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Green Synthesized FM-AgNPs Lead to Alterations in Hematology, Oxidative Stress Biomarkers, and Microanatomy of Liver and Spleen in Rats

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HIGHLIGHTS

- Green synthesized silver nanoparticles are not always safe for use.
- *Fortunella margarita* (FM-AgNPs) lead to alteration in hematological indicators.
- FM-AgNPs leads to elevation in SOD, CAT and MDA in liver and spleen.
- High doses of FM-AgNPs lead to degenerative changes in microanatomy of liver.

Abstract: Widespread use of green synthesized silver nanoparticles (AgNPs) in pharmaceutical and cosmetics demand for its toxicological assessment. The current study describes the influence of exposure to kumquat, *Fortunella margarita* (FM) synthesized AgNPs in albino rats. Animals (n=60) were randomly distributed into four groups. Group, I served as control while groups II-IV were exposed to FM-AgNPs by the single intraperitoneal dose of 1, 5, and 10 mg/kg body weight, respectively. Animals were sacrificed at two-week intervals up to 5 weeks. Hematology, tissue (liver and spleen) oxidative stress biomarkers (SOD, CAT, and MDA), and histopathological examination were performed. Significant elevation in hemoglobin (Hb), Erythrocyte count (RBC), hematocrits (HCT), and hematological indices and depletion in total leukocytes count (TLC) was recorded at different time points. Similarly, a significant increase in SOD, CAT, and MDA activity was observed in both liver and spleen. But changes ($p \leq 0.005$) in the spleen was recorded more consistently. Exposures to the high dose of FM-AgNPs lead to significant alterations in microanatomy of the liver and spleen. In liver vacuolization, enucleation, necrosis, congestion, and reduction in the size of cell, nucleus, portal vein, and elevation in cell density per unit area were recorded. In the spleen, except for enlargement in splenic sinuses, no other alterations could be recorded. It is concluded that FM-AgNPs leaves time and dose-dependent toxicity in rats but variations are more evident at high doses.

Keywords: *Fortunella margarita*; silver nanoparticles; hematology; oxidative stress biomarkers; histopathology.

INTRODUCTION

Nanoparticles (NPs) are nano-sized engineered materials that have diverse applications due to their unique characteristics *viz*., small volume, high surface area/mass ratio, and the possibility of attachment of surface reactive chemical groups [1]. The NPs from different metals have been recognized as biocompatible, anti-inflammatory, and antimicrobial and are used for effective drug delivery and tumor targeting [2-4]. Among different metals, the nanoparticles of silver have distinctive surface properties and are known for strong biocidal ability [5]. The engineered AgNPs are also used in coating hospital beds, medical implants, making antiseptic, and wound/burn dressings etc. [6,7]. They are also used in other fields e.g., electrochemical appliances, water decontamination, toothpaste, shampoos, baby nipples, treatment bottles, water filters, toys, kitchen utensils, and humidifiers [8]. Extensive use of AgNPs on the other hand, has enhanced human exposure to the metal and subsequent threat of toxicity [9]. Silver has been reported to produce reactive oxygen species (ROS) that interact with proteins, enzymes, and DNA, and disturb their functioning [10]. Silver ions have also been reported to induce toxic effects following bioaccumulation in the liver and spleen [1]. In short, the direct and indirect exposure of humans to AgNPs is quite frequent [11] and their safety has become a point of concern particularly, in treating human patients [12].

To avoid the issues related to the toxicity of metallic nanoparticles (M-NPs), their green synthesis was devised. Green synthesized NPs are considered less toxic and their production is believed eco-friendly and cost-effective [13]. Plants extracts obtained from plant leaf, root, seed, and stem are frequently applied for green nano-material production, as these extracts contain various polysaccharides, proteins, vitamins, or alkaloids, which are generally nontoxic, biodegradable, and can act both as reducing and capping agents, promote the formation and inhibit the agglomeration of nanoparticles [14, 15]. The synthesis from several plant bases has resulted in diverse sizes of AgNPs; these plant bases included bark of *Salacia chinensis* (40-80 nm), leaf extract of *Brassica rapa* (5.7–24.4nm), *Enicostemma axillare* (15-20 nm), and *Cannabis sativa* (20-40 nm), have been currently used for capping-agents [16]. Kumquat (*Fortunella margarita*) is a member of the family *Rutaceae*. The active-nano particles from this can be prepared and used as effective anti-bacterial agents even against multidrug-resistant bacteria. Similarly, FM-AgNPs are claimed to be safe with least toxic effect on non-target organisms [17]. However, this claim was not based on experimental outcomes of safety evaluation. The nano-particles can interact with cells or blood following entry into the body through any route [18]. Researchers also claim that NPs show concentration dependent toxicity [19]. Therefore, experiments on safety assessment/health risks are mandatory before claiming any practical utility. Although antimicrobial efficiency of FM-AgNPs against multi drug resistance (MDR) pathogens has been reported using *in vitro* assays but their safety evaluation through *in vivo* approach has not been performed so far. The present study was designed to evaluate the safety of FM-AgNPs. Changes in hematology, tissue antioxidant status (liver and spleen), and histopathology in liver and spleen of albino rats following exposure to different doses of FM-AgNPs were taken into account as biomarkers of toxicity.

MATERIALS AND METHODS

Chemicals

FM-AgNPs were provided by plant biotechnological laboratory, IMBB, UOL, Lahore. These particles were synthesized following Mubeen and coauthors [20]. The FM-AgNPs were spherical and crystalline in shape. The particles were having aromatic compounds, carboxylic acid, hydroxyl, amine and aliphatic groups on their surface. Di sodium hydrogen phosphate, nitro blue tetrazolium, Phenazine methosulphate, sodium dihydrogen phosphate, nicotinamide-adenine dinucleotide, Trichloro-acetic acid, Thiobarbituric acid, acetic acid, were purchased from Sigma Aldrich (Germany) through a local vendor. Ketamine was purchased from Indus Pharmaceuticals (Pvt.) Ltd, Pakistan. All other chemicals viz; formaline, chloroform, n-butanol; hydrogen peroxide used in the experiments were of analytical grade. Deionized water was used in experiments, where required.

Ethical Statement

Animal handling and experimentations were performed following rules as declared by Helsinki, permitted and granted by the Ethical Committee, IMBB/CRIMM, The University of Lahore; Lahore.

Dose Preparation

The FM-AgNPs were suspended evenly by sonication for 2 hours to avoid aggregation (Jinyunbao ultrasonic cleaner; DSA 100-SK1-2.5L). Three different concentrations (1, 5, and 10 mg/kg) of FM-AgNPs were prepared in normal saline.

Animals

In total 60 male albino rats with 120-150g weight were purchased from The University of Lahore, Lahore. Animals were acclimatized for 7 days. Afterward, animals were randomly divided into four groups (I-IV) having 15 rats with five replicates of 3 animals in each. The rats were housed in standard size (410 x 282 x 150mm) cages of polycarbonate and kept in a room with controlled temperature (23 ± 2^{o}C), humidity (55 ± 7%), and 12h light/dark cycle. Rats received poultry Feed#4 (Asia Poultry Feeds, Pvt. Ltd), and tap water *ad libitum*.

Exposure to FM-AgNPs

Group I served as negative control while, Groups II, III, and IV were exposed to FM-AgNPs at a dose equivalent to 1, 5, and 10 mg/kg of body weight, respectively, through the intraperitoneal route. Similarly, the control group was treated with normal saline through the same route. The rats were observed for five weeks' post-exposure to FM-AgNPs for physical and behavioral signs of toxicity.

Sampling

At 7, 21, and 35 days' post-exposure to FM-AgNPs, five animals from each group (one from each replicate) were randomly selected, blood samples were collected by direct cardiac puncture under mild anesthesia with (25 mg/kg) ketamine (Indus Pharmaceuticals (Pvt) Ltd, Pakistan). Later on, the animals were killed by a high dose of Ketamine and their liver and spleen were excised aseptically. The blood samples were kept in heparinized vacutainers and freshly used in hematological studies. The Liver and spleen were weighed and sliced into two parts. One part was quickly frozen in liquid nitrogen and later on kept at -80^{o}C till further use. While another half of the tissue was fixed in 10% formaline and processed for histopathological observation.

Hematology

Collected blood samples were used for leukocyte count, erythrocyte count, granulocyte to lymphocyte count and determination of hemoglobin concentration, hematocrits (HTC), and hematological indices (MCV, MCH, MCHC). Hematology was performed on an automated blood analyzer (SYSME KX-21 hematology blood analyzer).

Tissue Homogenate Preparation

The frozen organs were weighed and placed in PBS, later on, the tissue was homogenized using an electric homogenizer (PYREX® Potter–Elvehjem, US) and sonicated by (Jinyunbao ultrasonic cleaner; DSA 100-SK1-2.5L). The homogenate was centrifuged at 10,000g at 4^{o}C for 10 minutes using a refrigerated centrifuge (Biobase Bioindustry Shangdon BK-1032J). The supernatant was separated and then used in biochemical analysis.

Estimation of Oxidative Stress Biomarkers

Antioxidant enzymes, Super Oxide Dismutase (SOD), and Catalase (CAT), along with Malondialdehyde (MDA), a non-enzymatic stress biomarker were estimated in the liver and spleen and the activity was expressed as units/gram of the tissue. CAT, MDA, and SOD were estimated following Aebi and coauthors [21], Ohkawa and coauthors [22], and Kakkar and coauthors [23], respectively. All tests were performed in triplicate to avoid internal errors and used in statistical analysis.

Histopathological Examination

Organs were processed for histopathological examinations using a standard procedure [24]. Thin sections (6 µm) were prepared from paraffin-embedded samples using an automated microtome (Model 5020, Leica Biosystems, Germany). The hematoxylin and eosin-stained sections were examined under a compound microscope (Austria). Liver tissue was examined for hepatic architecture, necrosis, enucleation, vaculation, intracellular spaces, size of portal vein, while spleen tissues were examined for splenic architecture, red and white pulp, and splenic sinuses.

Statistical Analysis

Data were analyzed using one-way ANOVA followed by Dunnett's test for comparison of all experimental groups with control using SPSS software, version 21 (USA, Inc.). The differences at p-value < 0.05 were considered significant.

RESULTS

Hematological Parameters

The exposure to biosynthesized AgNPs from FM at low and medium doses lead to a significant elevation in Hb (7.2-16.8%), RBCs (4.05-25.67%), and HCT (2.54-22.90%). The change normalized after III-week of exposure at the low dose but remained consistent at the medium dose. On the other hand, at the high dose, no such change could be recorded. In contrast, a significant depletion in total leukocyte count was observed at all doses. At low dose, depletion was 62.79%, 26.12%, 51.02% while at medium dose significant depletion was observed at I and V week (20.84 %, 13.96 %), respectively. In the case of high dose, the reduction was 72.28%, 68.06%, 65.28% at 1, 3, and 5 weeks. Similarly, the elevation in the proportion of granulocytes was recorded throughout the experimental period at low dose (p < 0.000). The animals receiving the medium and high dose of FM-AgNPs displayed significant increase (p < 0.000) in the proportion of granulocytes at weeks III and V. The elevation was 42% & 28% at the medium dose and 26% & 78% at the high dose. In contrast to granulocytes, the lymphocyte count decreased significantly in animals receiving low dose (the week I), medium dose (week V), and high dose (week III and V) when compared to control. The values of hematological indices also varied, and that significant alterations were observed at different time points following exposure to all doses (Table 1).

Table 1. FM-AgNPs dose and exposure time associated changes in the hematological parameters of rats

Parameter	Week	Group				*P* F-test
		Control 0 mg/kg	Low 1 mg/kg	Medium 5 mg/kg	High 10 mg/kg	
Hb (g/dl)	1	12.60 ± 0.06	13.43 ± 0.07*	12.50 ± 0.06	12.47 ± 0.20	**0.01**
	3	12.47 ± 0.03	13.30 ± 0.21	14.57 ± 0.03*	12.83 ± 0.55	**0.00**
	5	12.60 ± 0.06	13.40 ± 0.17*	13.40 ± 0.06*	12.50 ± 0.12	**0.00**
RBC ($\times 10^6/\mu L$)	1	7.35 ± 0.01	9.30 ± 0.12*	7.90 ± 0.06*	7.93 ± 0.09*	**0.00**
	3	7.39 ± 0.01	7.87 ± 0.09*	8.77 ± 0.07*	7.27 ± 0.15	**0.00**
	5	7.33 ± 0.01	7.73 ± 0.15	8.00 ± 0.29	7.57 ± 0.15	**0.13**
HCT (%)	1	41.00 ± 0.58	48.27 ± 0.15*	45.33 ± 0.12*	41.67 ± 0.33	**0.00**
	3	39.33 ± 1.45	46.00 ± 1.15*	47.50 ± 0.44*	41.33 ± 0.33	**0.00**
	5	37.00 ± 3.51	40.30 ± 0.17	44.47 ± 0.87*	43.53 ± 0.29	**0.07**
TLC ($\times 10^3/\mu L$)	1	14.00 ± 0.58	8.60 ± 0.17*	11.10 ± 0.06*	9.27 ± 0.15*	**0.00**
	3	12.00 ± 0.58	9.93 ± 0.03*	12.50 ± 0.12	10.53 ± 0.29*	**0.00**
	5	14.00 ± 1.15	6.27 ± 0.15*	8.33 ± 0.15*	8.47 ± 0.29*	**0.00**
NP (%)	1	14.00 ± 0.58	22.00 ± 0.58*	14.00 ± 1.15	20.13 ± 0.88*	**0.00**
	3	12.33 ± 0.88	17.33 ± 0.88*	20.00 ± 0.58*	25.00 ± 1.15*	**0.00**
	5	11.00 ± 1.15	15.33 ± 0.33*	18.00 ± 0.00*	23.00 ± 1.15	**0.00**
LC (%)	1	86.33 ± 0.33	77.33 ± 0.33*	86.00 ± 2.31	90.33 ± 0.88	**0.00**
	3	84.00 ± 0.58	83.00 ± 2.31	80.33 ± 0.33	75.00 ± 2.89*	**0.03**
	5	84.33 ± 0.33	85.00 ± 2.89	70.00 ± 2.89*	67.33 ± 0.33	**0.00**
MCV (fL)	1	56.00 ± 0.58	50.33 ± 2.91	55.67 ± 0.88	54.00 ± 0.58	**0.11**
	3	53.00 ± 1.15	59.00 ± 2.31*	54.47 ± 0.29	66.00 ± 0.58*	**0.00**
	5	55.00 ± 0.58	52.00 ± 0.00	55.47 ± 0.29	57.33 ± 1.45	**0.01**
MCH (pg)	1	17.00 ± 0.58	14.00 ± 0.58	16.00 ± 1.73	16.00 ± 0.58	**0.26**
	3	17.00 ± 0.58	17.00 ± 1.15	16.57 ± 0.30	17.00 ± 0.00	**0.95**
	5	18.67 ± 0.88	17.13 ± 0.19	16.60 ± 0.31	16.00 ± 0.58*	**0.04**
MCHC (g/dL)	1	31.00 ± 1.15	28.10 ± 0.49	28.00 ± 1.73	27.47 ± 0.52	**0.17**
	3	28.00 ± 0.58	28.00 ± 0.58	30.67 ± 0.49	31.03 ± 1.13*	**0.03**
	5	28.00 ± 0.58	33.27 ± 0.32*	30.10 ± 0.62	28.03 ± 0.55	**0.00**

In all tables the values are presented in terms of Mean ± SE. Asterisks on values represent significant differences (each row) from respective control group. Data were analyzed using one-way ANOVA followed by Dunnett's test.

Oxidative Stress Biomarkers

The levels of oxidative biomarkers including SOD, CAT, and MDA were recorded in the liver and spleen. In the liver, significant elevation in SOD was recorded at the week I (61%) and III (80%), and V (46%) following exposure to low dose, while at medium (79%, 99%, and 53%) and high dose (46%, 52%, and 31%) was recorded at respective weeks (Table 2). A similar trend in the spleen was recorded throughout the experimental period with a *p*-value (0.000) at low, medium, and high doses. The elevation at low dose was 41.30%, 62.45%, 54.49%, at medium dose it was (62%, 49%, 21%) and at high dose was 60%, 21%, 46% after I, III, and V weeks of exposure to FM-AgNPs respectively (Table 3). The catalase level, in the liver, remained unaltered during the experiment, following exposure to all doses of FM-AgNPs except at high dose ($p < 0.000$) at week-I as shown in (Table 2). Conversely, in the spleen, a significant increasing trend in CAT level was recorded throughout the experimental period at all doses ($p < 0.05$). The variation was 46-53% at low, 15-45% at medium, and 39- 49% at high dose (Table 3). Similarly, The MDA activity in the rat liver remained unaltered throughout the experimental period at low and medium dose but at high dose it varied significantly at week III (15.49%) and week V (10.20%). However, an increasing trend was noticed in the spleen throughout the experimental period at all doses. The elevation was 28%, 28%, 26% at week I, 45%, 49%, 42% at week III, and 31%, 30%, 29% at week V following exposure to the low, medium, and high dose respectively (Table 2,3).

Table 2. Effects of Green Synthesized AgNPs, from *Fortunella margarita*, on Oxidative Stress Biomarkers of Liver in Rat

Parameter	Week	Group				*P* F-test
		Control 0 mg/kg	Low 1 mg/kg	Medium 5 mg/kg	High 10 mg/kg	
CAT ($Umin^{-1}g^{-1}$)	1	6.12 ± 0.27	7.14 ± 0.15	7.12 ± 0.09	7.59 ± 0.49*	**0.04**
	3	6.04 ± 0.19	7.35 ± 0.33	6.29 ± 0.60	7.55 ± 0.30	**0.05**
	5	6.30 ± 0.43	6.49 ± 0.31	7.39 ± 1.16	6.92 ± 0.70	**0.72**
SOD (Ug^{-1})	1	27.97 ± 0.87	45.86 ± 3.00*	50.29 ± 0.85*	51.86 ± 6.23*	**0.00**
	3	26.33 ± 0.84	47.65 ± 3.67*	40.38 ± 5.17*	55.46 ± 2.02*	**0.00**
	5	31.52 ± 0.63	36.89 ± 1.04	36.27 ± 2.47	45.85 ± 2.31*	**0.00**
MDA (μMg^{-1})	1	8.44 ± 0.36	9.42 ± 0.27	8.42 ± 0.27	8.67 ± 0.23	**0.12**
	3	7.96 ± 0.08	8.16 ± 0.18	7.06 ± 0.52	9.42 ± 0.36*	**0.00**
	5	9.24 ± 0.34	7.63 ± 0.14	9.42 ± 0.40	12.29 ± 2.69	**0.19**

Table 3. Effects of Green Synthesized AgNPs, from Fortunella margarita, on Oxidative Stress Biomarkers of Spleen in Rat

Parameter	Week	Group				*P* F-test
		Control 0 mg/kg	Low 1 mg/kg	Medium 5 mg/kg	High 10 mg/kg	
CAT ($Umin^{-1}g^{-1}$)	1	42.32 ± 0.78	90.76 ± 1.07*	78.07 ± 1.78*	70.13 ± 0.88*	**0.00**
	3	56.62 ± 1.13	58.82 ± 1.70	59.75 ± 2.59	65.35 ± 0.99*	**0.03**
	5	35.64 ± 0.45	66.71 ± 1.02*	42.32 ± 1.48*	70.95 ± 2.67*	**0.00**
SOD (Ug^{-1})	1	212.64 ± 1.18	362.25 ± 5.46*	566.31 ± 0.87*	467.33 ± 5.27*	**0.00**
	3	268.36 ± 4.62	368.79 ± 0.51*	238.12 ± 2.67*	343.47 ± 4.05*	**0.00**
	5	190.93 ± 2.63	417.22 ± 2.53*	375.94 ± 2.88*	354.27 ± 2.57*	**0.00**
MDA (μMg^{-1})	1	69.22 ± 1.35	245.34 ± 31.04*	152.86 ± 23.06*	217.46 ± 11.85*	**0.00**
	3	79.34 ± 3.03	275.66 ± 10.04*	161.13 ± 7.02	258.13 ± 21.88*	**0.00**
	5	80.90 ± 5.51	308.53 ± 15.61*	191.08 ± 7.94*	275.80 ± 14.11*	**0.00**

Histopathology

Liver

Histological examinations in control groups showed standard architecture of the liver without any damage. They had regular cell association with intact state normal liver tissues. Cells were arranged in a radiating way from the central vein. Exposures to FM-AgNPs lead to changes in microanatomy of the liver and spleen. In the liver, vacuolization was prominent along with enucleation and necrosis at the high dose (Figures 1, 2). A significant reduction in nuclear diameter (83.95%) and cell size (35.30%) was observed. At the high dose, the cell population per unit area was greater (48.88%) as compared to other groups. Similarly, the size of the portal vein also reduced (86%) in treated livers (Figure 3).

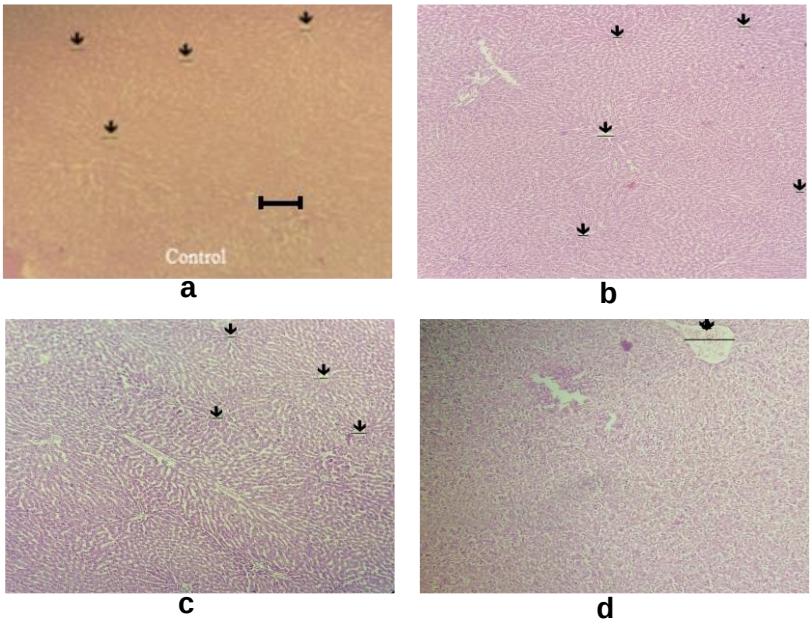

Figure 1. Histopathological alterations in the liver of albino rat exposed to different doses of FM-AgNPs at 100X. Control group (a), low dose 1mg/kg (b), medium dose 5mg/kg (c) and high dose 10mg/kg (d). Size of the central vein (black arrow) was recorded using Image J software.

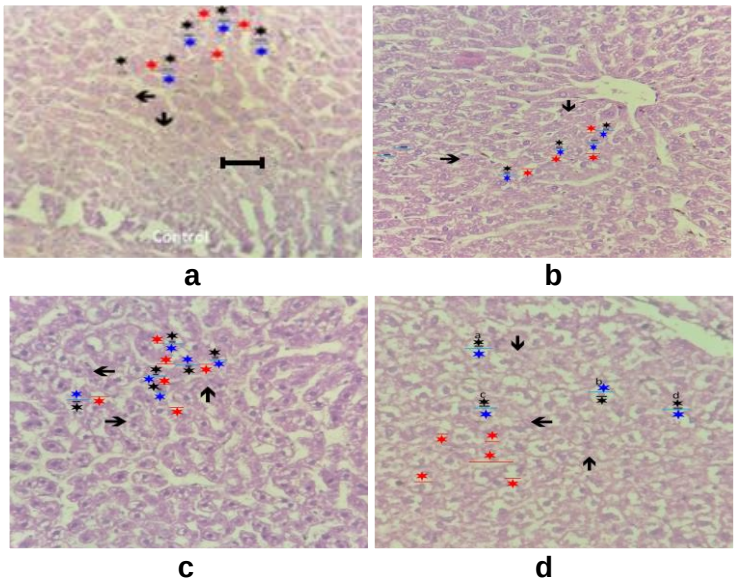

Figure 2. Histopathological alterations in the liver of albino rat exposed to different doses of FM-AgNPs at 400X. Control group (a), low dose 1mg/kg (b), medium dose 5mg/kg (c) and high dose 10mg/kg (d). The cell Size (blue star and line), nuclear diameter (black star and line), intercellular spaces (red star and line), was recorded using Image J software. In control, black arrow represents hepatocites Bar on control indicate 10µm.

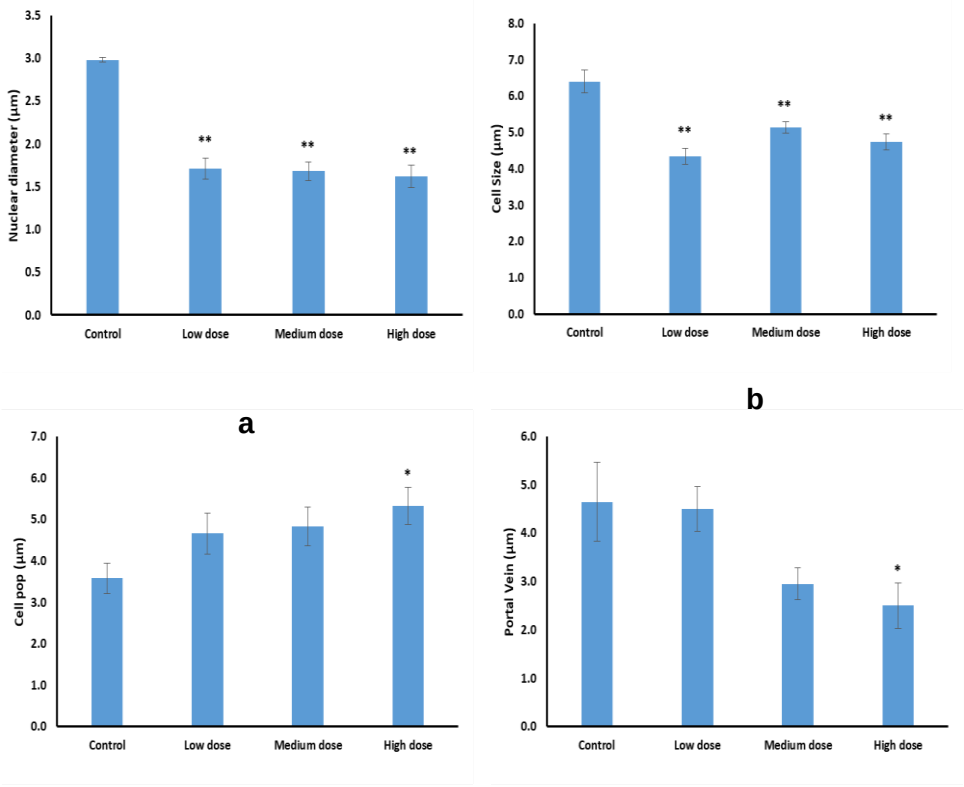

Figure 3. Alterations in the nuclear diameter, cell size, cell population, and portal vein of the rat liver following exposure to different doses of FM-AgNPs. (* $p \leq 0.05$; ** $p < 0.01$).

Spleen

The Control group revealed normal spleen architecture with its two major components; white pulps and red pulp, a marginal zone separating these two pulps. The white pulp consisted of a follicle with a pale germinal center and a peripherally-located central arteriole, surrounded by a sheath of lymphocytes; per arterial lymphatic sheath. Splenic sinuses were observed in the red pulp. The variations in microanatomy of the spleen were not much pronounced (Figures 4, 5). Sizes of red and white pulp did not vary significantly ($p > 0.05$) following exposure to FM-AgNPs. However, consistent with the results of liver morphometry, the cell population (64%) and splenic sinuses (58%) increased following exposure to FM-AgNPs (Figure 6).

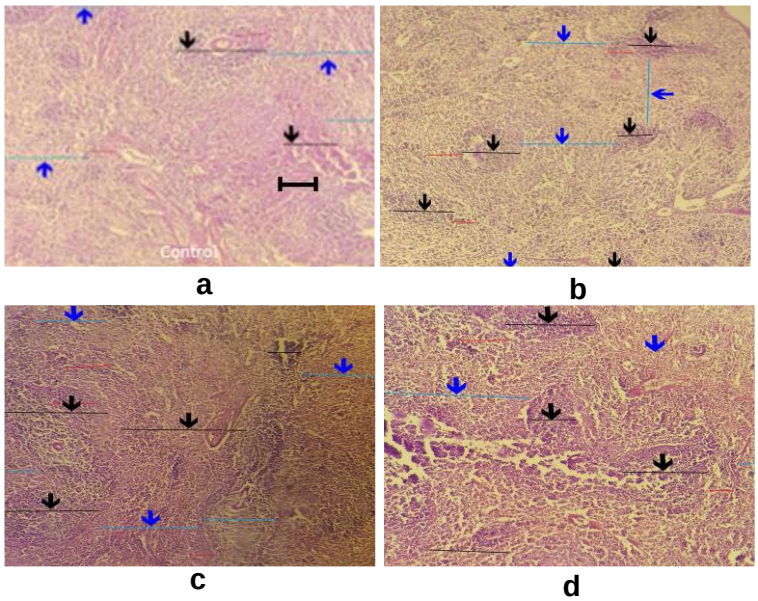

Figure 4. Histopathological alterations in the spleen of albino rat exposed to different doses of AgNPs at 100X. Control group (a), low dose 1mg/kg (b), medium dose 5mg/kg (c) and high dose 10mg/kg (d). The sizes of white pulp (black arrow and line) and red pulps (blue arrow and line) were recorded using Image J software. Bar on control indicate 10µm.

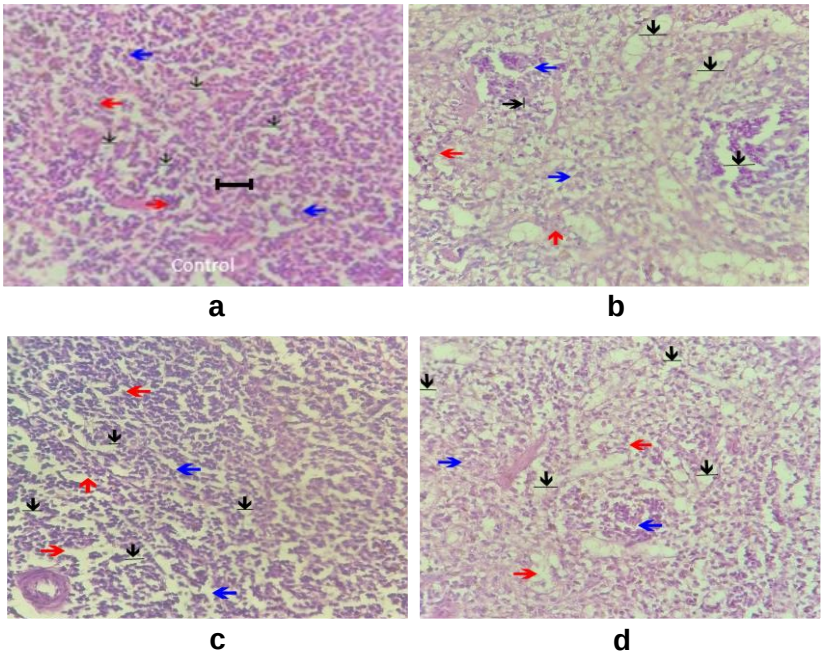

Figure 5. Histopathological alterations in the spleen of albino rat exposed to different doses of FM-AgNPs for five weeks, (400X). Control group (a), low dose 1mg/kg (b), medium dose 5mg/kg (c) and high dose 10mg/kg (d). In control, blue and red arrows indicate standard size while in treated groups, blue arrow represent congestion and red arrows represent vacuolization. Bar on control indicate 10µm.

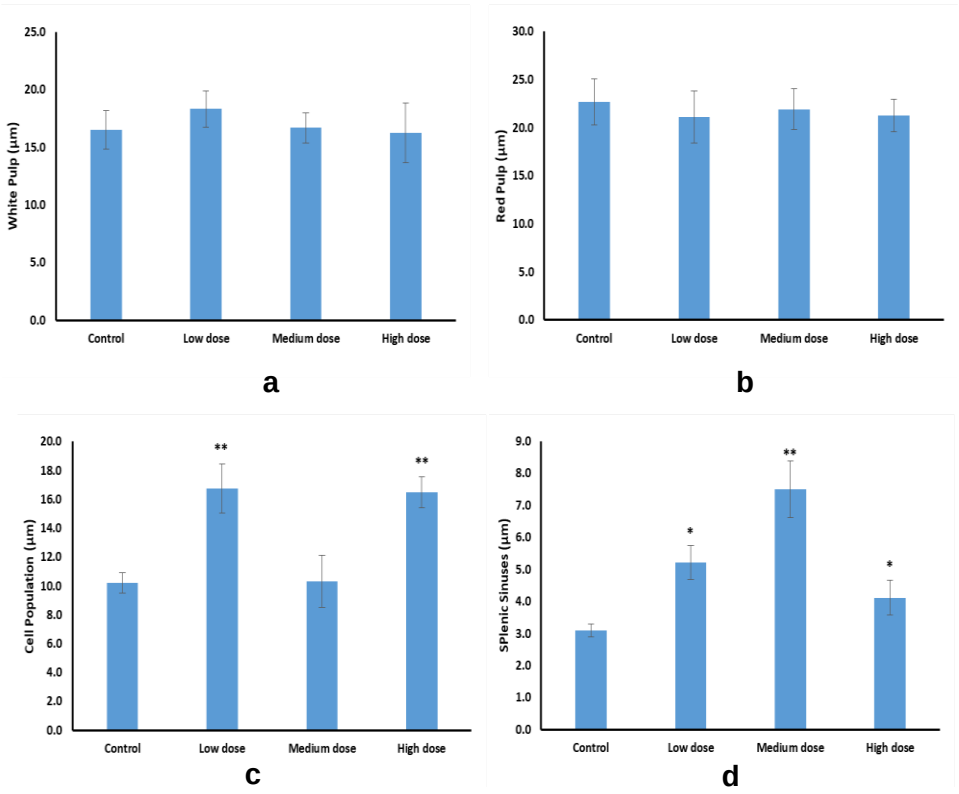

Figure 6. Alterations in the white pulp, red pulp, cell population, and splenic sinuses of the rat spleen following exposure to different doses of FM-AgNPs. (* $p < 0.05$; ** $p < 0.01$).

DISCUSSION

Chemically synthesized NPs react with biosystems more aggressively than biologically synthesized NPs which are considered less toxic [25]. In the last decade, numerous medicinal plant extracts were used for the synthesis of silver nanoparticles which exhibit favorable biological effects, *viz*., antioxidant activity, antimicrobial activity, anti-inflammatory activity, hypoglycemic effect, antitumor activity, and catalytic activity

[17, 26, 27]. Kumquat is an economically cultivated plant in South China, South Asia, and Asia–Pacific regions. It has distinctive fruit species in the citrus family and extremely popular because of its high medicinal value [28]. It is traditionally used as a folk medicine for treating common colds, coughs, allergies, and other inflammatory diseases [26, 29]. Furthermore, kumquat fruits have long been known as an excellent source of flavonoids, carotenoids, ascorbic acid, and essential oils, possess antioxidant, antimicrobial, and anticancer activity [26, 30]. The characteristic of Kumquat (*Fortunella margarita*) as a reducing agent has been exploited for preparing environmentally friendly silver nanoparticles. The silver nanoparticles synthesized by Kumquat (*Fortunella margarita*) have been documented to show effective antibacterial activity during *in vitro* studies [17, 31]. Al-kalifawi and coauthors [17] assumed that nanoparticles from FM can control human microbial infections. However, Data is scarce on the influence of green synthesized AgNPs particularly from *Fortunella margarita* on the general health of non-target organisms. The current study describes the *in-vivo* effects of FM-AgNPs on hematology, oxidative biomarkers (SOD, CAT, and MDA), and microanatomical alterations of liver and spleen in albino rats. Some studies on metallic and phytosynthesized AgNPs are available with contrasting results on hematological parameters depending on the nature of NPs. Ghareeb and coauthors [32] and El-Naggar and coauthors [33] have reported elevation in Hb, RBC, and HCT while Moreno and coauthors [34] and Ghareeb and coauthors [32] have reported adverse effects in terms of anemia. In the current study, we observed elevation in Hb, RBC, and HTC following exposure to FM-AgNPs at low and medium doses but no such change was observed at high doses. It could be an outcome of compensatory mechanisms that might have been stimulated after partial toxicity with low doses of FM-AgNPs or the influence of FM-AgNPs (in low doses) on the hematopoiesis to meet the higher need for oxygen transport [35]. Similarly, consistent with Recordati and coauthors [36] and Moreno and coauthors [34], a depletion in WBC and elevation in granulocyte to lymphocyte ratio was noticed at all doses in this experiment. The normal total leukocyte count and granulocyte lymphocyte ratio also indicate the immune status of the host [37]. The reduction in leukocyte count might be due to the suppression in leukocytosis or reduction in the half-life of leukocytes [38]. Current data further indicate that exposure to AgNPs, even at a low dose, may lead to immune compromised conditions [39]. In contrast to our findings, Rudi and coauthors [40] reported that leukocyte count and granulocytes to lymphocyte ratio remain unaltered after oral exposure to 1 mg/kg citrate coated AgNPs for 4 weeks. The variation could be linked with different types and sizes of AgNPs used in the studies.

Oxidative stress (OS) is caused by the production and accumulation of reactive oxygen, nitrogen or hydrogen molecules in cells and tissues. The ROS, attack the biomolecules leading to cell damage that results in multiple diseases and metabolic disorders [41]. The antioxidant system modifies the highly reactive oxygen species to the least reactive intermediate and reduces OS with the help of non-enzymatic (vitamin C, vitamin E, carotenoids, and glutathione) and enzymatic (Superoxide dismutase, glutathione peroxidase, and catalase) antioxidants. Changes in the level of enzymatic and non-enzymatic antioxidants in serum and tissue reflect the cellular response against oxidative stress [42]. SOD, CAT, GPX, and GSH have been used as biomarkers of OS for assessing the toxicity of xenobiotic by many researchers [33, 34, 42]. SOD is considered one of the most active enzymes which helps in the conversion of superoxide radicals into oxygen and hydrogen peroxide [43, 44]. Similarly, in the presence of oxidative stress, CAT plays a significant role in cell defense. During detoxification reaction, the H_2O_2 is generated which is converted to water and oxygen by the action of CAT and GPX [43]. The process of lipid peroxidation generates free radicals. While Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. The OS conditions leads to an increase in free radicals which results in the overproduction of MDA. Thus, excess in MDA indicates the presence of OS. Irrespective of the direction of change, the alteration in these parameters are indicators of OS. Alkadi [44]; El-Naggar and coauthors [33]; Magdy and coauthors [45] reported depletion in SOD, CAT following continuous exposure of M-AgNPs for 7-14 days at different doses. In the current study, an elevation in SOD, CAT, and MDA was observed at all doses in both liver and spleen at different time points following the exposure of FM-AgNPs. However, the changes were more consistent and pronounced in the spleen. Previously, alterations in these parameters/indicators as a consequence of exposure to metallic and green synthesized AgNPs have been reported by various authors [19, 32, 33]. Although, Green synthesized NPs are considered nontoxic and safe but controversial reports are available on them depending on the plant source. Husain and coauthors [46], mentioned that exposure to NS-AgNPs does not induce any change in OS biomarkers, while Mirmoeini and coauthors [47] mentioned phytotoxicity of CS-AgNPs that resulted in the elevation of SOD, CAT, and MDA in plant tissues the following spray with CS synthesized NPs. Previously Mohammed and coauthors [48] reported high antagonistic efficiency of FM-AgNPs against multiple human pathogens through *in vitro* assays and declared their therapeutic potential. The elevations in SOD, CAT, and MDA, observed in the current study may be linked with the toxicity of FM-

AgNPs. The higher levels of SOD, CAT, and MDA could be due to upregulation in the expressions of the enzymes at transcription and/or translation levels [42]. The change in the normal architecture of tissue is an indicator of toxicity. The toxicity of the FM-AgNPs was further confirmed in histopathological examination of the liver and spleen. The liver architectural changes may include hypertrophy, vacuolization, or invasion of lymphocytes in the tissue [33]. Similarly, alterations in the size of the red and white pulp of the spleen are used as important criteria of pathological alterations [49]. In the current study, we used a quantitative approach to assess changes in tissue architecture. Exposures to the high dose of FM-AgNPs led to significant alterations in microanatomy of the liver and spleen.

In liver, exposure to FM-AgNPs led to significant elevation in cell density per unit area, while significant depletion in size of cells, nuclear diameter, and portal vein along with vacuolization, enucleation, necrosis, congestion, and reduction in nuclear were recorded. However, the variations in microanatomy of the spleen were not much pronounced. Sizes of red and white pulp slightly varied, but consistent with results of liver morphometry, the cell population, and splenic sinuses increased significantly ($p < 0.05$); following exposure to the high dose of FM-AgNPs. Previously, El-Naggar and coauthors [33]; Magdy and coauthors [45]; Sampath and coauthors [50]; Hassanen and coauthors [51] reported several degenerative alterations viz., vacuolization, congestion, necrosis, hepatocyte degeneration in liver and spleen tissues. Results indicate that single exposure at 1, 5, 10 mg/kg does not sufficiently damage the tissue rather up regulates the compensatory mechanism. The data further confirms that FM-AgNPs even at low doses impose toxic effects and induce the production of OS.

CONCLUSION

It is concluded that FM-AgNPs cause only functional alterations at low dose while the high dose leads to micro anatomical changes along with functional alterations. In our study it is further confirm that FM-AgNPs are not always safe for use and highlight the importance of *in vivo* toxicity assessment.

Conflicts of Interest: The authors declare no conflict of interest.

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