EFFECT OF SILVER NANOPARTICLES SYNTHESIZED USING LEAVES EXTRACT OF OLIVE ON HISTOPATHOLOGICAL AND CYTOGENETIC EFFECTS IN ALBINO MICE

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ABSTRACT

This study was conducted to examine the possible effects of Ag-NPs synthesized using the olive leaf extract on histopathology and cytogenetic effct in mice. A total of thirty albino mice aged two months were divided into five treatment groups as follows: Group 1: served as control was administrated orally with 0.3 mg/kg bw of normal saline; Group 2 and 3 were administrated orally with varying doses of Ag-NPs synthesized on olive leaf extract (10 and 100 mg/kg bw, respectively) for 30 days; Group 4 and 5 were administrated orally with olive leaf crude extract (300 and 1000 mg/kg bw, respectively) for 30 days. At the end of experimental period, detection of possible chromosomal aberrations in blood samples and histopathology (liver, spleen, kidney, uterus and testes) were carried out. Statistically significant differences ($P \leq 0.05$) were observed for the chromosomal aberrations in all Ag-NPs groups compared to control and to crude olive leaf extract groups. Histopathological study revealed various alterations in internal organs at high dose of Ag-NPs group including: inflammatory reaction, blood congestion, degeneration, fibrosis, mononuclear cells lesion and necrosis. Slight changes were identified at both doses of crude olive extract treated groups. Based on these results, oral administration of Ag-NPs could cause genotoxic and inflammatory responses in mice and this could be representing a risk to both environment and human health.

Keywords: albino mice, chromosomal aberrations, liver, kidney, uterus

تأثير جسيمات الفضة النانوية المشتقة من خلاصة اوراق الزيتون على التشريح المرضي النسجي والتاثير الوؤاثي في الفئران البيضاء

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المستخلص

هدفت الدراسة الى تقييم تأثيرات جسيمات الفضة النانوية (Silver nanoparticles) التي تم تصنيعها باستخدام مستخلص أوراق الزيتون على التشريح النسجي والتاثير الوراثي الخلوي في الفئران. تم تقسيم 30 من الفئران البيضاء إلى خمس معاملات على النحو التالي: المعاملة 1: مجموعة السيطرة اعطيت عن طريق الفم المحلول الفسيولوجي بجرعة 0.3 ملغم / كغم من وزن الجسم؛ المعاملات 2 و 3 اعطيت عن طريق الفم مع جرعات متفاوتة من Ag– NPs التي تم اشتقاقها من مستخلص أوراق الزيتون (10 و 100 ملغم / كغم من وزن الجسم ، على التوالي) لمدة 30 يوما ؛ المعاملات 4 و 5 اعطيت عن طريق الفم مستخلص زيت الزيتون الخام (300 و 1000 ملغم / كغم من وزن الجسم ، على التوالي) لمدة 30 يوما. في نهاية الفترة التجريبية ، تم فحص الانحرافات الكروموسومية في عينات الدم وفحص التشريح المرضي النسجي (الكبد والطحال والكلى والرحم والخصيتين). وقد لوحظت فروقات ذات دلالة إحصائية ($P < 0.05$) في الانحرافات الكروموسومية في جميع معاملات الفضة النانوية مقارنةً بمجموعة السيطرة ومعاملات مستخلص أوراق الزيتون الخام. اظهرت نتائج التشريح المرضي النسجي تغيرات مختلفة في الأعضاء الداخلية في معاملات الجرع العالية من Ag–NPs تضمنت: التفاعل الالتهابي ، والنزيف واحتقان الأوعية الدموية والتنكس والتليف ، المتعددة والتنخر في معظم الاعضاء. تم تحديد تغييرات نسجية طفيفة في كل من جرعات معاملات زيت الزيتون الخام المستخلص يمكن أن يسبب تناول Ag–NPs عن طريق الفم استجابات التهابية وسمية جينية في الفئران والذي يمكن ان يمثل خطراً على كل من البيئة وصحة الإنسان.

الكلمات المفتاحية: الفئران البيضاء–الانحرافات الكروموسومية–الكبد–الكلية–الرحم

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INTRODUCTION

Nanotechnology is recently a field of passionate scientific attention owing to its wide variety of potential uses in industrial, biomedical and therapeutic related products (26). Regardless of the rapid development of nanotechnology, the adverse health impacts due to exposure at different levels of nanoparticles in environment and human beings have not yet been well-known. It has been estimated that the effects of nanoparticles on environment and in living organisms will increase extensively in the future (9, 19). Nanomaterials have unique structures such as very small size, good mobility, high reactivity, penetration ability and high surface area, (18, 30). As a consequently, the physicochemical and electrical characteristics of NPs are increasing the probability of interaction between these particles and environment (2, 18). Silver nanoparticles (Ag-NPs) have become one of the most nanomaterials that have been widely applied at various sectors. Recently, Ag-NPs have expanded much approval due to their unique anti-inflammatory, antifungal, antibacterial and antiviral activities (8). Various sources are available for biosynthesis of Ag-NPs such as; ultrasonic, violet radiation and lithography. But, these methods are not considered eco-friendly (25, 27). Hence, plant extract synthesis is considered an alternative method and to be a need of an hour, because it possesses several benefits compared to chemical methods (3, 29, 31). In this regard, olive leaf extract could have several potential health benefits such as protect from heart disease and lowering blood pressure. Also, contains potential antioxidants which are considered phyto-reductants for tailoring nanostructures like apigenin-7-O-glucoside oleuropein and oleuroside (16, 22). Thereby, in this study we attempt to examine the possible effects of Ag-NPs (synthesized using the aqueous leaf extract of olive, *Olea europaea*) on histopathology and cytogenetic in mice and to compare between a crude leaf extract of the olive and Ag-NPs derived using the same olive leaves extract.

MATERIALS AND METHODS

Preparation of olive leaves extract: Initially, fresh olive leaves were collected from olive trees, and then these leaves were cleaned by water and left them for drying in room temperature for 50 days. Then, the dry leaves were grinded well using electric grind until reach to fin powder. Olive powder was washed by sterile distilled water then centrifuged and analysis [report NO.1077 in project of liquidation of Muthanna stores (8/5/2017) chromatogram mar q C:/GCMS solution/ Data/project1/ marw.QGD]. After that olive powder dissolved in sterile distal water (1 g of olive powder added to 10 ml of distilled water) mixed the heated about 10 min till the color changed to yellowish. Then, the mixture was cooled at room temperature then filtrated by using filter paper and kept until use it (5).

Synthesis of silver nanoparticles (Ag-NPs)

Ag-NPs were synthesized using olive leaves extract according to method proposed by Awaad *et al.* (5). Firstly, olive solution extract was added to $AgNO_3$ (each 10ml of olive extract added to 0.5ml of $AgNO_3$ to produce Ag-NPs). Then, the extract put on magnetic stirrer until the color changed to faint yellow solution at room temperature. Later, the blend was heated in water bath at 40 to 60 °C. The color change for the blend was monitored at different time and temperature which result change in color, the color change of Ag-NPs during the day and to air exposure. Then, centrifugation of coordinated materials of Ag-NPs, the sediment redisposed in sterile DW to eliminate any of unw. After that, olive leaves were tested to identify the materials used for treated product.

Examination of art

Size of particles

Particles sizer and distribution was measured using zeta sizer (Nano-ZS- Malvern zeta sizer) according to the light scattering method which was 20nm.

X-Ray diffraction (XRD) pattern

XRD measurements were carried out according to the ASTM (American Society of Testing Materials) cards taken from Match program version 1.9b (2011).Using Philips pw 1050 X-ray diffractometer of 1.54 Å from Cu-k α, the XRD patterns of samples were recorded in the range $2\theta=10\text{-}70^{\circ}$.The diffractometer was operated at 20 kV and 30 mA, is incident on a specimen and is diffracted

by the crystalline phases in the specimen according to Bragg's law.

$$n\lambda = 2dsin\theta$$ Where d is the spacing between atomic planes in the crystalline phase and λ is the X-ray wavelength. The intensity of the diffracted X-rays is measured as a function of the diffraction angle 2θ and the specimen's orientation. This diffraction pattern is applied to identify the specimen's crystalline phases and to measure its structural properties. Nondestructive and does not require elaborate sample preparation which characterization.

Scanning Electron Microcopy (SEM)

The SEM study was carried out by at the University of Tehran, scanning electron microscope (Hitachi FE-SEM model S-4160, Japan) equipped with Energy dispersive X-ray (EDAX); determine the energy of the X-rays microanalysis.

Experimental procedure and conditions

A total of 30 adult of both sex albino mice aged two months were obtained from Veterinary laboratories /Ministry of Agriculture and were housed in the Unit of Small Animal Management and Care/ College of Veterinary Medicine/ University of Baghdad. Mice were acclimatized for two weeks before beginning the experimentation. Throughout this period and the experiment period, mice were kept in a plastic cages (3 mouse per each cage) in well ventilated room with regular light (12light/12 dark) and in room temperature and were fed commercial pelleted feed and given water. Then, the mice were divided randomly into five main treatment groups (six animal per group) as follows: Group 1: served as control (normal) was administrated orally with 0.3 ml/kg bw of normal saline; Group 2 and 3 were administrated orally with different doses of Ag-NPs synthesized on leaves olive extract (10 and 100 mg/kg bw, respectively) for 30 days; Group 4 and 5 were administrated orally with olive leaf crude extract (300 and 1000 mg/kg bw, respectively) for 30 days. The doses of the Ag-NPs and olive leaves crude extract were determined according to Mohammed (23). At the end of experimental period (i.e., 30 days), blood samples were collected from each group to detect the chromosomal aberration (21) and micronucleus assay (6). Also, selected organs were dissected out for histopathological study.

Histopathological study

At the end of experimental period, specimens of internal organs (viz; liver, spleen, kidney, uterus and testes) were collected after euthanizing the mice, fixed in 10% formalin. Then, tissue samples were dehydrated through ascending sequences of ethanol, after that, embedded in paraffin wax, sectioned (4–6 μm thickness) and stained by hematoxylin and eosin (24).

Data analysis

Data analysis was performed using the SPSS version 20 (SPSS, Richmond, VA, USA). One way analysis of variance (ANOVA) was applied to compare the effect of crude olive leaf extract and Ag- NPs doses. Less Significant difference (LSD) was used to compare the differences among means of treated groups. Probability (P) values less than 5% were considered significant difference.

RESULTS AND DISCUSSION

Micronucleus assay

The results of micronucleus assay are illustrate in Table 1. Mean of micronucleus frequency values revealed no significant ($P>0.05$) differences within all treated groups (G1, G2, G3, G4 and G5) and as well as among these treatment groups (Figure 1).

Chromosomal aberrations

After 30 days of experimental period, the total of chromosomal aberrations (CA) showed significant ($P<0.05$) increase within G2 and G3 in comparison to control group (G1). But, G4 and G5 were not significant ($P>0.05$) relative to G1. The highest value was seen in G3 which was significantly increase compared to other treated groups (G1, G2, G4 and G5) respectively. However, there are no significant ($P>0.05$) differences between G4 and G5 (Table 2). Results showed different chromosomal aberrations in the cells of all Ag-NP treated groups containing: Acentric Chromosome, Acentric Break Chromatid and Deletion Chromosome (Figure. 2A, B & C).

Table 1. Results of micronucleus frequency values in mice treated with different doses of Ag-NPs and crude olive leaves extract for 30 days.

Treatment groups	Values after30 days
G1	2.11 ± 0.00[a]
G2	2.26 ± 0.07[a]
G3	2.00 ± 0.05[a]
G4	2.08 ± 0.24[a]
G5	2.24 ± 0.06[a]
LSD	0.27

Data are means±SE. Different superscript letters indicated significantly different at *P*<0.05.

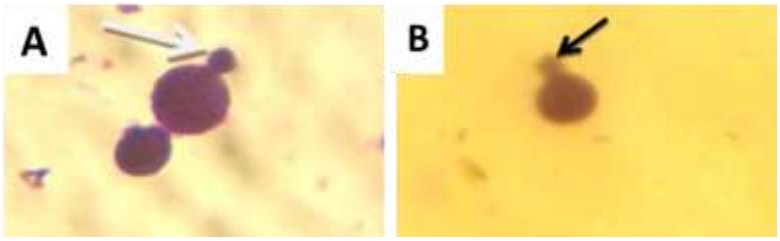

Figure 1. Micronucleus under microscope (x100), A &B showing nuclear bud formation.

Table 2. Results of the total values of chromosomal aberration in mice treated with different doses of Ag-NPs and crude olive leaves extract for 30 days.

Treatment groups	Values of Chromosomal aberrations (30 days
G1	0.37 ± 0.07[c]
G2	0.87 ± 0.07[b]
G3	1.18 ± 0.06[a]
G4	0.50 ± 0.14[c]
G5	0.56 ± 0.11[c]
LSD	0.31

Data are means±SE. Different letters superscript indicated significantly different at *P*<0.05.

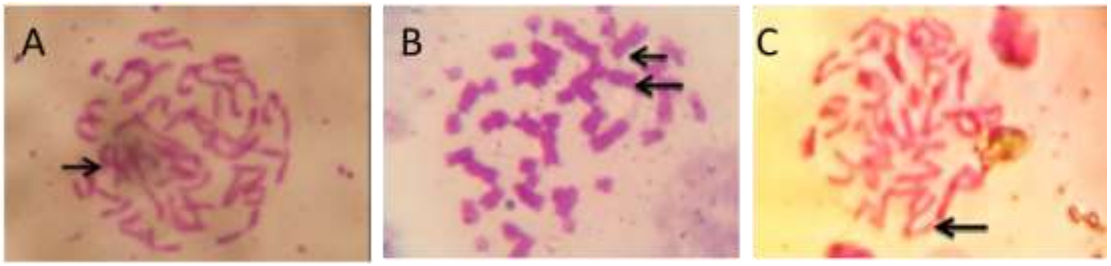

Figure 2. Chromosomal aberrations under microscope (100x) A. Acentric Chromosome. B. Acentric Break Chromatid, C. Deletion Chromosome, Giemsa stain.

Regardless of the rapid development of nanotechnology, however, enough toxicological information has not been yet elucidated (9). Moreover, most of the toxicity investigations of Ag-NPs have been conducted on mechanistic pathway using *in vitro* models, rather than aiming on toxicity and histopathological manifestations using *in vivo* model via possible exposure scenario. Exposure to Ag-NPs possibly occurs via different routes such as ingestion, inhalation and dermal contact. Ingestion route could be an important route in many consumer related products such as kitchen utensils, food containers, toothpaste, toys, cosmetics (8, 10).

The cytogenetic effect of Ag-NPs was indicated by different types of chromosomal aberrations (CA) that were dose dependent. High dose of Ag-NPs treated group showed significant increase of CA compared to control and to crude olive leaf extract groups, this could be owing to liberated silver ions could react with cellular DNA that lead to dysfunction of DNA backbone and result oxidative damage. Although, Asharani *et al.* (1) observed from human lung case (fibroblastoma and glioblastoma) the increase

in ROS production, DNA damage, decreasing ATP and mitochondrial damage which result dysfunction of energy-dependent DNA repair. But, the real mechanism for potential of Ag-NPs to induce genotoxicity still not well known. Several *in vitro* and i*n vivo* investigations confirmed the results of the current study (1,7, 11, 17).

Histopathological study

Liver

Liver sections of control animals showed normal hepatic architecture and there was no noticeable lesion. But, liver sections at high dose of Ag-NPs treated group revealed of aggregation of active macrophages and lymphocytes in parenchyma and mononuclear cells (MNCs) infiltration (Monocytes /macrophages and lymphocytes) in portal area around proliferation of bile duct and portal blood vessels. Other sections showed vacuolar degeneration and necrosis of hepatocytes. In addition, coagulative necrosis appeared in parenchyma surrounded by macrophages and lymphocytes as well as dilation of sinusoid and loss of hepatic cord pattern, these changes appeared to be less pronounced at low dose of Ag-NPs group. Only degenerative changes were observed in liver sections at high dose of crude olive leaf extract treated group (Fig. 3 A-D). These alterations representing the hepatotoxic impact of Ag-NPs including hepatic degeneration, dilatation of sinusoids, bile duct hyperplasia and necrosis were the most identified hepatic changes that were dose dependent. Sardari *et al.* (32) speculated the pathway of Ag-NPs induced hepatotoxicity by repeated oral administration and they concluded that macrophages removed nanoparticles from the liver due to phagocytosis and the repetition of this pathway induced higher reactive oxygen species (ROS) Several researchers have established that liver is the target organ for the impact of Ag-NPs (13,16, 32). Silver nanoparticles have ability to diminish the activity of mitochondria which could cause decrease of energy that available for cells. Furthermore, Hussain *et al.* (14) documented that Ag-NPs were highly toxic to hepatic cells.. The results of the present study are in agreement with previous studies by Sung *et al.* (33) and Kim *et al.* (20).

Spleen; The most predominant splenic alterations in Ag-NPs were found to be dose dependent. Group treated with high dose of Ag-NPs exhibited hyperplasia of lymphocytes in the white pulp and proliferation of MNCs around sinus in red pulp form cord-like appearance. Other sections showed amyloid like substance deposition (Fig. 4 A). While, the main lesion in group treated with low dose of Ag-NPs observed inflammatory cells mostly neutrophils infiltration in the congested red pulp with reduction of white pulp (Fig. 4. B). Slight proliferative changes were identified at high dose crude olive extract, but no pathological lesions were noticed at low dose of crude olive extract treated group. These findings are in consistent with study in albino mice exposed to magnetite nanoparticles carried out by Awaad (4).

Kidney

Kidney sections at high dose of Ag-NPs expressed MNCS infiltration in the interstitial tissue and atrophy of glomerular tufts with dilation of bowman space and fibrosis of their walls (Fig.5A). These changes appeared to be less pronounced at low dose of Ag-NPs group which exhibited MNCs infiltration, hyperplasia of fibroblast between renal tubules and acute cellular degeneration in epithelial cells of the renal tubules (Fig. 5 B). Also, high dose of crude olive leaf revealed MNCs infiltration between renal tubules and in the interstitial tissue (Fig. 5C), but these alterations diminished at low dose of crude olive leaf extract treated group (Fig. 5D). Tang *et al.* (35) showed that Ag-NPs translocated from the blood circulation to some organs, such as liver and kidney, then resulting renal toxicity. Nanoparticle toxicity is determined by inflammation resulting from oxidative stress (12). These findings are in consistent with observation of Roda *et al.* (28).

Uterus

Uterus from high-dose of Ag- NPs exposure demonstrated infiltration of neutrophils with fibrin net deposition in serosal layer, between muscular layer and in sub-epithelial. Another section, expressed infiltration of neutrophils, plasma cells, macrophages and lymphocytes between uterine gland and mucosal glands as well as, between muscular layers. Besides, there was proliferation of epithelial layer of

endometrium (Fig. 6A). Also, high dose of Ag-NPs showed hyperplasia of myometrium which was very clear at this dose (Fig.6B). In other animals, granulomatous lesion consisting from clusters of macrophages and lymphocytes was observed in sub-epithelial layer (Fig. 6C) as well as neutrophils and mononuclear cells in the lumen of dilated uterine glands (Fig. 6D). High dose of olive leave crude extract treated group revealed infiltration of MNCs around uterine glands (Fig. 6E). While, there were no remarkable histopathological alterations at low dose of crude extract. Previous investigations showed that NPs can pass via the blood–placental barrier and epithelial barrier, which protect reproductive organs, and then accumulate in reproductive tissues resulting damages and dysfunction to these organs (testis, epididymis, ovary, and uterus) by destroying germ cells, Leydig cells and Sertoli cells as reviewed by Wang et al. (36). Sodani, (33) suggested that low molecular weight of *Aloe vera* leaf extract induce effects on the histological features in mice and impaired their entire structures.

Testes

Histopathological observations in testes at high-dose of Ag- NPs exposure characterized by severe inflammation (orchitis), in addition to absence of spermatogenesis (Fig. 7A), but these alterations diminished at low dose of Ag-NPs and the congestion appeared very clearly (Fig.7B). Slight changes were found at high dose of crude extract and only congestion was observed (Fig. 7C). There were no remarkable histopathological alterations at low dose of crude extract (Fig.7D). In testes the severe inflammation and absence of spermatogenesis at high dose of Ag- NPs group may possibly suggest that genotoxic effects of Ag-NPs. These findings are in consistent with a study in albino mice exposed to Ag-NPs carried out by Iyiola *et al.* (15) who reported abnormal in sperms shape and decrease in sperms count which results disturbance of spermatogenesis. Based on these results, oral administration of high dose Ag-NPs had the ability for inducing chromosomal aberrations which indicating the genotoxicity of Nano silver particles. In addition to that, induce adverse histopathological alterations in selected tissues in contrast to mild changes in crude olive leaf extract which could be owing to low molecular weight can infiltrated and entering the cells and probably result oxidative stress. Thus, further cellular and molecular studies should be done to shed lights on the molecular pathways involved.

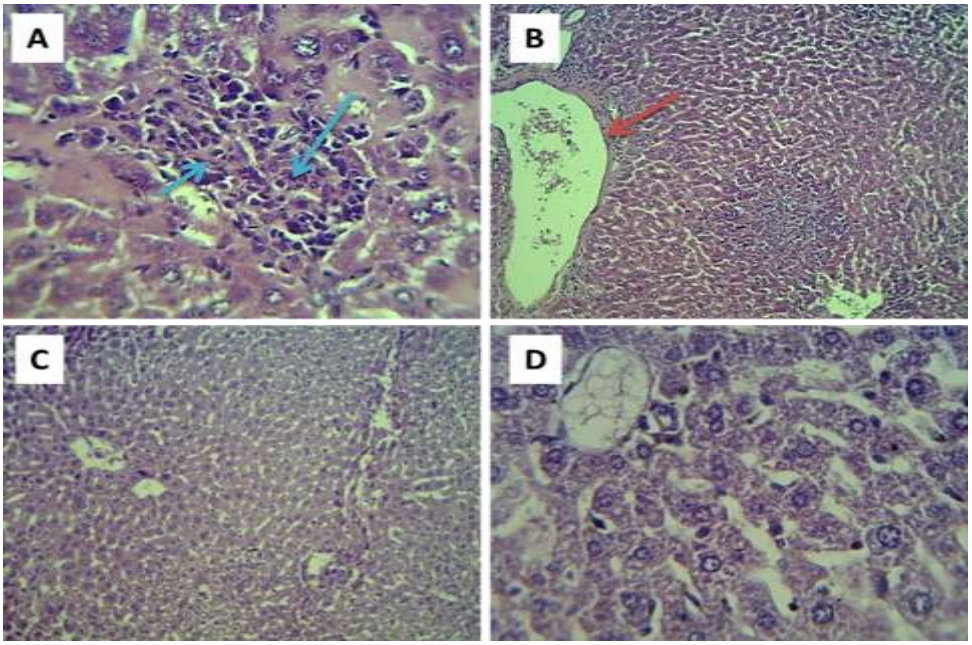

Figure. 3. Histopathological sections for liver in mice treated with different doses of Ag-NPs and crude olive leaf extract. (A& B) high dose of Ag-NPs (x 40) demonstrating of aggregation of macrophages and lymphocytes (blue arrow); (B) (x10) thickening of central vein wall (red arrow); (C) low dose of Ag-NPs (x10) showing degenerative changes of hepatocytes; (D) high dose of crude olive leaf extract (x40) demonstrating degenerative changes of hepatocytes. H&E. 4-6 µm thickness.

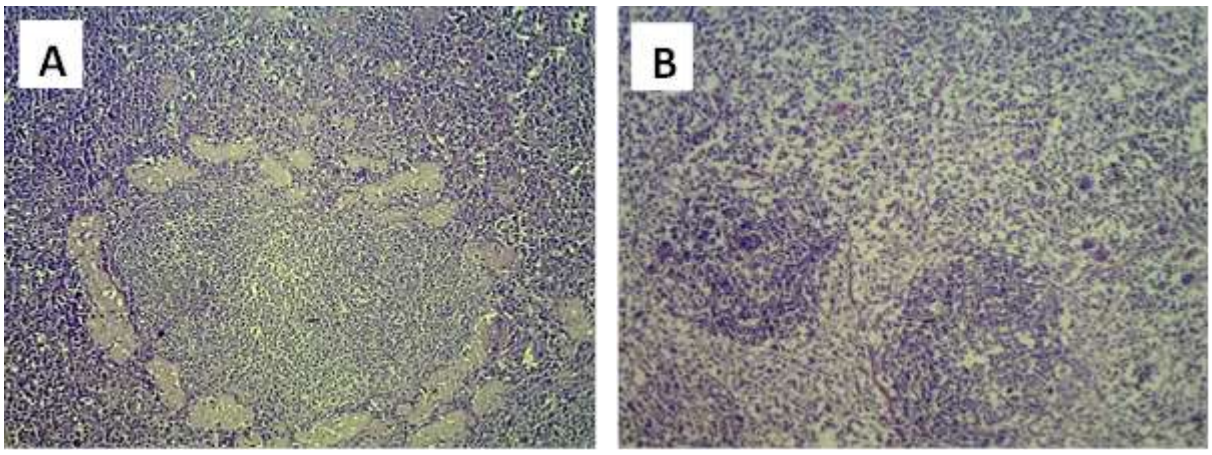

Figure 4. Histopathological sections for spleen in mice treated with different doses of Ag-NPs and crude olive leaf extract. (A) high dose of Ag-NPs showing hyperplasia of lymphocytes in the white pulp and proliferation of MNCs around sinus in red pulp with myeloid like substance deposition (B) low dose of Ag-NPs demonstrating inflammatory cells particularly neutrophils infiltration in the congested red pulp with depletion of white pulp and presence of megakaryocytes. (H and E X20). 4-6 µm thickness.

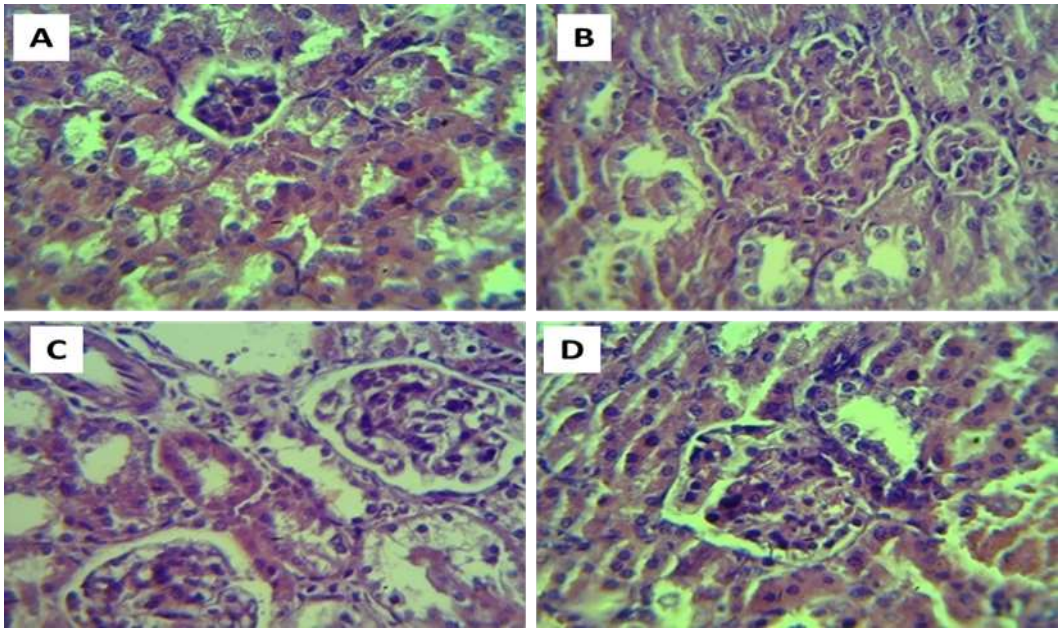

Figure 5. Histopathological sections for kidney in mice treated with different doses of Ag-NPs and crude olive leaf extract (A) high dose of Ag-NPs treated group expressed MNCS aggregation in the interstitial tissue and atrophy of glomerular tufts with dilation of bowman space and fibrosis of their walls; (B) low dose of Ag-NPs showing MNSC infiltration with hyperplasia of fibroblast between renal tubules with acute cellular degeneration of epithelial cells of renal tubules; (C) kidney section of mice treated with high dose crude olive leaf extract showing MNCS in the interstitial tissue and between renal tubules; (D) low dose of olive leaf extract treated group showing less congestion (H and E X20). 4-6 µm thickness.

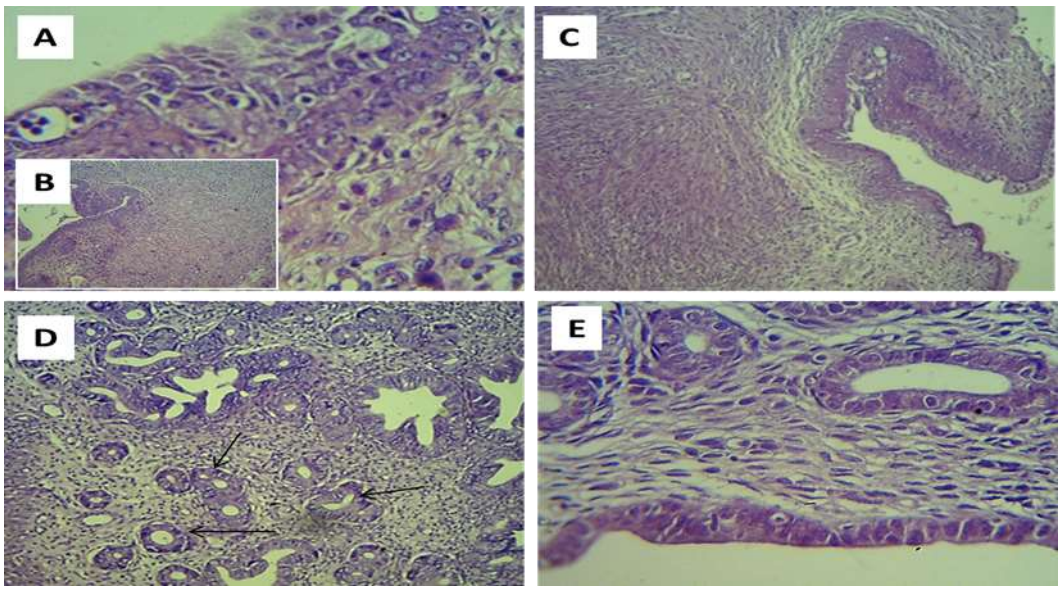

Figure 6. Histopathological sections of uterus in mice treated with different doses of Ag-NPs and crude olive leaf extract (A-D) high dose of Ag-NPs treated group (A) showing hyperplasia of epithelial layer of endometrium;(B) hyperplasia of myometrium; (C) showing granulomatous lesion consisting of aggregation of macrophages and lymphocytes in sub-epithelial layer; (D) proliferation of uterine glands surrounding by inflammatory cells (black arrow); (E) high dose of crude olive leaf extract demonstrating inflammatory cells surrounding uterine glands. (H&E X20). 4-6 µm thickness.

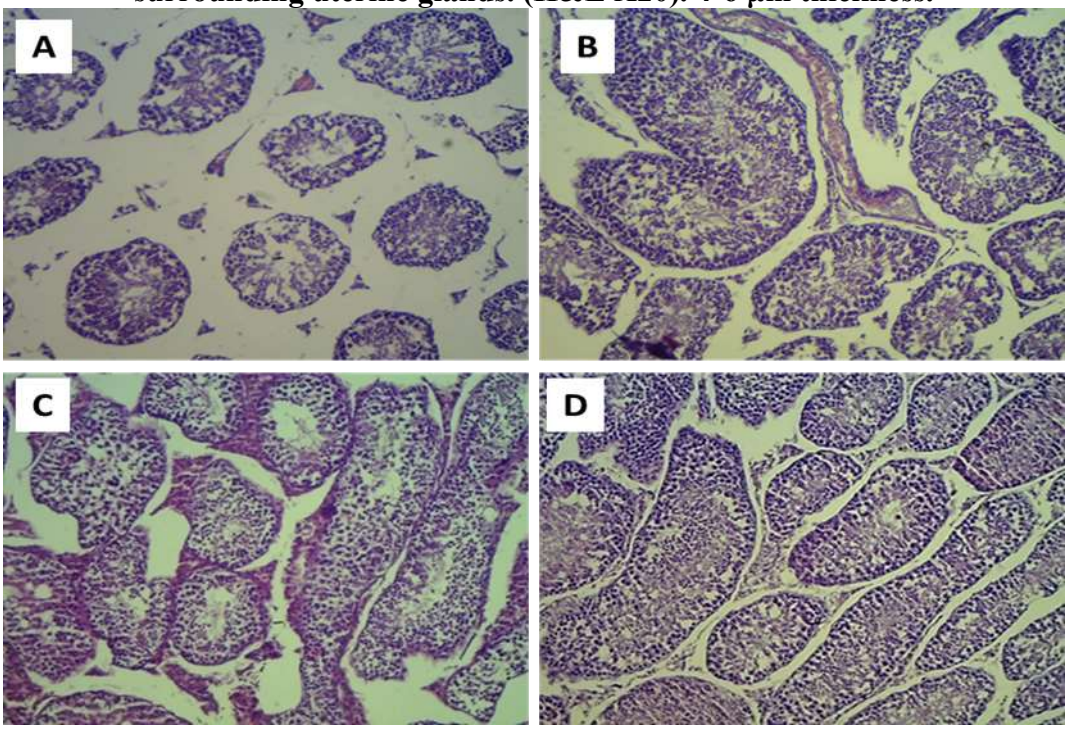

Figure 7. Histopathological sections of testes in mice treated with different doses of Ag-NPs and crude olive leaf extract (A) high dose of Ag-NPs treated group showing severe inflammation (orchitic) and absence of spermatogenesis; (B) at low dose demonstrating the congestion very clear; (C) high dose of crude extract of olive leaves extract showing the very clear congestion very clearly; (D) low dose displaying normal structure. (H&E X20). 4-6 µm thickness.

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