Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.154

RESEARCH ARTICLE

Protective Effects of *Moringa oleifera* Leaf Extract against Silver Nanoparticles and Arsenic Induced Hepatotoxicity in Rats

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ARTICLE HISTORY (23-513)

Received: November 16, 2023
Revised: March 10, 2024
Accepted: March 13, 2024
Published online: April 01, 2024

Key words:
Arsenic
Hepatotoxicity
Moringa oleifera
Nanoparticles
Oxidative stress

ABSTRACT

The present study was designed to investigate whether *Moringa oleifera* leaf extract (MOLE) modified the changes caused by interactive exposure of nanoparticles and heavy metals in various hepatotoxicity parameters. Sixty-three Sprague Dawley rats weighed 105 ± 5 g were assigned into seven groups (n=9) each. Group I was control (C). Groups II and III were given moringa with low 2% and high 3 % doses. Group IV consisted of low doses of silver nanoparticles (100 mg/kg) and arsenic (10.25mg/kg). Group V embraced high doses of silver nanoparticles (150 mg/kg) and arsenic (16.4mg/kg). Group VI comprised low and group VII consisted of high doses of chemicals and MOLE as described above. Doses were administered orally and daily for three months. Three rats from each group were euthanized and evaluated for various parameters after 4th, 8^{th,} and 12th weeks of the experiment. Results revealed that silver nanoparticles and Arsenic exposure at low and high doses induced severe necrotic histopathological changes in liver tissue accompanied by remarkably escalated levels of ALT, AST, ALP, and oxidative stress marker (TBARS). Furthermore, albumin, total protein, SOD, CAT, GPX, and GSH levels were significantly depleted in each successive three-month treatment. Concurrent treatment of silver nanoparticles and arsenic-intoxicated rats with MOLE prevented tissue injury through improving the liver's cellular integrity, correcting liver proteins/enzymes and inhibiting TBARS levels by activating the antioxidant enzymes in a dose-dependent manner. Therefore, MOLE administration demonstrated a therapeutic role against hepatotoxic effects of silver nanoparticles and arsenic, which are related to its antioxidant capabilities.

To Cite This Article: Bashir F, Sharif S, Manzoor F, Naz S and Rashid F, 2024. Protective Effects of *Moringa oleifera* Leaf Extract against Silver Nanoparticles and Arsenic Induced Hepatotoxicity in Rats. Pak Vet J, 44(2): 377-383. http://dx.doi.org/10.29261/pakvetj/2024.154

INTRODUCTION

Animals and humans are exposed to multiple pollutants from various sources, including food, water, and air. The bioaccumulation of chemicals in animal tissues through the food chain and drinking contaminated water are the main sources of animal and human exposure (Josende *et al.,* 2019). Therefore, in recent years, more consideration has been given to the interaction of chemicals with each other and their influence on animals and human health (Mahajan *et al.,* 2018). After their presence in the environment, these nanomaterials become capable of interacting with existing chemicals, especially heavy metals like cadmium, lead, arsenic, etc. Interactive exposure to these chemicals causes nutritional inadequacy, hormonal imbalance, cancer and autoimmune diseases (Andjelkovic *et al.,* 2019).

Due to their antimicrobial characteristics, silver nanoparticles (AgNps) are widely used in food technology, consumer goods, textiles, and healthcare (Gokulan *et al.,* 2018). The use of AgNps in drug delivery and therapy makes it essential to examine the possible cytogenotoxicity consequences linked to its capability to liberate free silver ions in the living system (Bouwmeester *et al.,* 2011). Arsenic (As) is a toxicant metalloid and cancer-causing trace element in the lithosphere resulting from anthropogenic and natural sources (Brinkel *et al.,* 2009). These silver ions and arsenic can produce excess reactive oxygen species (ROS) in the bodies of living organisms and damage different organs, including the skin, lungs, kidneys, and liver (Olugbodi *et al.,* 2023). The production of ROS causes inflammation, lipid peroxidation, mitochondrial and DNA damage, induction of apoptosis and changes in

the expression of antioxidative genes like SOD, GPx, CAT, etc. (Nowack *et al.*, 2011).

Moringa oleifera is a fast-developing deciduous shrub. It is broadly utilized as a source of nourishment and as a customary medication for treating different ailments like hypertension, diabetes, and cancer (Akunna *et al.*, 2012). *Moringa oleifera* embraces specific phytochemicals with established powerful antioxidant properties like antitumor, antidiuretic, anti-inflammatory, hypolipidemic, and hypocholesterolemic characteristics (Moodley, 2017). These properties make it a potential candidate for checking its antioxidant activities against toxicity created by heavy metals and nanoparticles.

The toxic effects of silver nanoparticles and heavy metal arsenic are individually known in mammals. However, there is a lack of research data about the chronic interactive influence of chemicals on the oxidative stress and histopathological parameters of mammalian model. Moreover, this is the first study that provides a comprehensive approach to the protective role of aqueous extract of *Moringa oleifera* leaves, which may influence oxidative stress and other parameters in rat tissue (liver) due to its antioxidant properties.

MATERIALS AND METHODS

Chemicals: Sodium Arsenite ($NaAsO_2$) was procured from (Merck, USA). Silver Nanoparticles (AgNps) powder of 20 nm diameter (CAS# 7440-22-4) was purchased from (Hongwu International Group Ltd) China.

Preparation of plant extract: The *Moringa oleifera* leaves were identified, collected, shaded dry, and pulverized into a fine powder using a laboratory homogenizer sealed into airtight plastic packets. The moringa aqueous extract of 2% and 3 % (2g and 3g of moringa leaf powder in 98ml and 97ml of distilled water, respectively) was prepared by adopting the procedure of (Nwamarah *et al.*, 2015). The mixture was kept overnight on an orbital shaker with constant stirring at room temperature. The extract was then filtered using Whatman filter paper and preserved in a refrigerator maintained at 4°C and administered to animals upon dosing.

Experimental design: Sixty-three Sprague Dawley rats weighed 105 ± 5 g were distributed into seven groups (n=9) each**.** The experiment was carried out in the animal house of the zoology department, Lahore College for Women University, Lahore, Pakistan, for three months under normal nutritional and environmental conditions. The group I control (C) administered distilled water with rodent feed. The rats in groups II and III received 2% and 3% of Moringa leaf extract, respectively. All the animals of group IV were supplemented with low doses of (AgNPs) 100mg/Kg and (As) 10.25 mg/Kg. Group V received a high dose of (AgNPs) 150 mg/Kg and (As) 16.4 mg/Kg. The VI and VII groups comprised low and high doses of silver nanoparticles, arsenic, and moringa, as described above. All rats received the treatment orally and daily for three months.

Collection of samples: Animals were euthanized after the 4^{th}, 8^{th} and 12^{th} week of the experiment. Blood samples (2 ml) for serum were collected through cardiac puncture into sterile glass tubes and allowed to stand for 30 min at room temperature. The serum was separated by centrifugation and stored at -20°C. After blood collection, three rats from each group were dissected. 100 mg of liver tissue was homogenized in 1 ml of HEEPS extract buffer. Tissue homogenate was collected by centrifuging the mixture for 15 minutes at 12000 rpm for histochemical analysis.

Hepatic markers estimation: Serum albumin, total protein, Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Alkaline Phosphatase (ALP) levels were evaluated following laboratory protocols using standard TERSACO lab kits, Switzerland.

Determination of oxidative stress marker (TBARS): Tissue lipid peroxidation was assessed by measuring the activity of the Thiobarbituric acid reactive substances (TBARS) following the procedure of Ohkawa *et al.* (1979). TBARS was calculated by using molar extinction coefficient of 1.56 × 105 M/cm.

nmTBRS/mg protein O. D x sample volume x total volume x 1.56 x 105 x mg protein/ml

Estimation of oxidative stress and antioxidant parameters: However, antioxidant enzyme catalase (CAT) activity was determined using the method of Aebi (1984). The technique of Klibet *et al.* (2016) had been used to evaluate the activity of superoxide dismutase (SOD). The methodology of Flohé and Günzler (1984) was used to estimate glutathione peroxidase activity (GPx). However, the activity of reduced glutathione (GSH) was evaluated through the procedure of Ellman (1959).

Histological examination: After dissection, liver tissue samples were fixed in a 10% formalin solution following the dehydration in ascending grades (80, 90 and 100%) of alcohol, clearing in xylene and then embedded in paraffin wax. Thin slices (5-6 µm) of tissues were cut out using a microtome and stained with haematoxylin-eosin (H&E). A binocular compound microscope (Optika B-150) was used for the microscopic examination of hepatic tissue. The observation and capturing of images of hepatic tissue were performed through Future Winjoe software.

Statistical analysis: Values expressed as Mean±SEM. One-way ANOVA followed by the Post Hoc Tukey test and two-way ANOVA followed by Bonferroni post-test were used to compare groups. P-values were < 0.05 and < 0.001.

RESULTS

Effect of MOLE, AgNps and Arsenic on albumin and total protein: The serum albumin and total protein levels remarkably declined ($P<0.001$) due to (AgNps+As) administration at low and high dose levels when compared to control and moringa-treated groups in a dose-dependent manner. While (AgNps+As+M) low and high dose coadministration significantly increases

Table 1: Protective effects of *Moringa oleifera* leaves extract against silver nanoparticles and arsenic induced changes in biochemical markers of liver after 4th, 8th and 12th weeks of treatment

Treatments	4th Week Biochemical Parameters				
	Total Protein (g/L)	**Albumin (g/L)**	**ALT (IU/L)**	**AST (IU/L)**	**ALP (IU/L)**
Control(C)	5.86±0.04	3.12±0.04	52±0.57	123.67±0.88	61.00±0.57
M(L)	6.23±0.02**	3.45±0.02**	47±0.88**	121.33±0.88	53.33±0.88**
M(H)	7.22±0.03**	4.34±0.03**	42±1.15**	117.33±0.88**	46.00±1.73**
(AgNps+As)L	3.61±0.04**	2.05±0.02**	125±0.57**	282.00±0.57**	107.00±1.15**
(AgNps+As)H	2.71±0.03**	1.19±0.02**	132±0.57**	288.67±0.88**	115.33±1.45**
(AgNps+As+M)L	3.85±0.02**	2.25±0.02**	87±0.57**	151.33±1.20**	74.67±1.76**
(AgNps+As+M)H	4.42±0.03**	2.46±0.01**	79±0.57**	139.00±0.57**	84.00±1.73**
Treatments	8th Week Biochemical Parameters				
	Total Protein (g/L)	**Albumin (g/L)**	**ALT (IU/L)**	**AST (IU/L)**	**ALP (IU/L)**
Control(C)	6.62±0.02	3.24±0.02	56.00±0.57	128.00±0.57	65.33±0.88
M(L)	7.49±0.03**	3.66±0.01**	51.67±1.20*	125.00±0.57**	57.33±0.88**
M(H)	7.87±0.03**	4.58±0.01**	44.67±0.88**	121.67±0.88**	51.00±1.52**
(AgNps+As)L	4.63±0.03**	2.16±0.01**	128.00±0.57**	284.33±0.88**	113.00±1.45**
(AgNps+As)H	3.37±0.02**	1.35±0.01**	133.67±0.88**	292.33±0.88**	125.33±1.45**
(AgNps+As+M)L	5.33±0.03**	2.36±0.01**	89.00±0.57**	154.33±1.20**	82.00±1.15**
(AgNps+As+M)H	6.22±0.03**	2.56±0.01**	80.67±0.88**	141.67±0.88**	87.67±0.88**
Treatments	12th Week Biochemical Parameters				
	Total Protein (g/L)	**Albumin (g/L)**	**ALT (IU/L)**	**AST (IU/L)**	**ALP (IU/L)**
Control(C)	7.79±0.05	3.36±0.01	60.33±0.88	131.67±0.88	69.00±0.57
M(L)	8.42±0.02**	3.85±0.04**	54.67±0.88**	127.67±0.88**	60.67±1.45**
M(H)	8.58±0.05**	4.85±0.04**	49.33±0.88**	123.00±1.15**	54.33±1.45**
(AgNps+As)L	5.41±0.08**	2.26±0.01**	131.33±0.88**	286.00±1.52**	126.00±1.73**
(AgNps+As)H	4.72±0.03**	1.48±0.02**	135.67±0.88**	294.67±0.88**	135.33±1.45**
(AgNps+As+M)L	6.46±0.04**	2.46±0.01**	92.00±0.57**	158.00±0.57**	87.33±0.88**
(AgNps+As+M)H	7.41±0.03**	2.66±0.01**	82.67±0.88**	145.33±0.88**	94.67±0.88**

Control(C) 0g/kg; M(L) 2%; M(H) 3%; (AgNps+As)L 100 mg/kg+10.25 mg/kg; (AgNps+As)H 150 mg/kg+16.4 mg/kg; (AgNps+As+M)L and (AgNps+As+M)H at low and high doses described in above treatments. Each value represents mean ± SEM, n = 3. p value < 0.05= *, < 0.001= **.

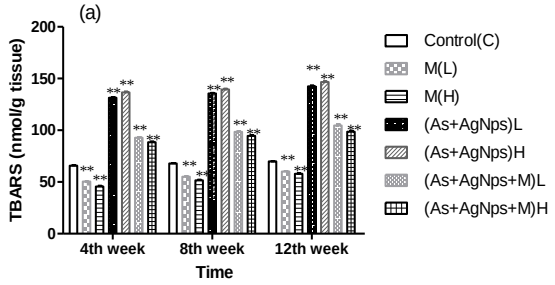
(P<0.001) the albumin and total protein activity in contrast to (AgNps+As) treated low and high dose groups. However, moringa supplementation alone normalized (P<0.001) the level of these parameters at both dosages when compared to control in successive 4th, 8th and 12th weeks of treatment (Table 1).

Effect of MOLE, AgNps and Arsenic on hepatic markers: The serum hepatic markers ALT, AST and ALP levels were augmented significantly (P<0.001) in the low and high doses of (AgNps+As) intoxicated rats when compared to control and moringa-treated groups. While, moringa co-treatment along with silver nanoparticles and arsenic (AgNps+As+M) at both dose levels significantly (P<0.001) improved hepatic markers in contrast to the (AgNps+As) intoxicated low and high-dose groups. However, moringa treated group at both dose levels considerably (P<0.001) lowered hepatic enzyme levels compared to the control in the 4th, 8th and 12th weeks of the experiment (Table 1).

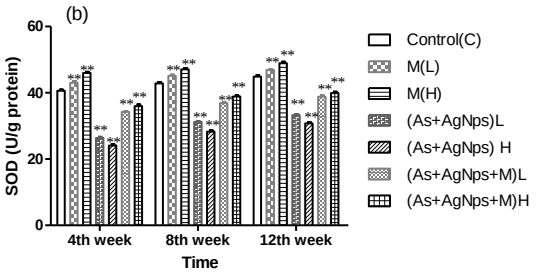
Effect of MOLE, AgNps and Arsenic on oxidative stress marker: Silver nanoparticles and arsenic exposure significantly (P<0.001) elevated the TBARS activity at low and high dose levels compared to the control. However, TBARS level was considerably depleted (P<0.001) after the co-treatment of (AgNps+As+M) at both dosage groups when compared to (AgNps+As) low and high dose groups. Moreover, Moringa alone administration significantly (P<0.001) reduced TBARS activity at both dosages when compared to control after the 4th, 8th and 12th weeks of treatment (Fig. 1 a).

Effect of MOLE, AgNps and Arsenic on antioxidant enzymes: Additionally, a striking decrease (P<0.001) in hepatic CAT, SOD, GPx and GSH levels were observed in AgNps+As intoxicated low and high dosage groups when compared to control and moringa-treated groups. However, coadministration of (AgNps+As+M) at low and high dose groups substantially improved (P<0.001) the antioxidant activities in contrast to (AgNps+As) intoxicated low and high dose group rats. However, moringa alone supplementation significantly (P<0.001) retained the normal activity of enzymes in comparison to a control group in each following 4th, 8th and 12th weeks of the experiment (Fig. 1 b, c, d and e).

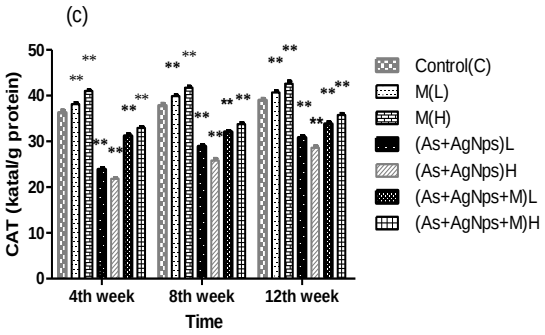
Effect of MOLE, AgNps and Arsenic on hepatic histology: Histopathological analysis of the control and moringa-treated low and high-dose groups represented the liver's normal structure. All these groups exhibited a regular lobular morphology with hepatocytes placed in cords, revealed proper vesicular structure, having apparent round one or two nuclei and rarely spaced hepatic sinusoids with a precise pattern of Kupffer cells. Perisinusoidal space (space of Disse) situated between the layers of hepatocytes and the sinusoidal endothelial cells was normal in appearance (Fig. 2 A4, A8, A12, B4, B8, B12 and C4, C8 & C12). However, histological studies showed that (AgNps+As) treatment at low and high dose levels resulted in severe hepatic injury including widespread deterioration of hepatic cord and hepatocytes with apoptosis and necrosis, vacuolization of Kupffer cells, distortion and dilatation of sinusoidal spaces and endothelial lining, vacuolated hepatocyte, nuclear pyknosis, amyloid, degenerated fatty change along with glycogen accumulation inside hepatocytes when compared with moringa treated and control rats (Fig. 2 D4, D8, D12, E4, E8 & E12). The histological alterations produced due to (AgNps+As+M) treatment at both levels were mitigated in the liver of rats given MOLE besides some degenerative changes caused by interactive exposure to chemicals after the 4th, 8th, and 12 weeks of treatment (Fig. 2 F4, F8, F12, G4, G8 & G12).



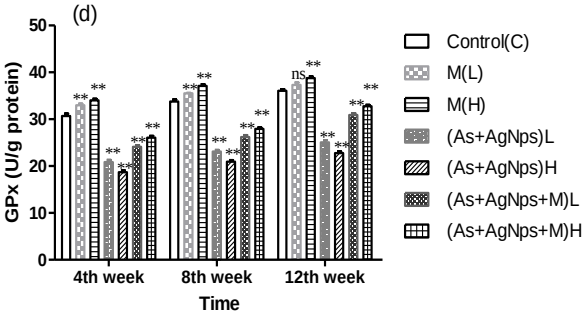
** showed *p<0.001* (highly significant) difference among control and all treatment groups



*** showed highly significant (p<0.001) difference among control and all treated groups*



** showed highly significant *(p<0.001)* difference between contril and all treatment groups



** showed highly significant *(p<0.001)* difference among control and treated groups
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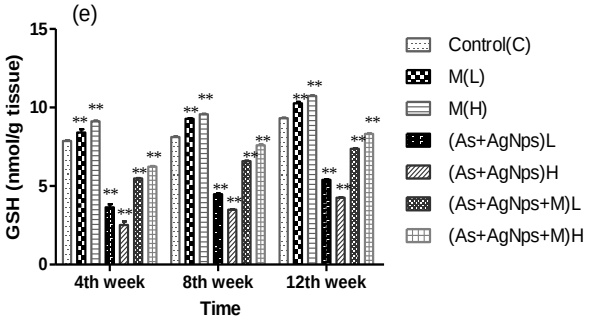

** showed highly significant (p<0.001) difference among control and treated groups

Fig. 1: Changes in oxidative stress (a) TBARS and antioxidant enzymes parameters (b) SOD, (c) CAT, (d) GPx, (e) GSH during three months treatment of silver nanoparticles, arsenic and moringa.

DISCUSSION

Pollution raises the levels of hazardous compounds in the environment, negatively impacting the lives of humans and animals. Silver nanoparticles (Panyala *et al.*, 2008) and sodium arsenite are ubiquitous chemical pollutants that contaminate the environment (Jang *et al.*, 2016). The liver is a vital organ for metabolism and detoxification, and the activity of the liver enzymes in the blood determines the liver's functional integrity (Lala *et al.*, 2022). According to the current study, interactive exposure to silver nanoparticles and arsenic indicated a considerable escalation in the ALT, AST and ALP levels besides decreased levels of albumin and total protein at both dose levels after the 4th, 8th, and 12 weeks of the experiment. Similar findings have been documented by other researchers (Shafik and El Batsh, 2016; Ramadhan and Ghareeb, 2021), where turbulences in the concentrations of albumin, total protein and liver enzymes reflect the diseased and pathological status of the liver. These alterations might be because, after hepatic damage, enzymes leak from the cytoplasm of the hepatocyte into the bloodstream, causing transaminases to rise (Hamza *et al.,* 2020).

Silver nanoparticles, arsenic and moringa co-treatment at both low and high-dose groups, and moringa treatment alone lowered the ALT, AST, and ALP levels by reducing hepatocellular damage in each consecutive 4th, 8th and 12th week of treatment. Our findings aligned with previous researchers' work, which found that oral administration of this plant extract could protect hepatocytes from injury (Sheikh *et al.,* 2014; Toppo *et al.,* 2015). One of the fundamental mechanisms through which these toxicants operate is oxidative stress, caused by increased free radical formation and an impaired antioxidant defense system (Ozougwu, 2016).

Previous studies have confirmed that silver nanoparticles (Miethling-Graff *et al.,* 2014) and arsenic cause oxidative impairment and several ailments in the liver and other body organs (Altikat *et al.,* 2015). Our results showed that after interactive exposure to these chemicals at both dose levels in each subsequent three-month treatment, a dramatic decrease in CAT, GPx, SOD, and GSH activities were observed. However, there was a striking increase in TBARS levels, suggesting lipid peroxidation, which causes oxidative stress. Hepatic functions decline due to an imbalance between oxidative stress markers and

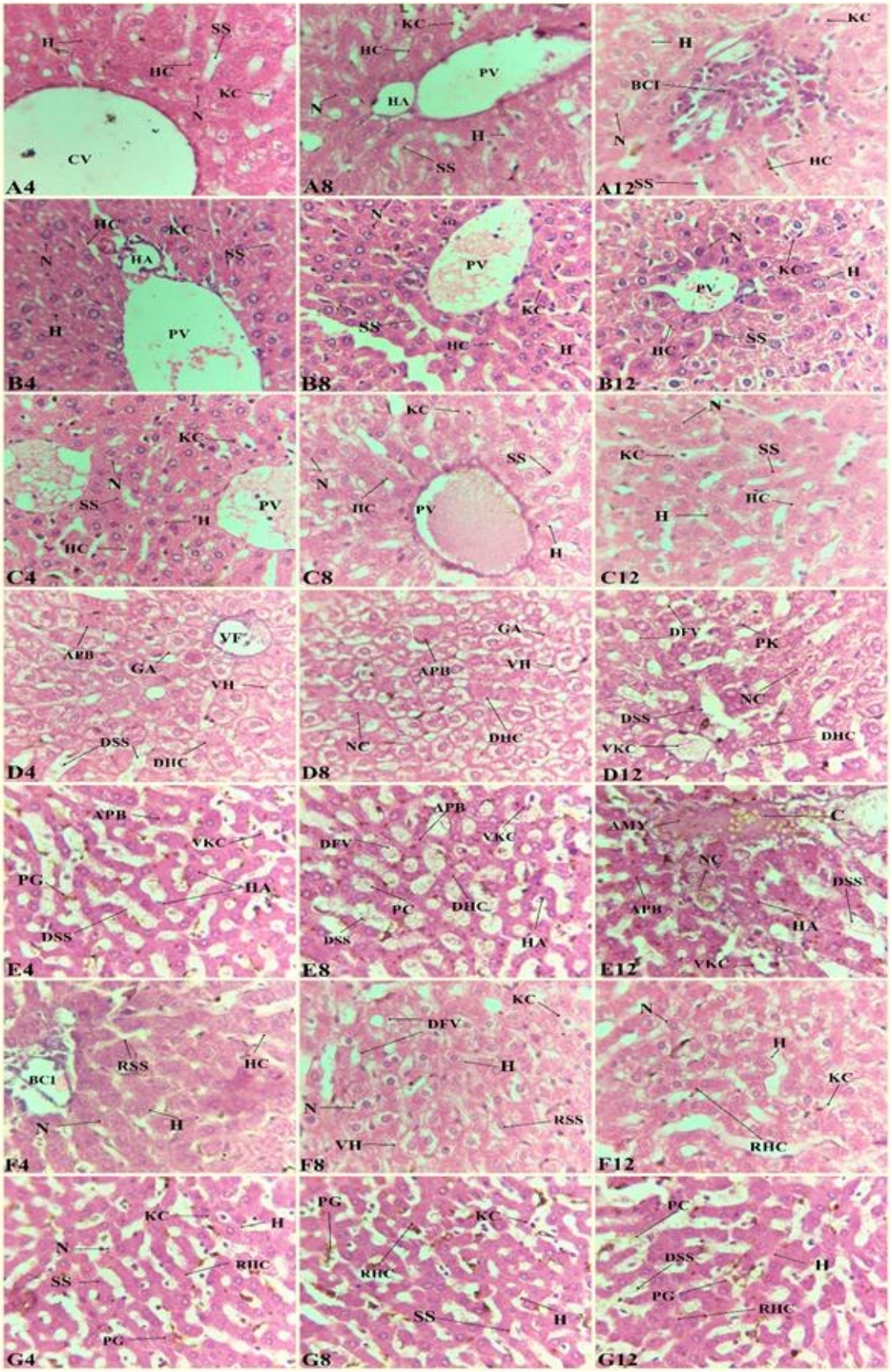

Fig. 2: Ameliorative impact of MOLE on silver nanoparticles and arsenic-induced histological impairments in liver tissues after 4th, 8th and 12th weeks (H & E/400X). Control(C) group (A4, A8, A12), M(L) B4, B8, B12 & M(H) C4, C8, C12; (AgNps+As)L group D4, D8, D12; (AgNps+As)H group E4, E8, E12; (AgNps+As+M)L group F4, F8, F12; (AgNps+As+M)H group G4, G8, G12), CV: Central Vein, H: Hepatocytes, HA: Hepatic Artery, HC: Hepatic Cord, KC: Kupfer Cells, VKC: Vacuolated Kupfer cells, N: Nucleus, PV: Portal Vein, SS: Sinusoidal Spaces, GA: Glycogen Accumulation, PG: Pigmentation, VH: Vacuolated Hepatocyte, DHC: Degenerated Hepatic Cord, AMY: Amyloid, APB: Apoptotic Body, NC: Necrotic Change, HAT: Hepatic Atrophy, DSS: Dilated Sinusoidal Spaces, PC: Protein Cast, RHC: Restored Hepatic Cord, BCI: Blood cell infiltration, DFV: Degenerative Fatty vacuole, VF: Vacuole Formation.

antioxidant enzyme activity (Li *et al*., 2015), which might lead to an increase in reactive oxygen species in the mitochondria of hepatocytes (Arroyave-Ospina *et al.,* 2021). Antioxidant enzymes like CAT SOD and GPx protect by removing the free radicals from the liver.

Peñalver *et al*. (2022) revealed that the antioxidant potential of moringa is associated with the occurrence of various antioxidant chemicals in its structure. The current analysis revealed that MOLE subjunction alone and with interactive exposure to chemicals at both low and high dose levels curiously lower the level of TBARS besides sustaining the normal levels of antioxidant enzymes when compared with (AgNPs+As) treated low and high dose groups after 4^{th}, 8^{th} and 12 weeks of the experiment. Due to its antioxidant characteristics, it is inferred that moringa might be utilized to lower the amount of oxidative stress.

The histomorphology of the liver was almost normal, like that of control in MOLE-administered rats, after the 4^{th}, 8^{th}, and 12^{th} weeks of the experiment. A similar finding about normal liver structure has been reported by Loha *et al.* (2019). Hepatic parameters such as hepatic atrophy, congestion, dilation and deterioration of sinusoidal spaces, pigmentation and degenerative fatty changes with hepatocyte cytoplasmic vacuolation were key indices of hepatic morphology observed in low and high doses of (AgNPs+As) treated groups after 4^{th}, 8^{th}, and 12 weeks treatment. Our findings about disordered hepatic architecture align with the study of Dkhil *et al.* (2020) in arsenic and silver nanoparticles (Yousof *et al.,* 2022).

These degenerative fatty changes produce a disorder of lipid metabolism. It may be assumed that the liver is impotent to transfer fat promptly, thus instigating fat to gather in hepatic cells and resulting in a fatty liver. Fat accretion in the liver disturbs hepatic function and abolishes liver cells, eventually lowering the liver's compensatory capability and triggering hyperplasia of connective tissue, liver damage and cirrhosis (Shtriker *et al*., 2018). The necrosis of hepatocytes produces many inflammatory components that further cumulate the generation of free radicals. In our study, The moderation of these parameters by the concomitant administration of (AgNPs+As+M) at both low and high dose levels in each successive three months is in agreement with the findings of others in arsenic (Ibrahim, 2007), silver nanoparticles (Mahmoudian *et al.,* 2016) and in Moringa (Abd Eldaim *et al*., 2017).

Therefore, this study revealed the effectiveness of *Moringa oleifera* leaf extract in lowering silver nanoparticles and arsenic harmfulness in rat models and can prove beneficial therapeutically in the future to reduce or avert the harmful impacts of toxic compounds in animals.

Conclusion: The combined exposure of chemicals enhances the risk of oxidative damage and disturbs the hepatic enzyme parameters, resulting in hepatocellular damage, liver tissue necrosis, and degenerative fatty changes, as observed in the histopathological examination. These degenerative fatty changes are key risk factors for cerebrovascular and cardiovascular diseases. Therefore, there is a need for the development of effective, affordable, and accessible therapies to help the vast majority of patients who have been exposed to heavy metals and nanomaterials. The findings of this study suggested that moringa leaf extract could be helpful therapeutically in the future as cost-effective cheap biomedicine, particularly in prevention and in diseased conditions.

Authors contribution: SS conceived and designed the idea of the study and helped in the analysis of data and writing of the manuscript. FB executed the experiment, analyzed the blood and tissue samples, wrote the manuscript, and performed data analysis. SN helps in methodology and study design. FM and FR reviewed and revised the manuscript. All the authors approved the manuscript before submission.

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