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Dermal Exposure of *Morinda Citrifolia* Linn. Fruit Extract Coated Gold Nanoparticles: Effects on Haematological and Biochemical Parameters in Wistar Rats

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Abstract

The present study was carried out to evaluate the effect of *Morinda citrifolia* fruit extract coated gold nanoparticles on sub-acute dermal toxicity in wistar rats. Gold nanoparticles (GNPs) were synthesized by biological reduction (green synthesis) of chloroauric acid ($HAuCl_4.3H_2O$) with *Azadirachta Indica* (Neem) 5% leaf extract[1]. The nanoparticles characterized and were confirmed by colour changes from pale yellow to ruby red. These nanoparticles were coated with *Morinda citrifolia* fruit extract (McFE)[1]. The toxicity was evaluated in 50 wistar rats of either sex and randomly assigned to five groups. One group was considered healthy, three groups received dermal application of the combination of 1 mg/kg GNPs with 150 mg/kg McFE (Low dose), 2 mg/kg GNPs with 300 mg/kg McFE (Medium dose) and 4 mg/kg GNPs with 600 mg/kg McFE (High dose) dermal application for 28 days. The last group was considered as satellite group which received 8 mg/kg GNPs with 1200 mg/kg McFE and animals were observed for extra 14 days beyond trial period for post withdrawal effect. There were no abnormal behavioral signs and no any alteration observed on skin throughout the experimental period. Hematological parameters showed no significant variations as a sign of toxicity except hike in neutrophil count and compensatory increase in TEC count on 28th day of entire treatment period but not in a dose dependent manner. Similarly, serum biochemical values showed a hike in ALT and AST in McFE coated GNPs groups. However, hike in BUN values in the experiment was observed to be non-dose dependent and a significant increase in creatinine dose independently on 28th days was observed. There was non-significant increase in total protein on day 14 was observed in the experiment. There was no significant toxicity in entire dosage, from hematology aspect but slight immune stimulation seen up-to 14th day of experiment (neutrophil) in McFE coated GNPs doses with no relation to dose and coating. However, in serum biochemical value slight liver functions tests affected on 14th day but no specific dose related toxicity is observed in any of the treatment. There is increase BUN and creatinine level on 28th day but proper excretion by kidney filtration units. A chronic toxicity trial is appealed to understand the extent of dermal toxicity.

Keywords: *Morinda citrifolia*, Sub-Acute Dermal Toxicity, Synthesis of Gold Nanoparticles

1. Introduction

Gold nanoparticles (AuNPs), an emerging nano-medicine, renowned for its promising therapeutic possibilities, due to its significant properties such as biocompatibility, high surface reactivity, resistance to oxidation and surface plasmon resonance[2]. Gold nanoparticles are compatible for a wide range of biological applications, because of their unique physical and chemical properties. Recently, the applications of gold nanoparticles have expanded into various biomedical fields (biosensors, immunoassays, genomics, photo thermolysis of cancer cells, micro-organism detection and control, targeted drug delivery, optical imaging, monitoring of biological cells and tissues by

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exploiting resonance scattering, or in vivo photo acoustic techniques[3,4]. The role of gold nanoparticles invading the treatment for various inflammatory diseases, other relative disorders and in modern medicine, gold nanoparticles find significant applications in drug delivery system as they are capable of encapsulating active drugs and targeting[5]. Gold NPs have very unique physico-chemical properties and capability of easy functionalization, binding to amine and thiol groups. All these characteristics possessed by gold NPs pave the way for surface modification and are being investigated as drug carriers in cancer and thermal therapy and as contrast agent. Gold NPs are considered to be relatively safe, as its core is inert and non-toxic[6]. Nano toxicology: An emerging discipline evolving from toxicity studies of ultra-fine particles. Widely accepted definitions of nanoparticles refer to a size range of 1-100 nm, where unique phenomena enable novel applications[7]. Nanoparticles are the versatile drug delivery system that can overcome physiological barriers and target the specific site. Nanoparticles can be used therapeutically as drug carriers either by dissolving, entrapping or encapsulating the active substance[8]. The smaller a particle, the greater it's surface area to volume ratio and the higher its chemical reactivity and biological activity. The properties of NPs depend on various factors such as size, shape, purity, crystallinity, electronic properties, type of surface function, solubility and stability[9].

Morinda citrifolia belongs to family Rubiaceae. Indian mulberry (Noni) is a small to medium-sized evergreen tree that is widely distributed in the tropics[10]. The leaves are 8-10 inches long, oval shaped; dark green and shiny with deep veins[11]. Noni is largely used in traditional medicine and has wide range of uses; including arthritis, atherosclerosis, boils, burns, cancer, chronic fatigue syndrome, circulatory weakness, cold sores, congestion, constipation, diabetes, gastric ulcers, gingivitis, heart disease, hypertension and infections[12]. Under favorable conditions plant can bear fruit about 9 months to one year after planting. Fruits are usually harvested at different stages, but most processors buy them at the "hard white" stage for juice production[13]. Several reports have described various potential health benefits of *M. citrifolia* fruit including immune modulation[14] and antioxidant activities[15,16] and *in vitro* and *in vivo* antihyperlipidemic effect[17]. The efficacy of *Morinda citrifolia* Linn. as anti-diabetic, antihypertensive, antioxidant, antihyperlipidemic were proved, the investigations on the efficacy of gold nano particle alone and *Morinda citrifolia* Linn. coated gold Nanoparticles was studied[1].

2. Materials and Methods

The present investigation was carried out on 50 wistar rats at Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Parbhani, MAFSU, Maharashtra.

3. Experimental Animals

The animals (Wistar rats) were purchased from Sanzyme, Ltd. Hyderabad. The present study was conducted on 50 wistar rats of either sex. The animals were selected on the basis of weight range (200-250 gm) of each sex and age (8-10 weeks). Wistar rats were housed in laboratory animal house of COVAS, Parbhani with standard laboratory conditions and *ad-libitum* food and water. The Institutional Animals Ethical Committee (IAEC) approved the experimental protocol vide Resolution no. IAEC/09/17 dated 18/02/2017. All necessary managemental procedures were adopted to keep the animals free from stress.

3.1 Acclimatization, Grouping and Identification of Experimental Animals

Wistar rats were acclimatized to experimental room condition for five days and were randomly divided into five groups, each containing 10 rats of either sex. All the animals were maintained for the experimental period of 28 days except the satellite group (administered with the highest dose), which was maintained for 14 more days (without treatment) to observe reversibility, persistence, delayed toxicity or to study the post withdrawal effect. Wistar rats were kept individually in separate cages, each cage was identified with a label, cage no, group details, treatment type and marking to identify the animals.

4. Drugs and Chemicals

4.1 Gold Chloride

Gold chloride was used to produce Gold nanoparticles (GNPs) by biological reduction method using *Azadirachta indica* (neem) leaf extract.

4.2 *Morinda Citrifolia* (Noni) Fruits Extract

Aqueous extract of *Morinda citrifolia* L. fruit was prepared by cold extraction method. Noni fruits were procured from the College of Food Science and Technology, VNMKV, Parbhani (Figure 3) and cut into small pieces to keep for shed dry. The dried fruit pieces were ground to powder with the help of

an electric grinder. Then, 20% aqueous solution was made by dissolving 200 grams of powder in double distilled water and the final quantity was made to 1 liter. It was mixed thoroughly and allowed to soak for 48 hours at 4^0C in refrigerator with intermittent shaking on flask shaker. Thus, resulting solutions was first filtered by using muslin cloth and then by filter paper. Then it was allowed to dry under shade and thus the aqueous extract of *Morinda citrifolia* fruit was obtained[1].

4.3 Synthesis, Coating and Characterization GNPs

Synthesis of gold nanoparticles was confirmed by color change, which was very well visible with the eyes. When 9ml of 1mM chloroauric acid solution (faint yellow) was reacted with 1ml of 5% Azadirachta *indica (neem)* leaves extract (also faint yellow colour), immediately colour was changed. After 30 minutes of incubation, the solution turned to ruby red to violet colour. When the tubes were left for 2 hours for incubation the colour intensified and tuned to reddish-black colour. To confirm this colour change was due to the formation of gold nanoparticles, one chloroauric acid control (1mM, 10ml) and another extract control (9ml distilled water ± 1ml 5% *Azadirachta indica* leaf extract) was taken to compared (Figure 1).[1]

4.4 Preparation of Site for Drug Application

Fur of all the experimental animals was clipped from dorsal area of the trunk under the inhalation anesthesia (diethyl ether) using an electric clipper, followed by manual shaving using razor blade. Based on OECD guideline 410[18], 10% of the body surface area was cleared for the application of the test substance. Animals were applied with the test substance for 6 hours per day on a 7-day per week basis, for a period of 28 days (Figure 2).

5. Experimental Design

Table 1: Experimental design

Group	Name of Group	Treatment	Dose	Route
I	Healthy control	Deionized water	1 ml	Dermal
II	*Morinda citrifolia* fruit extract Coated GNP Dose –I	Gold nanoparticles	@ 1 mg/kg Bwt.	Dermal
		Morinda citrifolia fruit extract	@ 150 mg/kg Bwt.	
III	*Morinda citrifolia* fruit extract Coated GNP Dose –II	Gold nanoparticles	@ 2 mg/kg Bwt.	Dermal
		Morinda citrifolia fruit extract	@ 300 mg/kg Bwt.	
IV	*Morinda citrifolia* fruit extract Coated GNP Dose –III	Gold nanoparticles	@ 4 mg/kg Bwt.	Dermal
		Morinda citrifolia fruit extract	@ 600 mg/kg Bwt.	
V	Satellite Group	Gold nanoparticles	@ 8 mg/kg Bwt.	Dermal
		Morinda citrifolia fruit extract	@ 1200 mg/kg Bwt.	

6. Collection of Sample

Blood samples were collected on the 1st day, 14th day & on the last day of experiment (28th day), from retro-orbital plexus with the help of capillary tube for estimations of haematological and biochemical parameters under inhalation anesthesia.

7. Parameters Studied

7.1 General Observations

All the animals in all the groups were daily examined for any abnormal behavioral changes, changes in water and feed intake if any.

7.2 Haematological and Serum Biochemical Parameter Estimations

In this investigation the hematological parameters were investigated to find the out any deviations in Hemoglobin (Hb)[19]; Total Erythrocyte Count (TLC) ; Total Leucocyte Count(TEC)[20]. Differential Leucocyte Count[21]. The serum biochemical parameters viz. AST (by UV kinetic method)[22]; ALT (by UV kinetic method); BUN (by Berthelot method)[23]; Creatinine (by Alkaline picrate method)[24] and Total Protein (by Biuret method)[25] also been investigated.

7.3 Statistical Analysis

The data obtained from various parameters from all the groups was analyzed by as per method suggested by Panse, U.G. and P.V. Sukhatme[26] Statistical methods for Agricultural workers, ICAR, Publication New Delhi using Factorial Randomized Block Design (FRBD) and

Completely Randomized block Design (CRD) as per requirement and interpreted.

8. Results and Discussion

8.1 Morinda Citrifolia Fruit Extract Recovery

For the preparation of *Morinda citrifolia* fruit extract 8.5 kg fresh fruit was collected. After shade drying and maceration, 3.2 kg fruit powder was obtained. Hence, by this procedure dry matter % was found to be 37.64%. All the powder was processed for extract preparation and 143 g aqueous extract was obtained. Percent extractability by this method was found to be 4.46%.

8.2 General Observations

During the period of acclimatization of all experimental wistar rats appeared alert, active and healthy without any untoward manifestations suggesting proper acclimatization. The animals from control group remained active and alive throughout the experimental period. Also, no visible signs of toxicity or no mortality was observed in any of the experimental group throughout the experimental period. All the experimental rats belonging to treatment group when observed daily post treatment did not reveal any observable behavioral changes or any abnormalities.



Figure 1. Confirmation of Gold nanoparticles formed by observing colour change through naked eye.

A : Natural colour of 1mM solution of chloroauric acid. B: Natural colour of 5% aqueous leaf extracts of *Azadirachta indica* (Neem) Plant. C: Ruby red color formed after addition of 1mM solution of chloroauric acid and 5% aqueous leaf extracts of

Azadirachta indica (Neem) Plant and 0.5 hour of incubation. D: Ruby red color darkens to reddish black after addition of 1mM solution of chloroauric acid and 5% aqueous leaf extracts of *Azadirachta indica* (Neem) Plant and 2 hour of incubation



Figure 2. Preparation of site for dermal application of drug.



Figure 3. *Morinda citrifolia* (noni) fruits.

8.3 Hematological Parameters

No toxicity signs observed in all the treated groups of *Morinda citrifolia* fruit extract coated GNP from blood Hb level aspect as the levels which were observed, to be within normal physiological limits. Hb levels in all the group animals treated with *Morinda citrifolia* fruit extract coated GNPs increase significantly throughout the exper-

iment when compared to the healthy control group on 28th day but the values were observed to be within normal physiological limits[27,28]. This increase in Hb value was due to a compensatory mechanism by body to avoid hypochromic anaemia as GNPs interact with RBC when come to systemic circulation.

Hemoglobin (Hb), Total Erythrocyte Count (TEC), Total Leucocyte Count (TLC), differential leucocyte count (DLC). Superscripts a,b,c,d,e shows significant difference within the column (between different groups on specific day) (p = 0.05). Superscripts p,q,r shows significant difference within the row (between different days in specific groups) (p = 0.05). Values are expressed as Mean±SE, Where NS=Non Significant, S=Significant, HS=Highly Significant.

On 1st day of experiment, a significant difference in the TEC values was observed in group II, III, IV and V in comparison to healthy group (group-I). This observation

Table 2. Hb values (Mean ± SE, g/dl) on different days in experimental rats of different groups

Group	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	$12.65\pm^{cq}$ 0.19	$13.25\pm^{ap}$ 0.31	$13.25\pm^{bp}$ 0.22	NS	
II	*Morinda citrifolia* fruit extract coated GNP dose-I	$12.72\pm^{cq}$ 0.34	$13.58\pm^{aq}$ 0.29	$15.66\pm^{ap}$ 0.89	HS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	$13.72\pm^{bq}$ 0.32	$13.87\pm^{ap}$ 0.29	$14.56\pm^{abp}$ 0.52	NS	At 5% 0.72
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	$14.90\pm^{ap}$ 0.41	$13.64\pm^{aq}$ 0.23	$15.72\pm^{ap}$ 0.48	HS	At 1% 0.98
V	Satellite group	$13.61\pm^{bp}$ 0.15	$13.82\pm^{ap}$ 0.21	$14.11\pm^{abp}$ 0.65	NS	
Stat		HS	NS	NS		
CD		At 5% 0.85 At 1% 1.13				

Table 3. TEC values (Mean ± SE, X 10^6 / µl) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E)	14th day (Mean±S.E)	28th day (Mean±S.E)		
I	Healthy control	$7.78\pm^{ap}$ 0.28	$7.21\pm^{ap}$ 0.20	$7.92\pm^{ap}$ 0.18	NS	
II	*Morinda citrifolia* fruit extract coated GNP dose-I	$7.66\pm^{ap}$ 0.20	$7.43\pm^{ap}$ 0.20	$8.46\pm^{ap}$ 0.43	NS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	$7.76\pm^{ap}$ 0.13	$7.64\pm^{ap}$ 0.15	$8.12\pm^{ap}$ 0.29	NS	At 5% 0.90
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	$7.97\pm^{apq}$ 0.45	$7.34\pm^{aq}$ 0.11	$8.60\pm^{ap}$ 0.26	S	At 1% 1.22
V	Satellite group	$7.68\pm^{ap}$ 0.06	$7.48\pm^{ap}$ 0.14	$7.88\pm^{ap}$ 0.41	NS	
Stat		NS	NS	NS		
CD		At 5% 0.73 At 1% 0.98				

was due to individual variation between animals of different groups. However, on 28th day of experiment, a significant increase in the TEC values was observed in the entire treated groups when compared to healthy group. But on 14th day of experiment, no significant difference in the TEC values was observed in entire treated groups except group V in comparison to healthy group. There was not toxicity observed in all the treated groups *Morinda citrifolia* fruit extract coated GNPs (different doses) from TEC values aspect, though the values were within normal physiological limits. TEC values in all *Morinda citrifolia* fruit extract Coated GNPs increased significantly on 28th day. But when the treatment continued up-to first 14th day, there were significant decrease in TEC values observed in all the groups in a higher degree. However, all the values were within normal indicated *Morinda citrifolia* fruit extract coated GNPs had excessive interaction when compared with healthy control group animals. This was due to change in the surface charge and functional groups but not in a dose dependent manner indicated *Morinda citrifolia* fruit extract had no dose related toxicity rather it had good coating and capping property which increased the interaction capacity of GNPs.

On 1st day of experiment, a non-significant differences in the TLC values were observed among the treatment groups in comparison to healthy group (group-I). However, on 14th day of experiment, a significant decrease in the TLC values was observed among all the treated groups in comparison to healthy group. But on 28th day of experiment, a significant increase in the TLC values was observed in group II in comparison to healthy group. There was absolute leucopenia in all treated groups *Morinda citrifolia* fruit extract Coated GNPs (different doses) up to 14th day from TLC values aspect indicated immediate reaction of body towards treatment. But at the end of study, TLC values did not reduce to normal indicative of immunostimulation by treatments on body or due to some inflammatory condition. Therefore, a chronic study is required to access the level to toxicity or immunemodulation property. However, all these changes observed were within normal physiological limits.

On 1st day of experiment, a no significant difference in the lymphocyte counts was observed among the treated groups in comparison to healthy group (group-I). However, on 14th day of experiment, a significant decrease in the lymphocyte counts except day 14th of group IV was observed among all the treatment groups in comparison to healthy group. But on 28th day of experiment, a significant difference in the lymphocyte counts was observed in the entire treated group comparison to healthy group. There was relative lymphocytopenia up to 14th day (except group IV) in entire treated groups observed. However, on 28th day, lymphocyte values achieved the normal status indicative of acclimatization of body. Lymphocyte counts in all *Morinda citrifolia* fruit extract Coated GNPs group decreased significantly up to 14th day except (group IV) of the experimental period from healthy group but no significant difference was noticed form GNPs doses aspect. So it can be inferred that McFE had no significant role in lymphocytopenia though it was having good interac-

Table 4. TLC values (Mean ± SE, 10^3/µl) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Health control	$10.08\pm^{a}$ 1.07	$10.97\pm^{a}$ 0.89	$11.85\pm^{b}$ 1.11	NS	
II	*Morinda citrifolia* fruit extract coated GNP dose-I	$13.52\pm^{ap}$ 1.71	$10.15\pm^{ap}$ 1.22	$17.28\pm^{ap}$ 3.00	NS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	$12.90\pm^{ap}$ 2.26	$10.61\pm^{ap}$ 1.06	$11.59\pm^{bp}$ 1.53	NS	At 5% 2.94
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	$11.65\pm^{ap}$ 1.07	$8.86\pm^{ap}$ 0.72	$10.75\pm^{bp}$ 0.82	NS	At 1% 3.95
V	Satellite group	$9.27\pm^{ap}$ 1.19	$8.48\pm^{ap}$ 0.40	$10.38\pm^{bp}$ 0.68	NS	
Stat		NS	NS	S		
CD		At 5% 4.75 At 1% 6.27				

Table 5. Lymphocyte values (Mean ± SE, %) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	66.17±ap 3.47	66.02±ap 3.75	66.20±ap 1.93	NS	At 5% 9.12 At 1% 12.37
II	*Morinda citrifolia* fruit extract coated GNP dose-I	65.56±ap 2.92	63.36±ap 4.27	68.06±ap 1.90	NS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	65.75±ap 5.10	65.71±ap 4.06	68.34±ap 2.26	NS	
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	68.83±ap 4.50	70.57±ap 5.04	68.43±ap 1.97	NS	
V	Satellite group	66.93±ap 2.72	63.02±ap 2.84	65.61±ap 2.54	NS	
Stat		NS	NS	NS		
CD		At 5% 10.91 At 1% 14.57				

tion property when coated over GNPs. However, at end of study, it achieved the normal status.

On 1st day of experiment, significant difference in the monocyte counts was observed among the treated groups in comparison to healthy group (group-I). However, on 14th day of experiment, a significant increase in the monocyte counts was observed among the treated groups except in comparison to healthy group. But on 28th day of experiment, no significant difference in the monocyte counts was observed among the treated groups except group III and IV in comparison to healthy group. Monocyte counts in almost all *Morinda citrifolia* fruit extract Coated GNPs groups decreased significantly up to 14th day of the experimental period from healthy group but at end of study, it achieved the normal status just similar to GNPs groups. So it can be inferred that body evenly reacted to the coated treatment doses just as GNPs alone in acute condition but in long term there was adaptation.

Table 6. Monocyte values (Mean ± SE, %) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	5.79±ap 0.77	4.60±ap 0.97	5.98±ap 1.42	NS	At 5% 3.15 At 1% 4.27
II	*Morinda citrifolia* fruit extract coated GNP dose-I	7.38±ap 1.06	4.90±ap 1.40	5.81±ap 1.41	NS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	5.50±ap 0.91	6.28±ap 1.20	5.17±ap 1.74	NS	
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	6.14±ap 0.56	5.14±ap 1.24	5.20±ap 1.35	NS	
V	Satellite group	4.75±ap 0.97	5.59±ap 1.31	5.83±ap 0.92	NS	
Stat		NS	NS	NS		
CD		At 5% 3.48 At 1% 4.64				

Table 7. Neutrophile values (Mean ± SE, %) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	23.30±ap 3.57	23.41±ap 3.10	23.48±ap 2.12	NS	At 5% 8.66 At 1% 11.72
II	*Morinda citrifolia* fruit extract coated GNP dose-I	23.31±ap 3.70	27.07±ap 3.61	21.22±ap 2.83	NS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	23.77±ap 4.54	24.58±ap 4.81	21.66±ap 3.62	NS	
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	20.28±ap 4.86	20.11±ap 4.92	21.08±ap 2.33	NS	
V	Satellite group	23.32±ap 2.62	25.94±ap 3.32	23.59±ap 2.57	NS	
Stat		NS	NS	NS		
CD		At 5% 11.15 At 1% 14.89				

On 1st day of experiment, a no significant difference in the neutrophil counts (except group-IV) was observed among the treated groups in comparison to healthy group (group-I). However, on 14th day of experiment non-significant increase in the neutrophil counts was observed among the treated groups in comparison to healthy group. But on 28th day of experiment, no significant difference in the neutrophil counts was observed in entire treated group comparison to healthy group. There was neutrophilia up to 14th day of treatment in all the groups observed. However, on 28th day, neutrophil values achieved the normal status indicative of good dermal absorption of treatment given and the reaction of immune system to *Morinda citrifolia* fruit extract Coated GNPs by treating them as foreign. Neutrophil counts in almost all *Morinda citrifolia* fruit extract Coated GNPs groups increased significantly up to 14th day of the experimental period from healthy groups but not in dose dependent

Table 8. Eosinophile values (Mean ± SE, %) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	4.55±ap 0.33	4.46±ap 0.31	4.03±ap 0.46	NS	At 5% 1.07 At 1% 1.45
II	*Morinda citrifolia* fruit extract coated GNP dose-I	4.38±ap 0.28	4.40±ap 0.27	4.16±ap 0.41	NS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	4.67±ap 0.33	4.41±ap 0.28	4.67±ap 0.33	NS	
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	4.10±ap 0.33	4.01±ap 0.31	5.07±ap 0.26	S	
V	Satellite group	4.45±ap 0.29	5.20±ap 0.29	4.75±ap 0.16	NS	
Stat		NS	NS	NS		
CD		At 5% 0.96 At 1% 1.28				

manner. However, at end of study, it achieved the normal status. There was no involvement of McFE as an extra agent to stimulate the body immunity. Though McFE was thought to be a potent capping and interaction enhancing agent, but no such signs were observed.

Between all the treated groups, on 1st and 14th day of experiment a no significant difference in the eosinophil counts was observed among the treated groups (except on day 14th of group-V) in comparison to healthy group (group-I). However, on 28th day, a significant increase was noticed in group III, IV and V when compared with healthy group (group-I). Eosinophil counts in all *Morinda citrifolia* fruit extract coated GNPs groups differed non-significantly throughout the experiment was in indicative of lack of interaction between McFE coated GNPs treatment and eosinophil. This indicated *Morinda citrifolia* fruit extract coated GNPs were non-allergic to the rats. There was no dose regarding association between eosinophil and treatment.

On 1st day of experiment, a no significant difference in the basophil counts was observed among the treated groups in comparison to healthy group (group-I). However, on 14th day of experiment, a significant increase in the basophil counts was observed among the treated groups in comparison to healthy group. But on 28th day of experiment, a non-significant increase in the basophil counts was observed in the all treated group II, III, and IV comparison to healthy group. There was increase in basophil count observed upto 14th day of treatment in all the groups. However, on 28th day, basophil values also increase than the normal status indicated treatments were having properties of inducing inflammation as they interact to the biological environment or some organs in acute dosing condition. But in long term treatment, gradual reduction was observed which described decrease in immune reaction towards treatments. However, the values at the end of experiment didn't reach normal was on indicative of a chronic toxicity trail should be undertaken. Basophil counts in almost all *Morinda citrifolia* fruit extract coated GNPs groups increased significantly till the end of the experimental period from healthy group. So it can be inferred that *Morinda citrifolia* fruit extract coated GNPs stimulated basophil count and tissue reaction to increase in both acute and sub-acute stage when compared to healthy group. This observation indicated *Morinda citrifolia* fruit extract had a basophil inducing potential when coated over GNPs as McFE was a very good interaction property. No concentration dependent effect for *Morinda citrifolia* fruit extract was observed indicated McFE had no potential toxicity.

On 1st day of experiment, a significant difference in the blood clotting time was observed among the treated groups in comparison to healthy control (group-I). However, on 14th day of experiment, a non-significant increase in the blood clotting time was observed among the treated groups in comparison to healthy group. But on 28th day of experiment, a highly significant increase in the blood clotting time was observed in group treated groups throughout the experimental period in comparison to healthy group. As there is statistically significant increase

Table 9. Basophile values (Mean ± SE, %) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	0.14±ap 0.02	0.15±ap 0.02	0.14±ap 0.02	NS	
II	*Morinda citrifolia* fruit extract coated GNP dose-I	0.14±ap 0.01	0.20±ap 0.02	0.18±ap 0.02	NS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	0.14±ap 0.01	0.19±ap 0.02	0.16±ap 0.02	NS	At 5% 0.07
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	0.15±ap 0.01	0.17±ap 0.02	0.19±ap 0.02	NS	At 1% 0.10
V	Satellite group	0.15±ap 0.02	0.20±ap 0.02	0.22±ap 0.02	NS	
Stat		NS	NS	NS		
CD		At 5% 0.06 At 1% 0.09				

Table 10. Blood clotting time (Mean ± SE, Seconds) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	$50.90\pm^{ap}$ 3.77	$52.50\pm^{ap}$ 2.71	$43.30\pm^{bp}$ 2.18	NS	At 5% 9.32 At 1% 12.50
II	*Morinda citrifolia* fruit extract coated GNP dose-I	$45.40\pm^{aq}$ 3.01	$54.80\pm^{aq}$ 3.29	$83.10\pm^{ap}$ 4.22	HS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	$48.50\pm^{ar}$ 2.61	$61.80\pm^{aq}$ 4.95	$84.10\pm^{ap}$ 4.18	HS	
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	$45.20\pm^{aq}$ 2.75	$53.40\pm^{aq}$ 1.31	$79.40\pm^{ap}$ 8.04	HS	
V	Satellite group	$50.80\pm^{aq}$ 3.52	$55.60\pm^{aq}$ 3.08	$71.00\pm^{ap}$ 1.79	HS	
Stat		NS	NS	HS		
CD		At 5% 13.47 At 1% 17.99				

in the blood clotting time in all the treatment group animals on day 28th of the experiment when compared with their day "0" values and on day 14th as against day '0' in group III animals. This may be because of some alteration in haemopoeitic mechanism.

8.4 Serum Biochemical Parameters

The various biochemical parameters viz. Alanine Transaminase (ALT), Aspartate Aminotransferase (AST), Total Protein (TP), Blood Urea Nitrogen (BUN) and Creatinine (CREA) values were analyzed from blood collected at 1st, 14th and 28th day of the experimental period. Table -11 shows the details of Serum AST value (IU/L) of all the groups on 1st, 14th and 28th day of experiment.

On 1st day off experiment, a no significant difference in the AST values was observed among the treated groups on comparison to healthy group (group-I). However, on 14th day of experiment, a significant increase in the AST values was observed among the treated groups except group-III in comparison to healthy group. On 28th day of experiment, a non-significant increase in the AST values was observed throughout the experimental period in

Table 11. Serum AST level (Mean ± SE, IU) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	$185.50\pm^{ap}$ 19.46	$206.30\pm^{ap}$ 18.66	$188.10\pm^{ap}$ 11.48	NS	At 5% 42.62 At 1% 57.66
II	*Morinda citrifolia* fruit extract coated GNP dose-I	$182.70\pm^{ap}$ 16.70	$211.00\pm^{ap}$ 23.26	$242.80\pm^{ap}$ 37.57	NS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	$183.10\pm^{aq}$ 14.30	$179.60\pm^{aq}$ 14.77	$236.80\pm^{aq}$ 15.06	NS	
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	$183.90\pm^{ap}$ 19.19	$186.30\pm^{ap}$ 25.99	$249.40\pm^{ap}$ 22.12	NS	
V	Satellite group	$175.20\pm^{ap}$ 16.15	$186.20\pm^{ap}$ 25.16	$235.80\pm^{ap}$ 18.43	NS	
Stat		NS	NS	NS		
CD		At 5% 64.52 At 1% 86.18				

comparison to healthy group. There was slight liver affection noticed in some of the treatment groups at the end of study period from AST values aspect indicated a chance of hepatocyte damage throughout the treatment period. But up to 28th day of study, AST values increased severely form normal indicative of treatments were approaching and accumulating in liver just after dermal application (acute stage).

On 1st day of experiment, a no significant difference in the ALT values was observed among the treated groups in comparison to healthy group (group-I). However, on 14th day of experiment, non- significant increase in the ALT values throughout experiment was observed among the treated groups in comparison to healthy group. But on 28th day of experiment, a significant increase in the ALT values was observed in group II and V in comparison to 1st day reading. There was slight liver affection noticed in some of the treatment groups at the end of study period from ALT values aspect indicated a chance of hepatocyte damage when the treatment continued for long term. But up to 14th day of study, ALT values reduced from normal indicative of treatments were approaching and accumulating in liver after dermal application but no interaction. ALT values in most of the *Morinda citrifolia* fruit extract Coated GNPs groups increased significantly at the end of study duration indicated that coated GNPs had more interaction and accumulation capacity so it had more liver damaging capacity. However, all the values were not at par to describe them as a sign of toxicity. For that, a long term toxicity trial is recommended to establish a concrete co-relation between toxicity and treatments. However different doses of McFE as a coating agent had no significant effect in the hike of ALT values indicated no dose related toxicity.

On 1st day of experiment, a no significant difference in the TP values was observed among the treated groups in comparison to healthy group (group-I). However, on 14th day of the experiment, non-significant increase in the TP values was observed among the treated groups in comparison to healthy group. On 28th day of experiment, non-significant increase in the TP values was observed in the entire treated group in comparison to healthy group. There was no decrease in TP values aspect indicated treatments had no effect on liver function and hepatocyte production capacity. In the same time, the hike in TP value was an indicative of rise in protein content of serum due to reaction of immune system to the treatments. This was the confirmation that rises in TLC count specifically neutrophil and basophil which stimulated immunoglobulin production would be the major factor of the hike in TP. Moreover, hike of serum enzymes may also be an effective factor for the rise in TP content. TP values in most of the *Moridna citrifolia* fruit extract Coated GNPs groups increased significantly throughout study duration. It indicated that coated GNPs had more immunity stimulation potency so it had more effect on immune system in long run. However, all the values were not at par to describe them as toxicity or beneficial effect of treatments given. For that, a long term toxicity trial is recommended to establish a concrete co-relation between toxicity and

Table 12. Serum ALT level (Mean ± SE, IU) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	$77.40\pm^{ap}$ 7.81	$77.20\pm^{ap}$ 13.58	$82.60\pm^{ap}$ 9.32	NS	
II	*Morinda citrifolia* fruit extract coated GNP dose-I	$76.20\pm^{ap}$ 6.42	$91.50\pm^{ap}$ 13.82	$78.60\pm^{ap}$ 4.18	NS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	$78.10\pm^{aq}$ 7.71	$118.00\pm^{ap}$ 10.46	$74.00\pm^{aq}$ 3.65	HS	At 5% 28.51
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	$75.90\pm^{apq}$ 6.79	$90.00\pm^{ap}$ 5.57	$71.00\pm^{aq}$ 2.51	S	At 1% 38.57
V	Satellite group	$76.90\pm^{ap}$ 10.13	$105.20\pm^{ap}$ 12.78	$81.80\pm^{ap}$ 4.94	NS	
Stat		NS	NS	NS		
CD		At 5% 32.96 At 1% 44.03				

Table 13. Serum TP level (Mean ± SE, g/dl) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	$7.51\pm^{ap}$ 0.33	$7.69\pm^{ap}$ 0.32	$7.37\pm^{ap}$ 0.28	NS	
II	*Morinda citrifolia* fruit extract coated GNP dose-I	$7.49\pm^{aq}$ 0.34	$8.84\pm^{ap}$ 0.46	$7.63\pm^{aq}$ 0.20	S	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	$7.68\pm^{aq}$ 0.29	$8.81\pm^{ap}$ 0.38	$7.88\pm^{aq}$ 0.19	S	At 5% 1.01
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	$7.44\pm^{ap}$ 0.36	$7.97\pm^{ap}$ 0.44	$7.96\pm^{aq}$ 0.39	S	At 1% 1.37
V	Satellite group	$7.59\pm^{ap}$ 0.51	$8.55\pm^{ap}$ 0.47	$7.65\pm^{ap}$ 0.32	NS	
Stat		NS	NS	NS		
CD		At 5% 0.82 At 1% 1.09				

treatments. However, doses of McFE had no significant effect in the alteration indicated dose variation had no role in toxicity

On 1st day of experiment, a non-significant decrease in the BUN values was observed among the treated groups in comparison to healthy group (group-I). However, on 14th day of experiment, a significant increase in the BUN values was observed among the treated groups except group II and V in comparison to healthy group. On 28th day of experiment, a highly significant increase in the BUN values was observed among the treated in comparison to healthy group. There was slight kidney

Table 14. Serum BUN level (Mean ± SE, mg/L) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	$73.25\pm^{ap}$ 9.00	$64.67\pm^{ap}$ 12.25	$64.72\pm^{bp}$ 4.19	NS	
II	*Morinda citrifolia* fruit extract coated GNP dose-I	$59.78\pm^{aq}$ 3.14	$62.89\pm^{aq}$ 8.48	$82.33\pm^{ap}$ 3.70	S	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	$62.18\pm^{ap}$ 7.07	$71.67\pm^{ap}$ 8.67	$81.40\pm^{ap}$ 3.16	NS	At 5% 16.34
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	$62.04\pm^{aq}$ 5.40	$90.00\pm^{ap}$ 5.57	$87.59\pm^{ap}$ 2.76	HS	At 1% 22.11
V	Satellite group	$64.53\pm^{a}$ 6.34	$55.60\pm^{a}$ 2.37	$66.23\pm^{a}$ 2.24	HS	
Stat		NS	NS	HS		
CD		At 5% 18.33 At 1% 24.49				

affection noticed in most of the treatment groups at the end of study period from BUN values aspect indicated a chance of renal tubular damage throughout the treatment period. But up to 14th day of study, BUN values hiked non significantly from normal indicative of treatments were approaching and accumulating in kidney for elimination (acute stage). However, towards 28th day, a gradual increase was noticed which indicated slight kidney affections reported in body when long term therapy. The measure of the amount of nitrogen in blood in the form of urea Is BUN and it is a very good indicator of kidney functionality status. Hypovolemia and decrease in GFR (Glomerular Filtration Rate) increases BUN value. In the present study, Gold nanoparticles interacted with protein and increased production of amino acid might be the cause of the elevation in BUN value. BUN values in most of the *Morinda citrifolia* fruit extract coated NPs groups increased significantly throughout study duration indicated that coated GNPs had more interacting capacity so it had more toxic effect on kidney than NPs alone. However, all the higher dose combinations had shown significant difference in BUN values from lower dose combination suggested, individual effect of McFE on toxicity was noticed. When correlated with the BUN values in the experiment animals it is observed that the BUN values in all the treatment groups increased as against their day '0' values and control group animals indicating damage to renal system.

On 1st day of experiment, a no significant difference in the Creatinine values was observed among the treated groups in comparison to healthy group (group-I). However, on 14th day of experiment, a significant decrease in the Creatinine values was observed among most of treated groups in comparison to healthy group. On 28th day of experiment significant increase in the Creatinine values was observed throughout the experiment in comparison to healthy group. There was slight kidney affection noticed in the treatment groups on day 28th of experiment period, from Creatinine values aspect indicated chances of renal filtration unit damage. However, up-to 14th day of study, Creatinine values decreased non significantly from normal indicative of treatments were effectively cleared out by kidney just after dermal application acute stage. But towards 28th day, a gradual increase was noticed which indicated adaptation of body to treatments in long term.

Creatinine values in most of the *Morinda citrifolia* fruit extract Coated GNPs groups decreased non-significantly on day 14th of study. However significantly increased throughout groups on 28th day of experiment indicated that coated GNPs were cleared form blood circulation properly without any significant accumulation or deposition in kidney. No significant dose dependent changes were observed between groups treated with variable doses of McFE coated GNPs which indicated *Morinda citrifolia* fruit extract did not have any toxic effect even in higher doses.

Aqueous extract of *Morinda citrifolia* fruit was made using cold extraction method, and the extractability was found to be 4.46%. Gold nanoparticles were synthesized

Table 15. Serum Creatinine level (Mean ± SE, mg/dl) on different days in experimental rats of different groups

Gr.	Treatment	Days			Stat	CD
		1st day (Mean±S.E.)	14th day (Mean±S.E.)	28th day (Mean±S.E.)		
I	Healthy control	0.32±^{aq} 0.04	0.29±^{aq} 0.06	0.36±^{bp} 0.06	NS	
II	*Morinda citrifolia* fruit extract coated GNP dose-I	0.34±^{aq} 0.05	0.37±^{aq} 0.05	0.79±^{bp} 0.05	HS	
III	*Morinda citrifolia* fruit extract coated GNP dose-II	0.35±^{aq} 0.04	0.34±^{aq} 0.08	0.94±^{bp} 0.12	HS	At 5% 0.14
IV	*Morinda citrifolia* fruit extract coated GNP dose-III	0.35±^{aq} 0.05	0.29±^{aq} 0.05	1.11±^{ap} 0.04	HS	At 1% 0.19
V	Satellite group	0.35±^{aq} 0.04	0.28±^{aq} 0.04	0.67±^{bp} 0.03	HS	
Stat		NS	NS	S		
CD		At 5% 0.19 At 1% 0.26				

by biological reduction (green synthesis) of chloroauric acid ($HAuCl_4.3H_2O$) with *Azadirachta indica* (neem) leaf extract, after coating the characterization of GNPs by colour changes deep yellow to ruby red was confirmed. These nanoparticles were coated with *Morinda citrifolia* fruit extract (McFE) which was used for the treatment.

Among haematological parameters, Hb value increased non-significantly from healthy rats and also between different days of a group whereas TEC and TLC values increase non significantly in entire treatment groups on 28th day of experiment. Similarly, immune stimulation was observed from TLC value and neutrophil count aspect and a compensatory decrease in other WBC count particularly lymphocyte, monocyte and eosinophil count aspect in the experiment. There was no allergic reaction observed from eosinophil value aspect. However, there might be some inflammatory reaction and immune stimulation observed from basophil count aspect.

The serum biochemical estimations for liver function test (ALT & AST) in the entire treated groups showed non-significant increase indicating interaction and accumulation of coated GNPs in liver. However, there was slight difference observed in total protein value indicating no loss in functionality of hepatocyte rather an increase in TP values were observed. The kidney function was slightly affected though BUN value in serum increased because destruction of protein due to interaction of GNPs might be a cause in hike of BUN. Similarly, creatinine value reported to increase in kidney damage and reduction in glomerular filtration was not severely noticed rather effective creatinine clearance was found indicating proper filtration unit performance after GNPs administration. However, this slight difference was not at par to describe it a sign of toxicity[28]. There was no significant toxicity in entire dosage, from hematology aspect but slight immune stimulation seen upto 14th day of experiment (neutrophil) in McFE coated GNPs doses with no relation to dose and coating. From serum biochemical value aspect, slight liver functions tests affected on upto 14th day but no specific dose related toxicity was observed in any of the treatment. For kidney function tests- increase BUN and CREA on 28th day but proper excretion by kidney filtration units. Present investigations can be concluded that *Azadirachta indica* leaf extract gold nanoparticles have no adverse effect so it can be exploited for further therapeutic purpose.

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