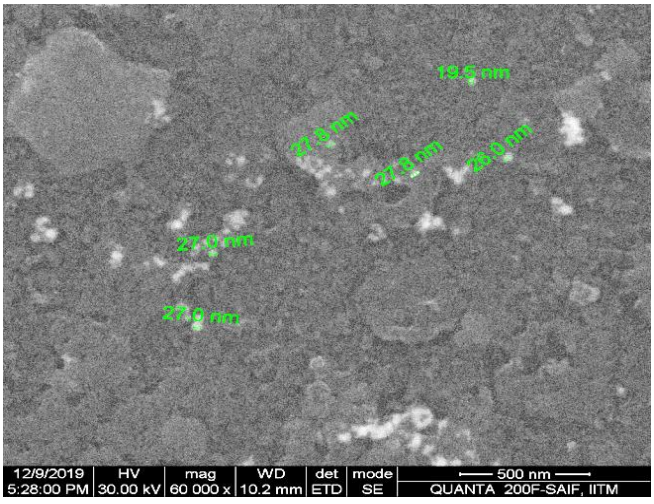


Fig. 4b: Size of nanoparticles in HRSEM

The size of the NPs was 19 nm upwards, and an average of 27nm, which was a commendable nano dimension achieved through plant mediated synthesis. The NPs

were spherical in morphology and evenly distributed. The significance on the size of the nanoparticles has been similarly stated by previous report [26].

3.5. *In vitro* Cytotoxicity study

The *in vitro* cytotoxicity of the biosynthesized AuNPs was tested in an epithelial cell lineage such as Vero cell line through MTT assay. Cytotoxicity was determined for varying concentrations of the synthesized gold nanoparticles such as 1.3, 0.65, 0.325, 0.1625, 0.08125, 0.040625, 0.0203125, 0.01015625, 0.0050781 & 0.0025390µg/ml. The minimum percentage of viability was observed to be 81% and maximum viability was 100%. The results implied that the AuNPs indicated very low cytotoxicity at all the assessed concentrations.

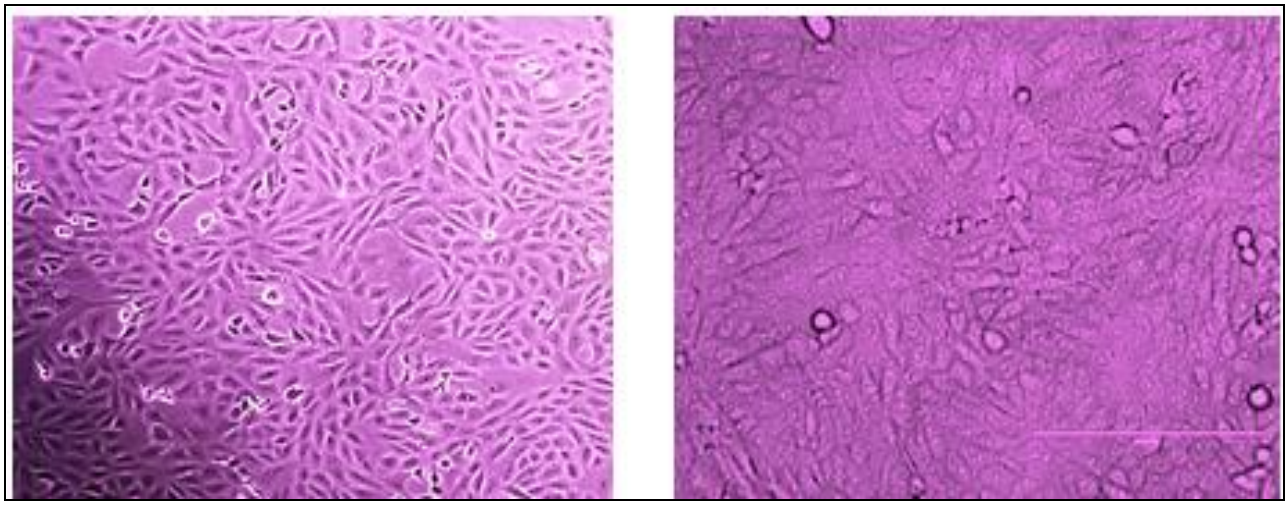


Fig. 5a: Control Vero cell line Fig. 5b. Vero cell line treated with AuNPs

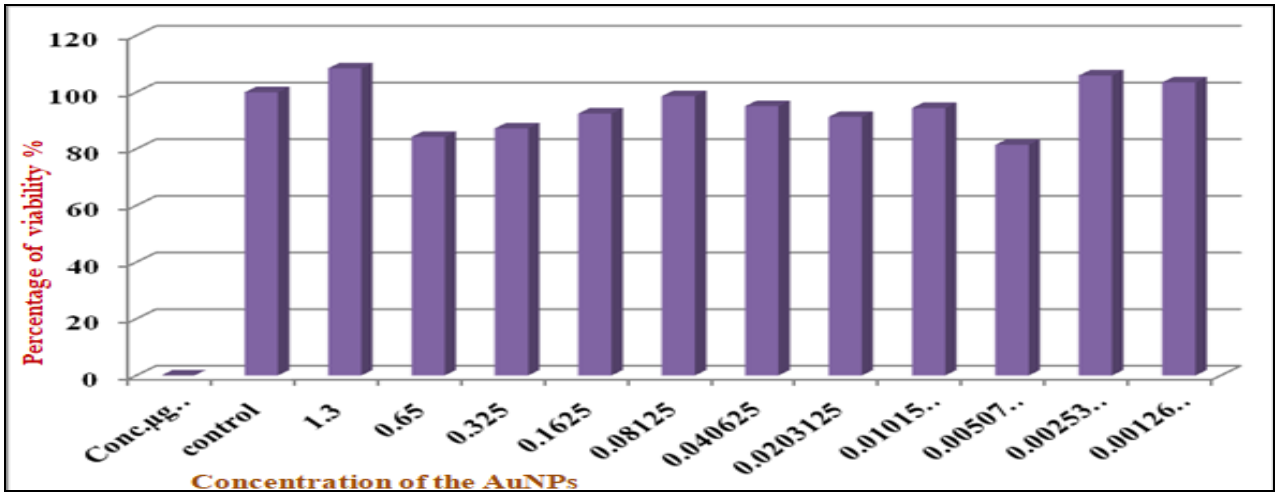


Fig. 6: Cytotoxicity assay - % of viability in Vero cell line

3.6. Acute Study

The animals were orally administered with single dose of AuNPs. The dosage was calculated as 100µg/kg body weight and administered. After administration of single dosage, all the animals were observed for the next 14 days. The animals were weighed every day and checked for the various body parameters like lacrimation, salivation, fatigue, loss of appetite, change in fur color, and weight loss. After 14 days of continuous observation, the animals were assessed on the concluding day. All the animals survived the entire duration of study. The animals did not show any adverse signs or symptoms and changes in behavior. The AuNPs were found to be non-toxic to the animal model system at the tested concentrations. Further, Chronic toxicity study was followed for 28 days.

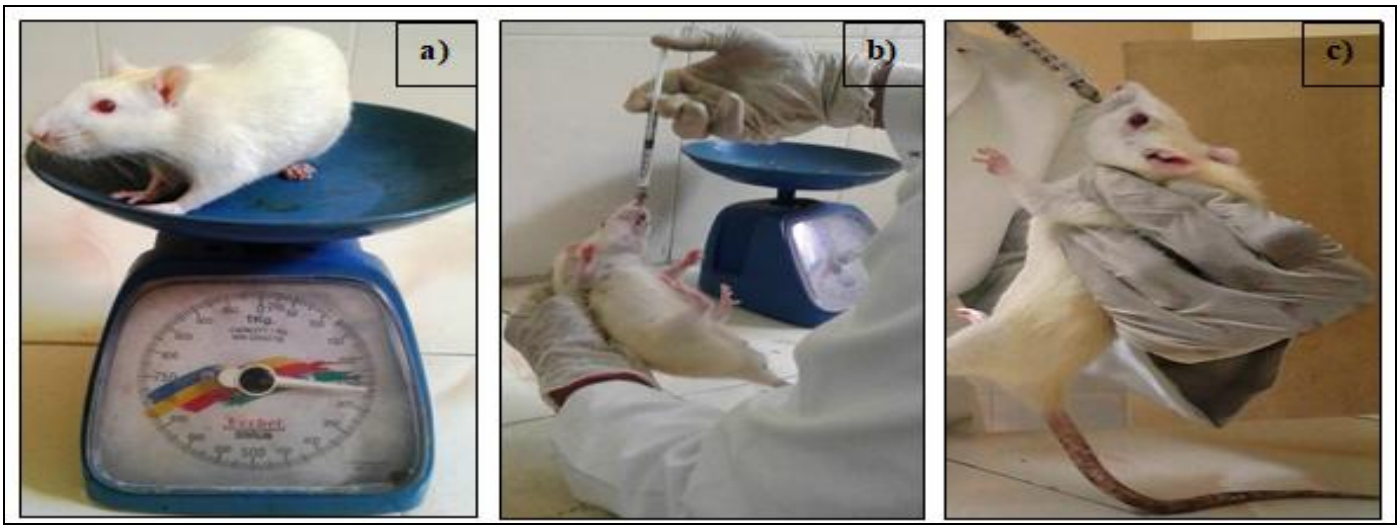


Fig. 7: a) Experimental setup b) Body weight assessment c) Oral dosage administration to animals

Table 2: Body weight assessment

S. No.	Body weight	Group 1-1A	Group 1-1B	Group 1-1C	Group 2-2A	Group 2-2B	Group 2-2C
1.	DAY 1	200 g	200 g	200 g	180 g	210 g	250 g
2.	DAY 2	200 g	200 g	200 g	180 g	210 g	250 g
3.	DAY 3	200 g	200 g	200 g	180 g	210 g	250 g
4.	DAY 4	215 g	215 g	200 g	200 g	210 g	250 g
5.	DAY 5	200 g	210 g	200 g	200 g	210 g	250 g
6.	DAY 6	210 g	215 g	200 g	200 g	210 g	260 g
7.	DAY 7	210 g	215 g	200 g	200 g	210 g	260 g
8.	DAY 8	210 g	220 g	210 g	210 g	210 g	260 g
9.	DAY 9	220 g	230 g	210 g	210 g	210 g	260 g
10.	DAY 10	220 g	230 g	220 g	220 g	230 g	260 g
11.	DAY 11	230 g	240 g	220 g	220 g	230 g	260g
12.	DAY 12	240 g	250 g	230 g	230 g	230 g	270g
13.	DAY 13	240 g	250 g	230 g	230 g	230 g	270g
14.	DAY 14	240 g	250 g	230 g	230 g	230 g	270g

3.7. Effects on the Body weight

The animals were administered with a single oral dose of biosynthesized gold nanocompounds and observed for the following 14 days. The weight chart was recorded for 14 days between the treated and control group. It was found that the animals were gaining healthy weight with normal development. There was no significant weight loss in treated animals and all the animals were healthy, active and displayed normal behavior throughout the study.

3.8. Acute Toxicity Study: Parameters under Observation

3.8.1. Effects on Body parameters

All the 14 body parameters of the treated group were in concordance with the control group including body weight, assessments of posture, signs of convulsion, body tone, lacrimation, salivation, and change in skin colour, piloerection, defecation, sensitivity response, locomotion, muscle gripness, rearing and urination. The results of the acute toxicity study indicated that the biosynthesized gold nanocompounds were non-toxic to the treated animals. Based on the results of acute study, the chronic study was succeeded with high and low dosages of the nanocompounds.

3.9. Chronic Toxicity Study

The animals were orally administered with a single dose of AuNPs for a consecutive 28 days. The high dose was calculated at 100µg/kg body mass and the low dose was

calculated at 10µg/kg of body mass. After the dosage, all the animals were under observation for the next 28 days. The animals were constantly observed for various body parameters like fatigue, loss of appetite, changes in fur color, and weight loss. On the 15th day of study, blood was collectedfrom the treatedand control group and compared for different blood parameters. Similarly, a comparative blood analysis was followed at the 28th day of study. After the end of 28 days, the animals were anaesthetized with Chloroform and sacrificed to precede with histopathological analysis. In the tissues samples, the major organs, such as the brain, the liver, the kidney, the spleen, and the heart were excised, stained, and examined. Fromthe outcome of the study, the toxicological implications of the biosynthesized AuNPs were inferred.

Table 3: Various Body Parameters under observation in the Wistar rats

S. No.	Parameters under observation	Control Group	Group 3 - a	Group 3 - b	Group 3 - c	Group 4 - a	Group 4 - b	Group 4 - c
1.	Body weight	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2.	Assessments of posture	Normal	Normal	Normal	Normal	Normal	Normal	Normal
3.	Signs of convulsion	Normal	Absence of sign	Absence of sign	Absence of sign	Absence of sign	Absence of sign	Absence of sign
4.	Body tone	Normal	Normal	Normal	Normal	Normal	Normal	Normal
5.	Lacrimation	Normal	Absence	Absence	Absence	Absence	Absence	Absence
6.	Salivation	Normal	Absence	Absence	Absence	Absence	Absence	Absence
7.	Change in skin color	No significant color change	No significant color change	No significant color change	No significant color change	No significant color change	No significant color change	No significant color change
8.	Piloerection	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9.	Defecation	Normal	Normal	Normal	Normal	Normal	Normal	Normal
10.	Sensitivity response	Normal	Normal	Normal	Normal	Normal	Normal	Normal
11.	Locomotion	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12.	Muscle gripness	Normal	Normal	Normal	Normal	Normal	Normal	Normal
13.	Rearing	Mild	Mild	Mild	Mild	Mild	Mild	Mild
14.	Urination	Normal	Normal	Normal	Normal	Normal	Normal	Normal

3.10. Effects on the Complete Haemogram of Control & Treated animals

There were previous reports on the *in vivo* toxicological effects of silica nanoparticles on bio-distribution, clearance and biocompatibility adapting mice as animal model system [26]. It was found that the standard hematological analysis including red blood cell count (RBC), haemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count (PLT) and white blood cell count (WBC) were mostly within normal ranges. It was found that the silica nanoparticles did not induce toxicity in the treated animals.

In the present study, standard hematological analysis was carried out between the control and treated animals. The hematology analysis reported on white blood cell count (WBC), red blood cell count (RBC), platelets, Polymorphs, Lymphocytes, Monocytes, Eosinophils, Basophils, haemoglobin (HG), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and packed cell volume (PCV) of the control and treated animals. The results of the treated group were mostly in concordance with the control group and within the normal ranges indicating normal body metabolism in the treated animals. It was found that the biosynthesized gold nanocompounds did not elicit any adverse effects over the treated animals.

3.11. Liver function test

There were previous reports on the toxicological effects of silica nanoparticles [26]. There was an increased activity of Total Bilirubin indicating an abnormal biliary function and abnormal levels of CREA and BUN indicating altered kidney function due to the effect of the nanoparticles. In the present study, all the

biochemical parameters were found to be within the normal ranges in the animals treated with gold nanocompounds. The results of the treated animals were in concordance with the control group.

Table 4: Complete Haemogram of control & treated group

S. No.	Blood parameters	Control	Treated (High Dose)	Reference value
1.	Total WBC count	11,900 cells/cumm	8400 cells/cumm	4000 to 11000
2.	Polymorphs	67 %	78 %	35 to 65
3.	Lymphocytes	29 %	15 %	30 to 55
4.	Monocytes	00 %	00 %	0 to 6
5.	Eosinophils	04 %	07 %	0 to 6
5.	Basophils	00	00 %	0 - 1
6.	Haemoglobin	9.3 gms %	9.4 gms %	11.5 to 17.0
7.	Total RBC count	4.07 millions/cumm	4.28 millions/cumm	3.0 to 5.5
8.	PCV	25.7 %	24.8 %	38 - 47
9.	MCV	63.1 fL	57.9 fL	76 - 96
10.	MCH	22.9 pg	21.7 pg	27 - 32
11.	M.C.H.C.	36.2 grams/dl	37.5 grams/dl	31 - 35
12.	Platelet count	1.66 Lakhs/cumm	2.65 Lakhs/cumm	1.5 to 4.0

Table 5: Liver function test of control and treated group along with reference values

S. No.	Blood parameters	Control	Treated (High Dose)	Reference value
1.	Bilirubin (T)	0.2 mgs/dl	0.3 mgs/dl	Upto 1.2 mg/dl
2.	Bilirubin (D)	0.1 mgs/dl	0.1 mgs/dl	Upto 0.4 mg/dl
3.	Bilirubin (ID)	0.1 mgs/dl	0.2 mgs/dl	Less than 0.9 mg/dl
4.	SGOT	263 U/L	202 U/L	10 - 40 u/l
5.	SGPT	76 U/L	61 U/L	10 - 40 u/l
6.	Alkaline Phosphatase	247 U/L	209 U/L	Upto 270 u/l
7.	Total Protein	6.8 gms/dl	6.2 gms/dl	6.0 - 8.8 gms/dl
8.	Albumin	2.9 gms/dl	2.9 gms/dl	3.5 - 5.2 gms/dl
9.	Globulin	3.9 gms/dl	3.3 gms/dl	2.0 - 4.0 gms/dl
10.	A/G Ratio			Less than 1.0

Table 6: Biochemistry & Lipid Profile of control & treated group along with reference values

S. No.	Blood parameters	Control	Treated (High Dose)	Reference value
		Biochemistry		
1.	Urea	37 mg/dl	48 mg/dl	15 - 45
2.	Creatinine	0.56 mg/dl	0.79 mg/dl	
3.	Uric acid	2.17 mg/dl	1.50 mg/dl	0.4 - 1.4 2.4 - 7.0
		Lipid Profile		
4.	Total Cholesterol	59 mg/dl	56 mg/dl	Less than 220 mg/dl
5.	Triglycerides	114 mg/dl	62 mg/dl	
6.	HDL	21 mg/dl	18 mg/dl	Less than 150 mg/dl 35 - 65 mg/dl

3.12. Histopathology

In the Histopathology investigation, the major organs such as the brain, the heart, the kidney, the liver and the spleen were excised from the control and treated animals. The tissue samples were sectioned, stained with eosin red and viewed under microscope. The microscopic examination of all the tissues represented normal cellular organization without any distinctive tissue degeneration and organ damage.

In the present study, the treated animals did not show marked differences in signs or symptoms compared to the control group. Hematology reports also suggested that the treated group did not vary significantly with the control group in all the metabolic parameters. All the

hematology reports implied that the biosynthesized gold nanocompounds did not elicit adverse effects on the animal model system. The results were in contrast to various reports on the *in vivo* toxicity of gold nanoparticles, where high toxicity was reported to the animal model system.

Fig. 8 a, b: Animals under anesthetics in Chloroform

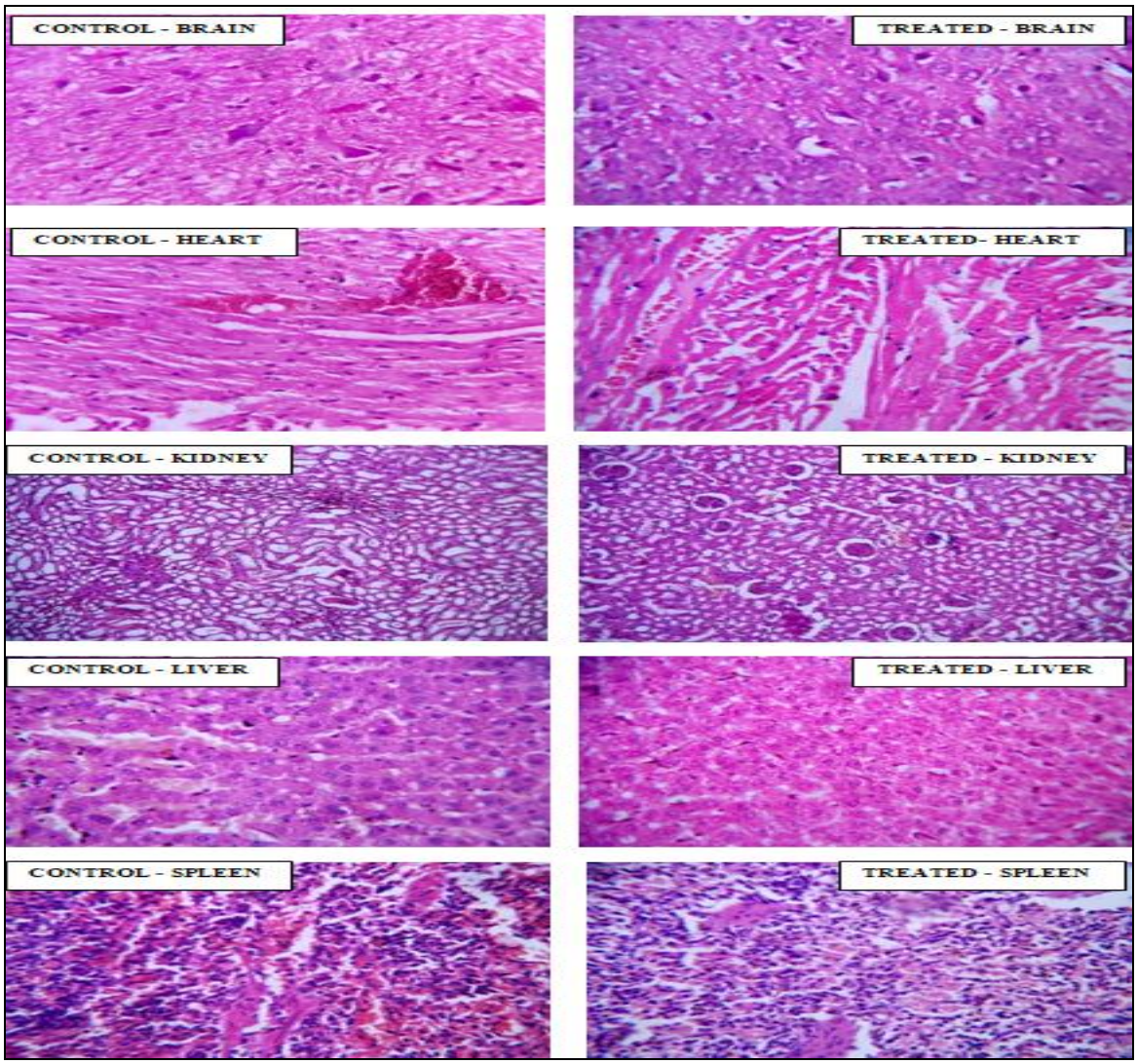


Fig. 9 a, b: Brain tissue, c, d: Heart tissue, e, f: Kidney tissue, g, h: Liver tissue i, j: Spleen tissue

3.13. Determination of gold nanoparticles concentration in the tissue samples: ICP-OES

The quantitative estimation of elemental gold was carried out in Spleen and Kidney tissue. The estimated concentration of the gold nanoparticles indicated the percentage of gold nanoparticles in the tissues/organs at the end of the study. The difference between the total dosage administered and final concentration accumulated will help to determine the relative elimination/retention of the gold nanoparticles in the animal system. The total dosage of gold nanoparticles administered was 359.52µg/ml for 28 days. The final concentration of gold nanoparticles was estimated to be 0.196µg/ml in Spleen and 0.172µg/ml in Kidney. It was observed that only 0.05% and 0.04% of gold nanoparticles was found in the Spleen and Kidney tissues. The ICP-OES analysis indicated the concentration of the gold nanoparticles after the completion of the study.

There were previous reports on the approximate permissible limits of gold content in biological samples as 1-10µmol/L [23]. There were previous reports on the biodistribution and bioaccumulation of gold nanoparticles in Wistar rats. The animals were treated with 0.4 mL/day of AuNPs in various nano dimensions such as 10, 30, 60 nm for 9 days. The bioaccumulation of gold in Kidney and Spleen was reported as 595 and 945 µg/kg of body weight respectively [28]. In the present study, the concentration of gold nanoparticles were found to be very low and within the permissible limits compared to the previous report. The biosynthesized gold nanoparticles can be further exploited for various *in vivo* applications.

Table 7: ICP-OES analysis of tissue samples

S. No.	Concentration of total Dosage administrated-High dose(AuNPs) (28 days)	Tissue Samples analyzed	Conc. of gold in ppm (µg/ml)/ (mg/L)	Approximate concentration/ permissible levels of gold In therapy
1.	359.52 µg/ml	Spleen	0.196 µg/ml	0.0196 µg/ml - 19.66 µg/ml
2.	359.52 µg/ml	Kidney	0.172 µg/ml	0.0196 µg/ml - 19.66 µg/ml

4. DISCUSSION

Various studies have reported on the *in vivo* toxicity of the metallic nanoparticles in animal models. In one of the *in vivo* studies, chronic toxicity was checked at two doses 1 mg/kg and 2 mg/kg which showed dose dependent effect of AuNPson animal system. There were acute symptoms, damage to the major organs and eventually leading to death of the animals. On long term exposure, there were some physical changes such as change in fur color, skin texture and skin flaccidity in the treated animal groups. The death of majority of mice in high dose group within 30 days of treatment indicated high toxicity of the treated gold nanoparticles. The results indicated high toxicity of the gold nano-particles [29]. On a contrary, the gold nanoparticle under study was observed to be non-toxic, making it more suitable for *in vivo* applications.

There were previous reports on the biodistribution and toxicity effects of gold nanoparticles in a single-dose intravenous administration adapting Wistar rats as animal model system. The effects were observed in short term after 30 minutes post administration and in long term after 28 days post administration of gold nanoparticles. It was found that there were no behavioral changes and body weight variations in the animals throughout the duration of the study [30]. In the present study, the biosynthesized gold nanoparticles were administered for 28 days and found that the gold nanoparticles did not induce any abnormal changes on all the 14 parameters under consideration. No significant variations were recorded in body weight among the treated and control group of animals. All the animals exhibited normal growth and healthy weight throughout the experimental study.

In the present study, Wistar rats were adapted as the animal model system to determine the toxicity of gold nanoparticles. Some studies have used zebrafish as *in vivo* animal model to understand the toxicity profile of AuNPs. It was reported that functionalized AuNPs caused abnormalities in the eye development and

affected pigmentation in the eyes leading to behavioral and neuronal damage. Another study assessed the toxicity mapping of AuNPs of different sizes and surface charges. It was found that the mortality and developmental disorders were closely related to the morphological and chemical characteristics of AuNPs.The correlation was emphasizing the importance on controlled synthesis of nanomaterials to reduce adverse effects. In a different study, real time *in vivo* imaging was adapted to determine the size-dependent transport and toxicity of AuNPs in zebrafish embryos. AuNPs were traced inside the embryos throughout the developmental period. Another study reported on the exposure of AuNPs on zebrafish for a period of 20 days at two concentrations. It was found that AuNPs induced alterations in oxidative stress, mitochondrial metabolism and neurotransmission [31]. In all the above studies, AuNPs were reported to be toxic to the animal model system. The biosynthesized AuNPs were found to be non-toxic to the treated animals.

Functionalization of nanoparticles was carried out to enhance the workability, stability and efficiency of nanomaterials. Particularly in biomedicine to achieve better therapeutic effects of drug and delivery molecules. In some instances these functionalized nanomaterials become more toxic than the native nanomaterials. A previous study has reported on toxicological implications of functionalized AuNPs adapting *Daphnia magna* as the model species. It has been found that the functionalized gold nanorods were more toxic than native nanomaterials [32]. In the present study, the biosynthesized AuNPs were proven to be non-toxic and much effective in the native form without any additional functionalization.

There were earlier reports on the toxicological effects of silica nanoparticles on Balb/C mice. The animals were monitored for 14 days on body weight and various body parameters post administration of silica nanoparticles. No abnormal signs or symptoms like hypopnea, tremor, arching of back, any symptoms of poisoning, loss of appetite, diarrhea, vomiting were observed on various dosages for 14 days of treatment [33]. In the present study, chronic toxicity was carried out to elucidate the toxicological effects of biosynthesized gold nanoparticles on Wistar rats for 28 days. All the animals were healthy and did not exhibit any abnormal signs and symptoms throughout the period of study. The biosynthesized gold nano-compounds were found to be non-toxic over the treated animal model system. The biosynthesized gold nanocompounds can be further exploited for various biomedical applications as lead drug molecules and or drug delivery molecules.

5. CONCLUSION

Nanomaterials play a pivotal role in some of the prominent fields such as tissue engineering, drug delivery, diagnostics, biosensors, and*in vivo* replacements. Preferably, gold nanoparticles have attracted more interest for *in vivo* applications, theranostics and therapy. In this context, the present study has been successfully carried out to check the *in vivo* toxicological effects of the biosynthesized gold nanoparticles on Wistar rats as animal model system. In acute toxicity, the animals were administered with single dose of AuNPs and were observed for 14 days. Further, Chronic toxicity study was followed for 28 days. In Chronic toxicity, two doses such as high dose and low dose were administered. On the 14th day of Chronic study, hematological analysis of different blood parameters like complete haemogram, biochemistry, lipid profile and liver function test was compared between the control and treated group. Similar hematological analysis was carried out on the 28th day of study. At the end of the study, there were no significant changes noted upon all the parameters under investigation. In the Histopathology investigation, the major organs, such as the brain, the liver, the kidney, the spleen, and the heart were excised, stained, and examined. The microscopic examination of all the tissue samples represented normal cellular organization without any distinctive tissue degeneration and organ damage. The outcome of the study emphasized that the biosynthesized AuNPs were non-toxic to the treated animal model system. Further the study will focus on other toxicological implications such as immuno-toxicity, neurotoxicity, reproductive toxicity and genotoxicity. The biosynthesized gold nanocompounds have promising role as versatile nanomaterials towards multiple applications.

Abbreviations

NPs - Nanoparticles
AuNPs - Gold nanoparticles
IAEC - Institutional Animal Ethics Committee
CPCSEA- Committee for the Purpose of Control and Supervision of Experiments on Animals
OECD - Organization for Economic Co-operation and Development
HRSEM-High Resolution Scanning Electron Microscopy

SPR - Surface Plasmon Resonance
FFPE - Formalin-fixed Paraffin embedded

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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