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Experimental Trial to Reduce Toxicity of Heavy Metals Mixture by Ferulic Acid: Histopathological and Biochemical Studies on Rats

Rasha A. Shalaby, Abeer F. Ahmad and Gihan M. Hammoud

Regional Center for Food and Feed (RCFF), Agricultural Research Center (ARC), Giza, Egypt.

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ABSTRACT

The issue of toxic substance in our food and intervention trial has started floating on the surface. Heavy metals (HMs) are persistent non-degradable globally wide environmental contaminants and pollutants of tremendous adverse effect on health. Seven metals (Al, Pb, As, Hg, Ni, Cr and Cd) were previously recorded in Baladi bread from rural area in Egypt. Biological experiment was conducted in order to evaluate the dynamic toxic interaction of those metals along with ameliorating effect of ferulic acid (FA, natural polyphenol). Two groups of male albino rats were fed on heavy metals mixture (HMMs) at dose level detected in bread and 1/10 of $LD_{50}$of same metals with total corresponding salts of 215.7 mg/kg diet and 284.7 mg/kg body weight (bw), respectively for 12 weeks. The systemic toxicity of HMMs was manifested by anemia, immunosuppression, decrease of blood ferritin, Zn and Fe levels, impairment of liver and kidney functions and histopathological alteration of liver, kidney, brain and intestine associated with oxidative stress. These effects were more pronounced with1/10 of LD_{50} dose. Fortunately, co treatment of intoxicated rats orally with ferulic acid (40 mg/kg bw) resulted in significant amelioration of adverse effect of HMMs on biological system through reduction of oxidative stress and restoration of antioxidant defense of living body.

Keywords: Ferulic acid, heavy metals, toxicity, rats.

1. Introduction

Since late of 20th century detection of food contaminants and designing mitigation strategies has become scientist's great concern. Heavy metals (HMs) are natural constituent of earth's crust which extensively mining and massively used in various industries and leaded fuel causing public health threats in developed and developing countries (Lu *et al.,* 2017 and Anyanwu *et al.,* 2018). HMs contaminate food, water and air through many sources (ground water, industrial effluents, sewage discharge, suspended particles and during fuel and coal combustion) causing acute and chronic poising (Wu *et al.,* 2016 and Balali-Mood *et al.,* 2021). Al (aluminum), As (arsenic), Cd (cadmium), Cr (chromium), Cu (cupper), Hg (mercury), Ni (nickel) and Pb (lead) are recorded as most environmental heavily loaded contaminants which are persistent, not easily excreted from body and bioaccumulated in different body organs leading to increase their concentration and hence tremendous damages (Cooper *et al.,* 2017; Masindi and Muedi, 2018 and Yokel and McNamara, 2001). Recently, researchers focused on assessment of toxicological interaction of heavy metals mixture (HMMs) rather than one element (Lin *et al.,* 2016). HMs are reported to induce hematological abnormalities, renal and liver damage, neurological and reproductive disruption and genetic alterations (Kenston *et al.,* 2018; Balali-Mood *et al.,* 2021 and Choudhuri *et al.,* 2021) mainly through binding with vital macromolecules and DNA, induction of reactive oxygen species (ROS), depilation of antioxidant defense system and excises production of lipid peroxidation, disruption of damage- repair process and oxidative stress (Jan *et al.,* 2015 and Balali-Mood *et al.,* 2021).

Seeking safe natural plant derived antioxidant to use as remedy for many oxidative stress disorders has become a modern obsession and produced and promoted by pharmaceutical companies as a dietary supplement.

Corresponding Author: Rasha A. Shalaby, Regional Center for Food and Feed (RCFF), Agricultural Research Center (ARC), Giza, Egypt. E-mail: rasha_shalaby@hotmail.com

Ferulic acid (FA) is a versatile antioxidant molecule found in abundant plant tissues such as grain, fruits and vegetables and popular food and beverages as popcorn, whole grain, corn, pasta, fruits juice and coffee (Kumar and Pruth, 2014 and Gohil *et al*., 2012). FA a peerless polyphenol which possess a substantial antioxidant and pharmaceutical properties and recorded to be beneficial in reliving many oxidative stress related injuries such as metabolic syndrome (Zhang *et al.,* 2022), liver, kidney, intestinal and brain damage (Gohil *et al.,* 2012; Hammoud *et al.,* 2018 and Tawfik *et al.,* 2018) and cardiotoxicity (Pandi *et al.,* 2022).

In our previous study, Shalaby *et al.,* (2022) compared the HMs content in Baladi bread in Egypt from multi district using different sources of fuel (wood, compressed natural gas and solar) and found that, bread produced by wood fuel have the highest HMs content. Herein we investigate the toxic interaction of seven HMs found in bread (based on previous evidence from last work (Shalaby *et al.,* 2022) as well as 1/10 of LD_{50} in order to evaluate accumulative effect of HMs of same metals on rats hematological and biochemical parameters and liver, kidney, brain and intestine histology. Also assess the antioxidant power of FA as intervention trial to inhibit HMs toxicity.

2. Materials and Methods

2.1. Chemicals

Heavy metals **(**Aluminum Chloride ($AlCl_3$), Analytical Reagent (AR) ≥ 99.0%; Lead Acetate ($Pb(C_2H_3O_2)_2$), AR ≥ 99.5%; Sodium Arsenate (Na_3AsO_4); Mercuric Chloride ($HgCl_2$), AR ≥ 99.5%; Nickle Pure (Ni); Potassium dichromate (K_2Cr2O_7), AR ≥ 99.8%; and Cadmium dichloride ($CdCl_2$) AR ≥ 99.0%, all analytical grade), ferulic acid (purity >98%) and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Kits used in determination of lipid peroxidation product (malondialdehyde, MDA), total antioxidant capacity (TAC) were purchased from Biodiagnostic, France. While ELISA kits used to determine ferritin supplied by San Francisco, CA, USA.

2.2. Animal' husbandry and Experimental protocol

Sixty adult male albino rats (150±10g) were supplied and housed by Food Technology Research Institute (FTRI), Agricultural Research Center, Giza, Egypt. Animal house facility and accommodations were confirmed with Directive of the European Counsel 86/609/EEC (1986) regarding protection of experimental animals. Rats were housed under controlled environmental condition (12-h light/dark cycle and 22±2^{O}C) and supplied with basal diet (prepared according to guidelines of National Research Council, 1995) and deionized water throughout adaptation period (2 weeks) and experimental period (12 weeks).

Animals were randomized and six groups were formulated (10 rats in each) as follow:

G1 (vehicle treated): served as a control and received basal diet + 3 ml DMSO: deionized water (1:3) /kg bw daily by gavage.

G2 (FA): rats received daily dose of FA 40 mg/kg bw by gavage dissolved in 3 ml DMSO: deionized water (1:3) /kg bw (Hammoud *et al.,* 2018).

G3 (bHMMs)

Rats received HMMs (215.7 mg/kg diet) as a recommended dose of Shalaby *et al.,* (2022) represent the following metal salts: aluminum chloride, lead acetate, sodium arsenate, mercuric chloride, nickle pure, potassium dichromate and cadmium dichloride (208.563, 0.644, 2.741, 0.218, 0.091, 3.423 and 0.006 mg/kg diet, respectively) +3 ml DMSO: deionized water (1:3) /kg bw daily by gavage.

G4 (bHMMs+FA): rats received HMMs + FA at same previous dose levels.

G5 (1/10 LD_{50})

Rats received 1/10 of LD_{50}/kg bw of HMMs (284.7 mg/kg bw) in diet with following concentration: aluminum chloride, lead acetate, sodium arsenate, mercuric chloride, nickle pure, potassium dichromate and cadmium dichloride (40 (Abdel-Wahab, 2012), 200 (Zhu *et al.,* 2014), 10(Zheng, 2012), 5.5 (Zhu *et al.,* 2014), 3.82 (Toya *et al.,* 1997), 9.6 (Zhu *et al.,* 2014) and 15.8 (Zhu *et al.,* 2014) mg/kg bw, respectively) + 3 ml DMSO: deionized water (1:3) /kg bw daily by gavage.

G6 (1/10 LD_{50}+FA)

Rats received 1/10 of LD50 of HMMs + FA at same previous dose levels.

At the end of experimental period (12 weeks) rats were starved and blood samples were collected from rat's eye (retro-orbital venous plexus) under CO_2 anesthesia. Aliquot of fresh blood were collected from

each rat into two heparinzed tubes, one for further hematological analysis and zinc (Zn) and iron(Fe) determination and other kept in order to obtain plasma for MDA, TAC determination. Other aliquot of blood were collected in tubes with no heparin to obtain serum which separated by centrifugation and kept at -20ºC for biochemical analysis and ferritin.

2.3. Hematological analysis

Automated Hematology Analyzer 5 (XT-2000i, Sysmex Corporation, KOBE, JAPAN) was assigned to determine Red Blood Cells count (RBCs), White Blood Cells count (WBCs), hemoglobin (Hb), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and platelets(PLT) in fresh blood.

2.4. Biochemical analysis

Biochemical Blood Analyzer (Alfa Wassermann Dignostic Technologies, LLC, ACE, Alera, USA) was assigned to determine serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), albumin (Alb), urea and creatinine.

2.5. Zn and Fe concentration

Zn and Fe in whole blood were analyzed using Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) – (Optima 2000 DV- Perkin Elmer, Germany) and guideline of methods provided by AOAC (2012).

2.6. Estimation of serum Ferritin: Serum ferritin level was quantitated by ELISA kits anti-ferritin according method described by Zhang *et al.,* (2021).

2.7. Estmiation of plasma MDA and TAC

Malondialdehyde (MDA, a lipid peroxidation product) was estimated by spectrophotometer in serum at 534 nm according to method of Onkawa *et al.,* (1979). While TAC was estimated spectrophotometrically in plasma at 593 nmaccording method described by Benzie and Strain (1996).

2.8. Tissue specimen and Histopathological studies

After blood sampling rats were sacrificed by cervical dislocation and dissected. Livers, kidneys, brains and intestine were removed and washed by normal saline then fixed in 10% buffered formalin. The tissues were subjected to histological processing routine described by Banchroft *et al.,* (1996) then stained with haematoxylin and eosin (H&E) and examined under light microscope.

2.9. Statistical analysis

Results of current studies were introduced in form of mean±SE and statistically analyzed using Duncan and least significant difference test (LSD, at 5% of probability) (Snedecor and Cochran, 1980) by CoStat program (version 6.400 1998-2008 CoHort software).

3. Results and Discussion

Exposure to multiple HMs simultaneously could be more hazardous than single metal and resulted in massive toxicity and serious oxidative stress on body organs of human and animals (Lin *et al.,* 2016 and Choudhuri *et al.,* 2021). Usually human exposed to heterogeneous metal mixtures pollutants; therefore, herein we aimed to assess the toxicodynamic interaction of seven HMs which reported earlier in Baladi bread by Shalaby *et al.,* (2022) as well as role of FA in detoxification of HMMs.

It worthy to note that oral administration of FA for 12 weeks (G2) didn't alter any of tested parameters comparing with control ($P<0.05$).

3.1 Hematological analysis

Tabulated results in table (1) revealed that administration of rats with HMMs and 1/10 of LD_{50} of HMMs for period of 12 weeks (G3 and G5) resulted in significant and highly significant reduction of RBCs, WBCs, Hb, MCHC, MCV), MCH and PLT count, respectively comparing with control (G1) ($P<0.05$). However, simultaneous administration of HMMs-intoxicated rats with FA (G4 and G6)

resulted in significant amelioration of adverse effect of HMMs on hematological parameters and was able to restore RBCs in blood of both intoxicated group (G4 and G6) and Hb, MCHC and PLT count in blood of bHMMs group (G4) to normal, comparing with control (*P<0.05*).

Table 1: Hematological parameters in blood of control and treated rats (means ± SE).

Parameters / Groups	RBCs (10^6/µL)	WBCs (10^3/µL)	Hb (g/dl)	MCV (fL)	MCH (pg)	MCHC (g/dl)	PLT (10^3/µL)
G 1 (Control)	8.30±0.04^{a}	16.08±0.33^{a}	15.10±0.20^{a}	53.68±0.55^{ab}	19.66±0.17^{ab}	37.82±0.19^{ab}	646.00±16.93^{a}
G 2(FA)	8.33±0.05^{a}	16.20±0.29^{a}	15.22±0.23^{a}	54.56±0.68^{a}	19.88±0.22^{a}	37.92±0.23^{a}	660.00±17.85^{a}
G 3 (bHMMs)	7.81±0.04^{b}	9.86±0.30^{d}	13.56±0.18^{c}	46.74±0.32^{d}	17.58±0.16^{d}	36.66±0.16^{c}	525.60±15.48^{c}
G4 (bHMMs+FA)	8.24±0.04^{a}	13.40±0.30^{b}	15.02±0.19^{a}	52.42±0.55^{b}	19.26±0.15^{b}	37.64±0.20^{ab}	623.60±14.96^{ab}
G5 (1/10 LD_{50})	7.27±0.05^{c}	8.08±0.24^{e}	11.96±0.29^{d}	40.28±0.57^{e}	16.70±0.22^{e}	34.98±0.22^{d}	436.00±15.31^{d}
G6 (1/10 LD_{50}+FA)	8.19±0.05^{a}	11.64±0.25^{c}	14.34±0.19^{b}	49.34±0.45^{c}	18.34±0.12^{c}	37.24±0.18^{bc}	592.20±12.77^{b}
$LSD_{0.05}$	0.13	0.84	0.62	1.55	0.51	0.58	45.70

Within the same column, various superscript letters indicate significant differences (Duncan, *P <0.05*).

These finding strongly suggested that, exposure to HMMs can cause anemia, immunosuppression, and coagulation disorder. Such abnormalities in hematopoietic system were noted in male and female rats treated with eight HMs (Zn, Cu, Mn, Cr, Ni, Cd, Pb and Hg) (Kenston *et al.,* 2018). Oral administration of Cd and Pb mixture resulted in microcytic hypochromic anemia and Lecuocytopenia in rats (Jaiswal *et al.,* 2018 and Andjelkovic *et al.,* 2019). Choudhuri *et al.,* (2021) reported disturbance in hematopoietic system after treatment of rats with chronic low dose of Pb, Cd, and as. The recorded microcytic hypochromic anemia in our study could be explained by destruction of RBCs by most of metals. Once Cd has absorbed, it enter blood stream and bind to plasma albumin causing destruction of RBC membrane and induction of ROS (Pawaiya *et al.,* 1998). Moreover, Cd prevents absorption of Fe from gastrointestinal tract and adversely influencing haem synthesis (ATSDR, 2000). Meanwhile, Pb (which represents a great portion (200 mg/kg bw) of HMMs of G5 in our experimental model) interferes with haem biosynthesis through inhibition of essential enzymes for synthesis process especially, Aminolevulinic Acid Dehydratase (ALAD) and ferrochelatase activity (Klassen, 2001). Also, Fazio *et al.,* (2019) reported hematological changes as a result of toxicity which bind to RBC and WBC upon absorption causing microcytic anemia. The noted anemia in our study could be also attributed to decrease of Fe in blood which noted in current study (table 2) as a result of the depressing effect of aluminum chloride which represent the greatest portion (208.563 mg/kg diet) in HMMs of G3 on Fe level and transferring (Tf) saturation rate (Mahieu *et al.,* 2000). Immunosuppressive effect of HMMs could be related to decrease production of WBCs from lymphoid organs which observed microscopically in spleen by Jaiswal *et al.,* (2018) and Jadhav *et al.,* (2007) and/or increase its destruction. Fortunately, in our study FA effectively protect hematopoietic system against HMMs toxicity. Such effect was previously recorded against cyclophosphamide toxicity in mice (Song *et al.,* 2016) and monosodium glutamate toxicity in rats (Hammoud *et al.,* 2018) through enhancing antioxidant status of body and reducing of inflammation (Song *et al.,* 2016).

3.2. Ferritin and Micronutrients in rat blood

Tabulated results in table (2) revealed that the levels of ferritine, Zn and Fe were significantly decreased in blood of G3 and G5 in dose dependant manner comparing with control. While co-treatment of rats with FA and HMMs (G4 and G6) resulting in restoration of their levels especially with lower HMMs dose (G4) comparing with control and corresponding groups (*P<0.05*).

Current study provides evidence on iron-deficiency anemia induced by exposure to HMs (table 1 and 2). Such effects were previously recorded due to decrease absorption of Fe from intestine after intoxication with Al (Mahieu *et al.,* 2000) and Cd (ATSDR, 2000). Our histological finding showed massive alteration of intestinal wall of rats treated with HMMs at both doses which augment this hypothesis. The decrease of Fe was adversely reflected on ferritin concentration whereat ferritin is a blood protein contains Fe (Shalaby *et al.,* 2022). Zn a macronutrient plays an important role in biological system and one of component of metalloenzymes in living cells. It could be antagonized and replaced by Pb (Moshtaghie *et al.,* 2007) and Cd (Satarug *et al.,* 2018). In our intervention strategy, FA alleviates

toxic effect of HMMs on ferritin and micronutrient due to its antioxidant activity and cytoprotective power. FA enhances and regulates defense mechanism of cell such as heme oxygenase-1, heat shock protein 70, and extracellular signal-regulated kinase and inhibits of cytotoxic enzymes such as nitric oxide synthase (Mancuso and Santangelo, 2014).

Table 2: Serum Ferritin and blood Zn and Fe levels in and treated rats (means ± SE).

Parameters Groups	Ferritin (ng/ml)	Zn (mg/L)	Fe (µg/dl)
G 1 (Control)	110.82±9.71^{a}	6.21±0.31^{a}	130.76±4.61^{a}
G 2 (FA)	125.18±10.68^{a}	6.49±0.37^{a}	135.96±4.06^{a}
G 3 (bHMMs)	61.50±8.27^{c}	4.28±0.27^{b}	98.73±3.31^{b}
G4 (bHMMs+FA)	97.65±9.28^{ab}	6.21±0.40^{a}	125.41±2.83^{a}
G5 (1/10 LD_{50})	31.52±7.21^{d}	2.76±0.32^{c}	76.30±3.29^{c}
G6 (1/10 LD_{50}+FA)	79.82±11.99^{bc}	5.99±0.43^{a}	105.68±3.21^{b}
$LSD_{0.05}$	28.21	1.03	10.52

Within the same column, various superscript letters indicate significant differences (Duncan, *P <0.05*).

3.3. Liver function parameters

Obtained data of liver function parameters (table 3) revealed that, feeding of rats of G3 and G5 with HMMs resulted in significant and highly significant increase in AST, ALT and ALP activities, and a significant and highly significant decrease in TP and albumin concentrations, respectively (comparing with control *P<0.05*). Such alterations of liver function parameters were successfully reduced by intragastric intubation of FA, moreover, activity of AST and concentration of albumin in blood of both HMMs groups (G4 and G6) and activity of ALT and concentration of TP in blood of bHMMs (G4) were restored to normal (comparing with control ((*P<0.05*)).

Table 3: Liver function parameters in serum of control and treated rats (means ± SE).

Parameters Groups	AST (U/L)	ALT (U/L)	ALP (U/L)	TP (g/dl)	Alb (g/dl)
G 1 (Control)	82.00±3.39^{cd}	51.80±2.97^{d}	105.20±5.60^{e}	6.44±0.16^{a}	3.72±0.10^{a}
G 2 (FA)	79.60±2.25^{d}	54.40±2.29^{d}	108.40±5.23^{e}	6.36±0.17^{a}	3.78±0.11^{a}
G 3 (bHMMs)	117.80±2.35^{b}	89.00±2.51^{b}	210.00±8.05^{b}	5.40±0.14^{c}	3.20±0.06^{b}
G4 (bHMMs+FA)	85.80±2.35^{cd}	57.60±2.54^{d}	144.20±5.51^{d}	6.22±0.19^{ab}	3.70±0.09^{a}
G5 (1/10 LD_{50})	182.00±3.59^{a}	109.80±3.36^{a}	283.40±7.41^{a}	4.84±0.15^{d}	2.84±0.09^{c}
G6 (1/10 LD_{50}+FA)	88.40±2.31^{c}	75.20±2.24^{c}	182.80±9.65^{c}	5.84±0.14^{bc}	3.56±0.09^{a}
$LSD_{0.05}$	8.073	7.84	20.74	0.47	0.26

Within the same column, various superscript letters indicate significant differences (Duncan, *P <0.05*).

3.4. Histopathological examination of liver

Microscopically, liver of rats from G1 (control, untreated rats) and G2 (FA treated rats) revealed the normal histological architecture of hepatic lobules, normal central vein, portal triad and hepatocytes (Fig. 1). On the other hand, liver of rats from G3 (bHMMs) which treated with dose of HMMs mimic to those found in bread revealed Kupffer cells activation and portal infiltration with inflammatory cells (Fig. 2) and few strands of fibroblasts in the portal triad around slightly dilated bile duct (Fig. 3). Meanwhile, liver of rats from G4 which treated with bHMMs + FA showed slight Kupffer cells activation only (Fig. 4). On contrary, liver of rats from G 5 which treated with 1/10 of LD_{50} of HMMs exhibited focal hepatocellular necrosis associated with inflammatory cells infiltration (Fig. 5), marked cystic dilatation of bile duct (Fig. 6), focal hepatocellular steatosis (Fig. 7), and binucleation of hepatocytes (Fig. 8). Examined sections from G 6 which treated with 1/10 of LD_{50} + FA revealed hepatocellular vacuolations (Fig. 9) and hepatocellular steatosis (Fig. 10).

In our experimental model, HMMs was found to be injurious to liver which concluded by degenerative changes of hepatic tissue and functional disruption. The severity of lesions was found to be correlated to HMMs dose. Similar toxic effects of HMs on liver were observed after administration

of rats with Pb and Cd mixture (Jaiswal *et al.,* 2018 and Andjelkovic *et al.,* 2019), Pb, Cd and As mixture (Choudhuri *et al.,* 2021), Zn, Cu, Mn, Cr, Ni, Cd, Pb and Hg mixture (Kenston *et al.,* 2018), Cr, Mn, Ni, Cu, Zn Cd, Hg and Pb (Wang *et al.,* 2020) and Al (Hammoud and Shalaby, 2019). Such increased concentration of serum ALT, AST and ALP could be attributed to leakage of enzymes into blood as a result of hepatocellular damage (Choudhuri *et al.,* 2021).

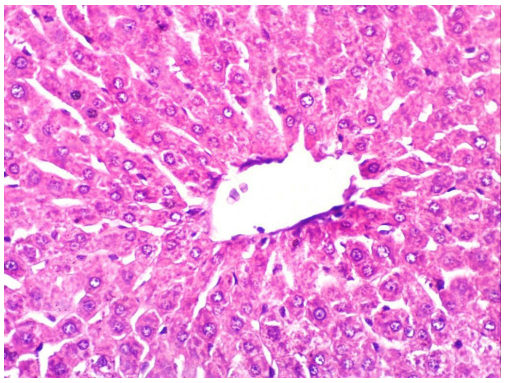

Fig. 1: Liver of rat from G2 showing the normal histological structure of hepatic lobule (H & E X 400).

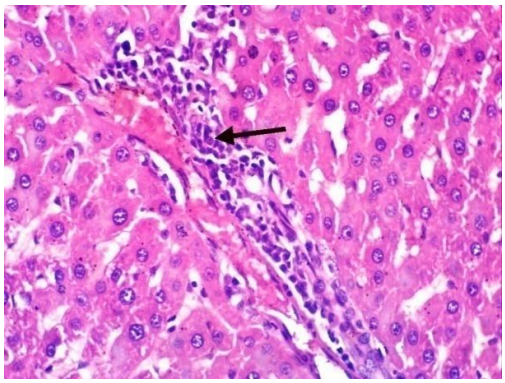

Fig. 2: Liver of rat from G3 showing portal infiltration with inflammatory cells (H & E X 400).

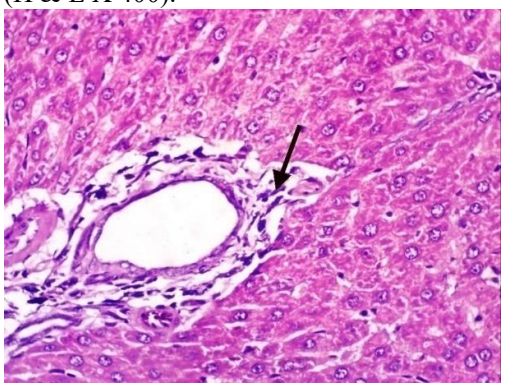

Fig. 3: Liver of rat from G3 showing few strands of fibroblasts in the portal triad around slightly dilated bile duct (H & E X 400).

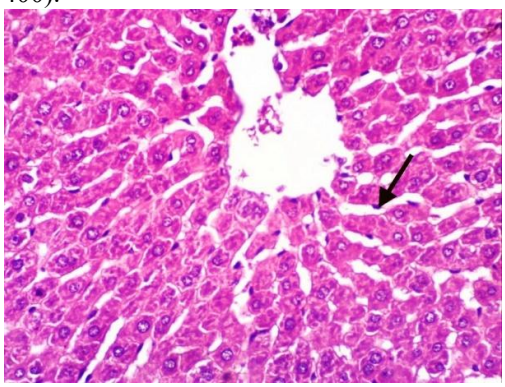

Fig. 4: Liver of rat from G4 showing slight Kupffer cells activation (H & E X 400).

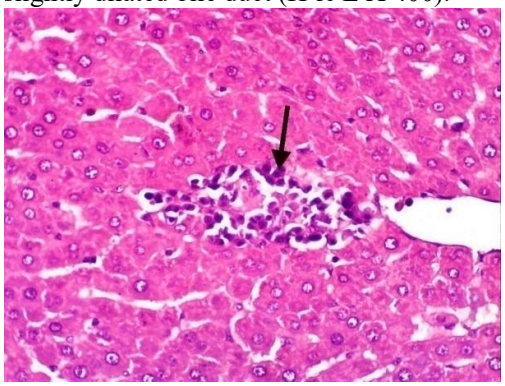

Fig. 5: Liver of rat from G5 showing focal hepatocellular necrosis associated with inflammatory cells infiltration (H & E X 400).

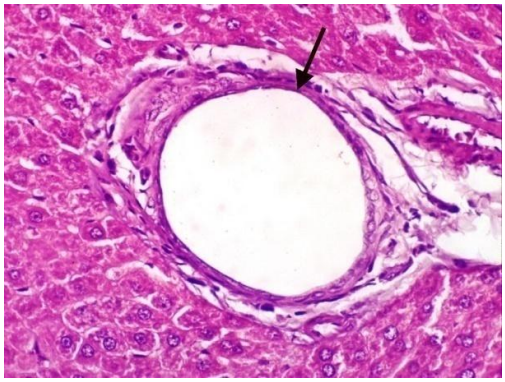

Fig. 6: Liver of rat from G5 showing marked cystic dilatation of bile duct (H & E X 400).

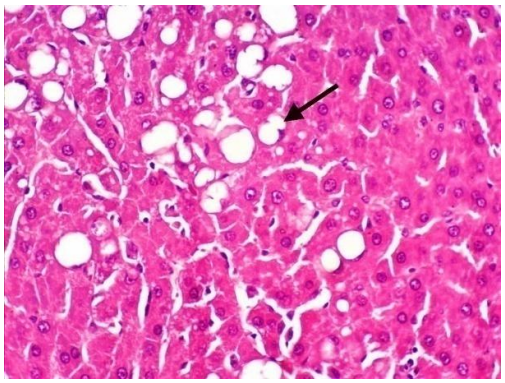

Fig. 7: Liver of rat from G5 showing hepatocellular steatosis (H & E X 400).

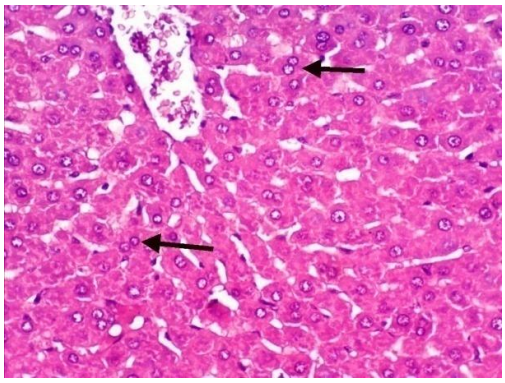

Fig. 8: Liver of rat from G5 showing binucleation of hepatocytes (H & E X 400).

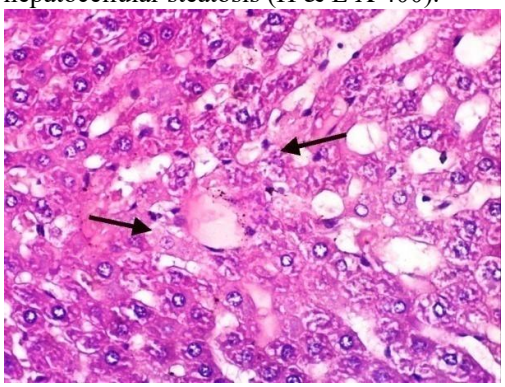

Fig. 9: Liver of rat from G6 showing hepatocellular vacuolations (H & E X 400).

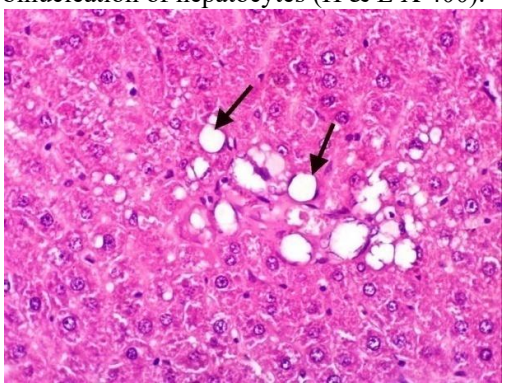

Fig. 10: Liver of rat from G6 showing hepatocellular steatosis (H & E X 400).

Meanwhile noticed decrease in serum albumin and protein could be resulted from binding of metals to plasma albumin and hence decrease total protein (Pawaiya *et al.,* 1998). Other explanation of decreasing of serum albumin and protein was disturbance of protein synthesis and metabolism associated with hepatic and renal damages in animals treated with HMMs (Choudhuri *et al.,* 2021). Destructive effect of HMs on liver could be attributed to generation of ROS and lipid peroxidation (Barnes *et al.,* 2020).

The improved liver function and histological architecture noted by FA administration were previously reported by (Hammoud *et al.,* 2018 and Tawfik *et al.,* 2018) through inhibition of excess lipid peroxidation and ROS production and preservation of integrity of cell membrane.

3.5. Kidney function parameters

Statistical analysis of variance of urea and creatinine illustrated in table (4) showed significant and highly significant elevation of their concentrations in serum of rats treated with bHMMS (G3) and 1/10 of LD_{50} (G5), respectively (comparing with control, $P<0.05$). While, co-treatment of rats with FA and both HMMs doses (G4 and G6) ameliorating the adverse effect of HMs on kidney function parameters. This effect was more pronounced with bHMMs group (G4) proved by restoration of urea and creatinine to normal (compared with control ($P<0.05$).

3.6. Histopathological examination of kidney

Microscopically, kidneys of rats from G1 (control) and G2 (FA) revealed the normal histological structure of renal parenchyma (Figs. 11). Meanwhile, kidney of rats from G 3 (bHMMs) treated rats) exhibited cytoplasmic vacuolization of epithelial lining renal tubules and endothelial lining glomerular tuft (Figs. 12& 13) as well as focal necrosis of renal tubules and atrophy of glomerular tuft (Fig. 13). On the other hand, Kidneys of G4 which co-treated bHMMs and FA revealed apparent normal renal parenchyma (Fig. 14). In contrary, kidneys of rats from G 5 (rats treated with 1/10 of LD_{50} of HMMs) showed more sever lesions manifested by cytoplasmic vacuolization of epithelial lining renal tubules

and intertubular inflammatory cells infiltration (interstitial nephritis) (Fig. 15) as well as hyaline droplets in the lumen of renal tubules (Fig. 16). However, kidneys of rats from G 6 (co-treated with 1/10 of LD_{50} of HMMs and FA) revealed no histopathological changes except cytoplasmic vacuolization of epithelial lining some renal tubules and endothelial lining glomerular tuft (Fig. 17).

Table 4: Kidney function parameters in serum of control and treated rats (means ± SE).

Parameters Groups	Urea (mg/dl)	Creatinine (mg/dl)
G 1 (Control)	27.14±1.02^{de}	0.36±0.02^{d}
G 2 (FA)	25.02±1.29^{e}	0.36±0.02^{d}
G 3 (bHMMs)	39.04±1.50^{b}	0.56±0.02^{b}
G4 (bHMMs+FA)	29.28±1.14^{d}	0.38±0.02^{cd}
G5 (1/10 LD_{50})	52.18±1.83^{a}	1.10±0.03^{a}
G6 (1/10 LD_{50}+FA)	33.80±1.21^{c}	0.44±0.02^{c}
$LSD_{0.05}$	3.97	0.07

Within the same column, various superscript letters indicate significant differences (Duncan, *P <0.05*).

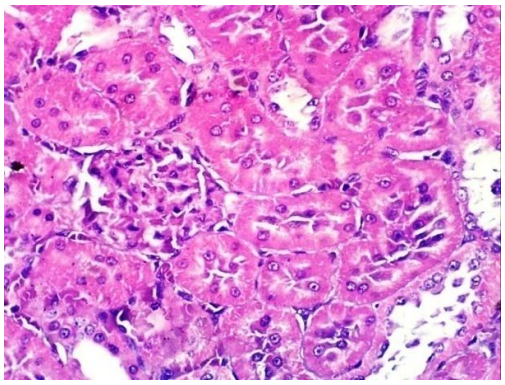

Fig. 11: Kidney of rat from G2 showing no histopathological alterations (H & E X 400).

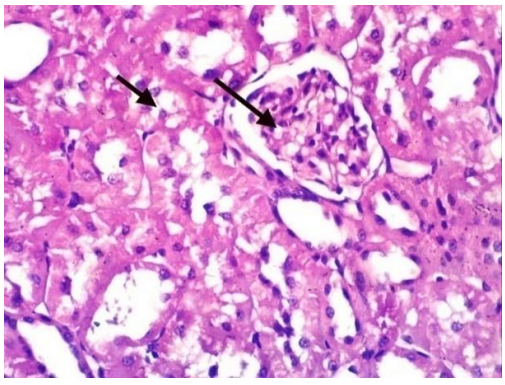

Fig. 12: Kidney of rat from G3 showing cytoplasmic vacuolization of epithelial lining some renal tubules and endothelial lining glomerular tuft (H & E X 400).

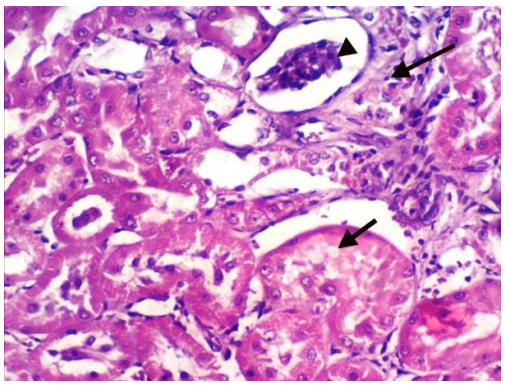

Fig. 13: Kidney of rat from G3 showing cytoplasmic vacuolization of epithelial lining renal tubules, focal necrosis of renal tubules and atrophy of glomerular tuft (H & E X 400).

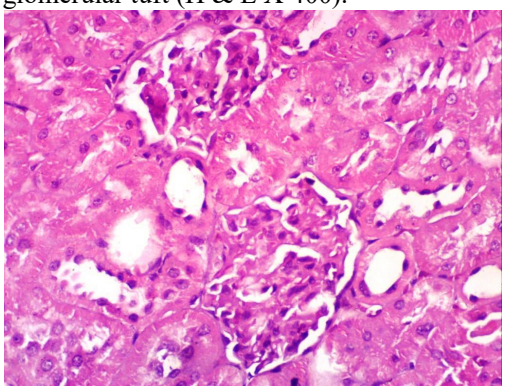

Fig. 14: Kidney of rat from G4 showing apparent normal renal parenchyma (H & E X 400).

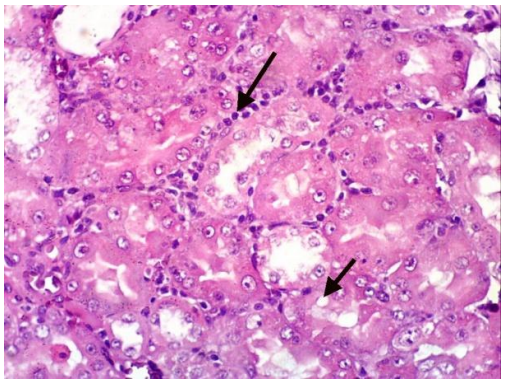

Fig. 15: Kidney of rat from G5 showing cytoplasmic vacuolization of epithelial lining renal tubules and intertubular inflammatory cells infiltration (interstitial nephritis) (H & E X 400).

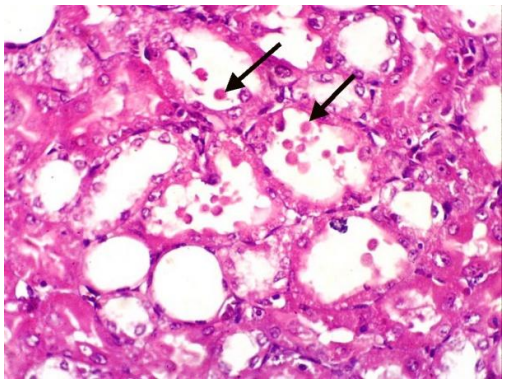

Fig. 16: Kidney of rat from G5 showing hyaline droplets in the lumen of renal tubules (H & E X 400).

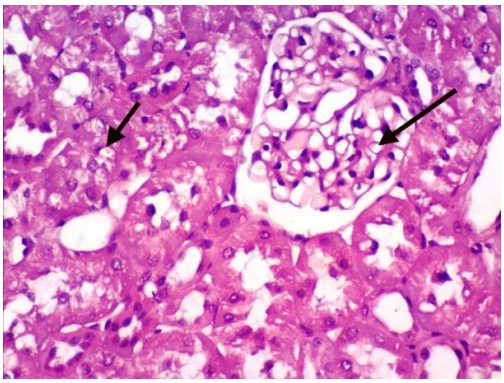

Fig. 17: Kidney of rat from G6 showing cytoplasmic vacuolization of epithelial lining some renal tubules and endothelial lining glomerular tuft (H & E X 400).

In the current study, HMMs exhibit deleterious effect on kidney function parameters and renal histology. Our results were in agreement with Barnes *et al.,* (2020) who reported that increase of urea and creatinine concentrations in serum of rats treated with Cu and Pb mixture were accompanied by necrosis, tubular degenerative changes and disrupted Bowman's capsule. Same results obtained by Andjelkovic *et al.,* (2019) in rats treated by Pb and Cd mixture, Wang *et al.,* (2020) in rats treated with Cr, Mn, Ni, Cu, Zn Cd, Hg and Pb mixture and Hammoud and Shalaby (2019) in rats treated by Al. In contrary, Kenston *et al.,* (2018) reported decreased renal function after oral administration of single dose of eight HMs. Noticed negative changes in kidney function and histology could be explained by in balance between antioxidant defense mechanism and free radicals production caused by HMs (Barnes *et al.,* 2020 and Choudhuri *et al.,* 2021).

Fortunately, FA successfully protects kidneys against toxic effect of HMs and preserves renal tissue due to its powerful antioxidant properties (Ramar *et al.,* 2012 and Hammoud *et al.,* 2018).

3.7. Histopathological examination of intestine

Microscopically, examined sections from Gs 1 & 2 (control and FA treated rats, respectively) revealed no histopathological changes (Fig. 18), with maintaining the normal histological archeticture of intestinal layers (mucosa, submucosa, musculosa and serosa). On the other hand, intestine of rats treaded with dose of HMMs mimic to those found in bread from G3 showed inflammatory cells infiltration in the lamina propria (Fig. 19) and activation and hyperplasia of mucous secreting cells (Fig. 20). Examined sections from intetine of G4 which co-treated with bHMMs and FA revealed normal histological architecture of intestinal layers (Fig. 21). Meanwhile, intestine of rats from G5 which adminstrated with 1/10 of LD_{50} of HMMs showed hyperplasia of mucous secreting cells, inflammatory cells infiltration in lamina propria (Fig. 22) and shortening of the intestinal villi (Fig. 23). However,

regressed lesions were noticed in intistine of rats from G6 (co-treated with 1/10 of LD_{50} of HMMs and FA) which revealed no histopathological changes except slight hyperplasia of mucous secreting cells and slight submucosal oedema (Fig. 24). HMs are passing into blood stream after absorption from intestine (Pawaiya *et al.,* 1998 and Mahieu *et al.,* 2000) causing sever lesions in intestinal wall during these pathway. Ali and Zinad (2020) recorded pathological changes in villi and goblet cells of intistine of femal rats treated with Pb for 90 days. However, FA in our intervantion trial was able to protect intistine against HMs toxicity. Similar to our results Tawfik *et al.,* (2018) reported that FA protect intestine against electromagnetic waves.

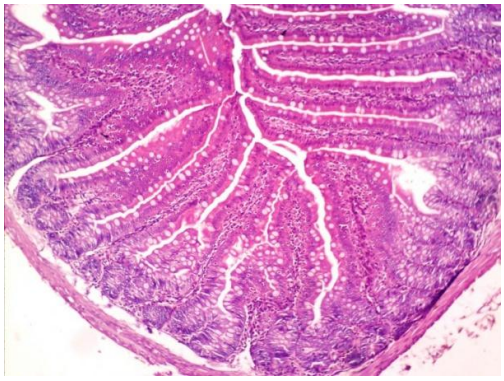

Fig. 18: Intestine of rat from G2 showing the normal histological architecture of intestinal layers (H & E X 100).

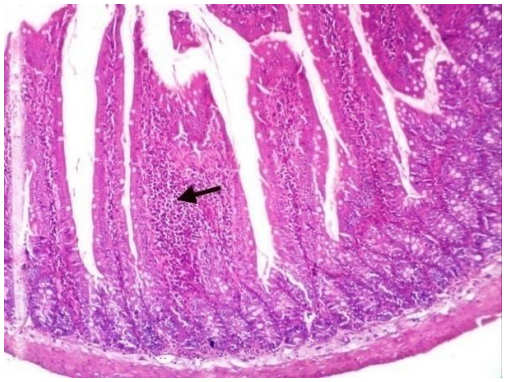

Fig. 19: Intestine of rat from G3 showing inflammatory cells infiltration in the lamina propria(H & E X 100).

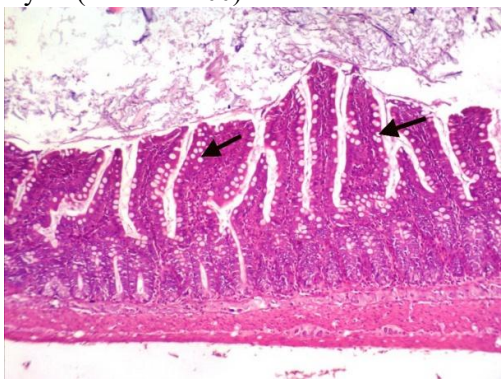

Fig. 20: Intestine of rat from G3 showing activation and hyperplasia of mucous secreting cells (H & E X 100).

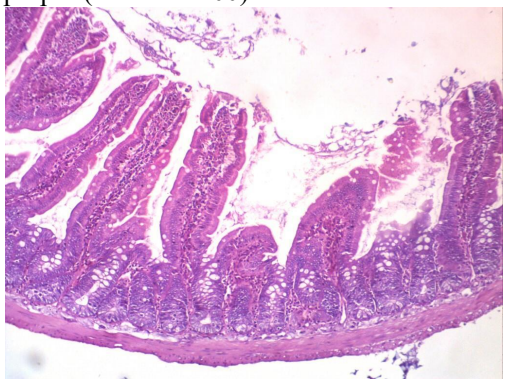

Fig. 21: Intestine of rat from G4 showing the normal histological architecture of intestinal layers (H & E X 100).

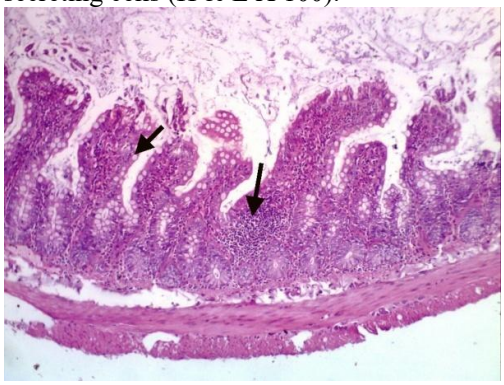

Fig. 22: Intestine of rat from G5 showing hyperplasia of mucous secreting cells and inflammatory cells infiltration in lamina propria(H & E X 100).

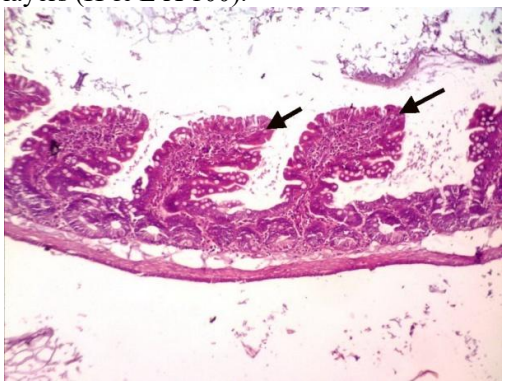

Fig. 23: Intestine of rat from G5 showing shortening of the intestinal villi (H & E X 100).

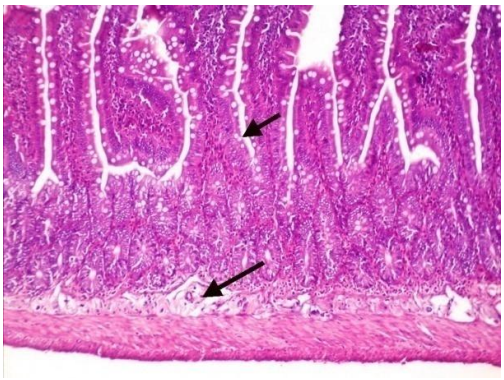

Fig. 24: Intestine of ratfrom G6 showingslight hyperplasia of mucous ecreting cells and slight submucosaloedema(H & E X 100).

3.8. Histopathological examination of brain

Brain (Cerebral cortex) of rats from Gs 1& 2 (control and FA treated rats, respectively) showed no histopathological alterations (Fig. 25). Meanwhile, sections from G3 (bHMMs group) revealed necrosis, shrunken and pyknosis of neurons associated with neuronophagia (Fig. 26). However, brain of rats from G 4which co-treated with bHMMs and FA showed apparent normal structure (Fig. 27).

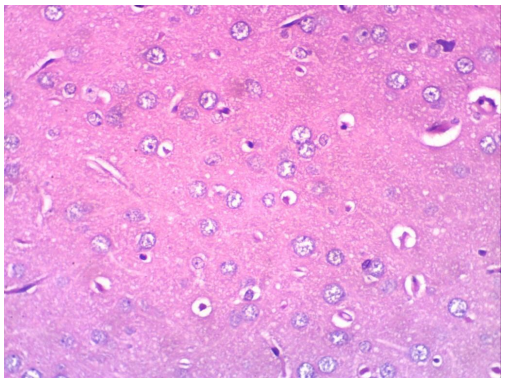

Fig. 25: Cerebral cortex of rat from G2 showing no histopathologicalalterations (H & E X 400).

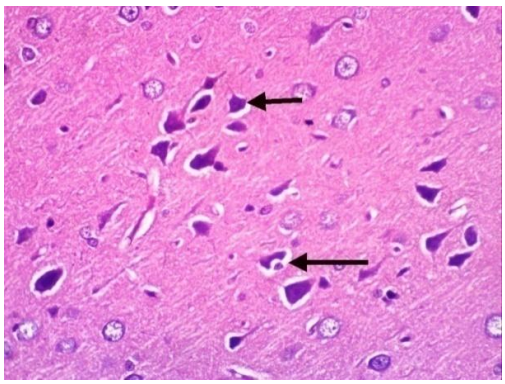

Fig. 26: Cerebral cortex of rat from G3 showing necrosis, shrunken and pyknosis of neurons associated with neuronophagia (H & E X 400).

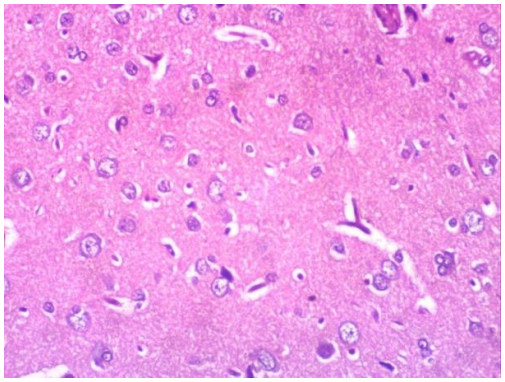

Fig. 27: Cerebral cortex of rat from G4 showing no histopathological alterations (H & E X 400).

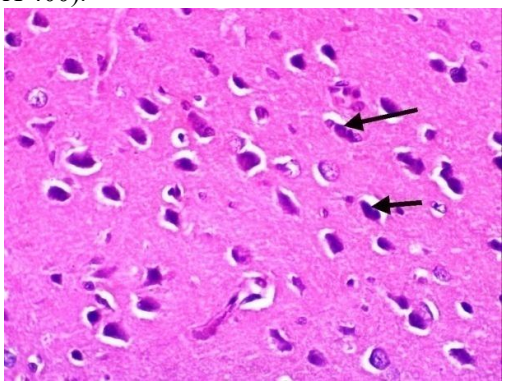

Fig. 28: Cerebral cortex of rat from G5 showing marked necrosis, shrunken and pyknosis of neurons associated with neuronophagia (H & E X 400).

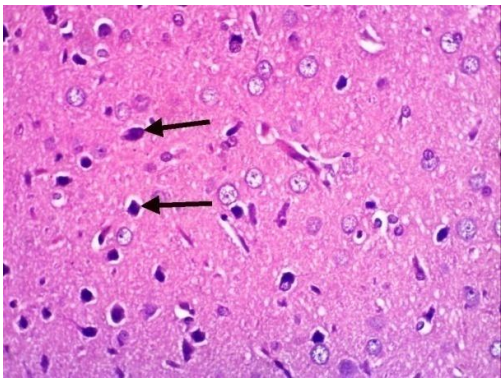

Fig. 29: Cerebral cortex of rat from G6 showing necrosis of some neurons (H & E X 400).

On the other hand, brain of rats from G5 which adminstrated 1/10 of LD_{50} of HMMs exhibited marked necrosis, shrunken and pyknosis of neurons associated with neuronophagia (Fig. 28). However, regressed lesions were noticed in brain from G 6 (co-treated with 1/10 of LD_{50} of HMMs and FA), examined sections revealed no changes except necrosis of some neurons (Fig. 29).

HMs induces injurious oxidative damage associated with archetictural alterations of brain. Similar finding was explined by Anyanwu *et al.,* (2020) who observed that adminstration of low dose of Pb, Cd and Hg mixture cause neurnal neccrosis, gliosis, prolifration of glial cell through agumant of pro-inflammatory processes and inhibition of anti-inflammatory cytokines in brain accompanied with increase of MDA and disruption of antioxidants. Moreover, Hammoud and Shalaby (2019) reported severe brain damage in rats treated by Al. In the view of the current study FA fully protect of brain against HMMs toxicity. Adefegha *et al.,* (2016) and Hammoud *et al.,* (2018) reported same protective effect of FA on brain tissue against lipid peroxidation induced by monosodium glutamate and Cd, respectively.

3.9. Antioxidant and oxidative stress markers

Impairment of antioxidant defense mechanism by HMs was evidenced by depletion of TAC and initiation of MAD (table 5). Treatments of rats with HMMs dose mimic to those found in bread (G3) and 1/10 of LD_{50} of same HMMs (G5) resulted in significant and highly significant decline of plasma TAC and significant and highly significant increase in MDA, respectively (compared to control ($P<0.05$)). In the current study simultaneous administration of FA and both HMMs dose (G4 and G6) resulted in significant enhancement of TAC and attenuation of MDA production.

Table 5: TAC and MDA concentrations in plasma of control and treated rats (means ± SE).

Parameters / Groups	TAC (mM/L)	MDA (nmol/ml)
G 1 (Control)	3.58 ± 0.16^{a}	18.20 ± 1.66^{d}
G 2 (FA)	3.88 ± 0.15^{a}	16.42 ± 1.74^{d}
G 3 (bHMMs)	0.90 ± 0.05^{d}	30.72 ± 1.57^{b}
G4 (bHMMs+FA)	2.02 ± 0.06^{b}	20.44 ± 1.06^{d}
G5 (1/10 LD_{50})	0.41 ± 0.07^{e}	42.44 ± 2.05^{a}
G6 (1/10 LD_{50}+FA)	1.47 ± 0.11^{c}	25.88 ± 1.23^{c}
LSD 0.05	0.36	4.63

Within the same column, various superscript letters indicate significant differences (Duncan, *P <0.05*).

In the point of view of the above results, it seems to be the HMs induces toxicity through disruption of balance between antioxidant defense of body and free radicals production (Choudhuri *et al.,* 2021). Increase of lipid peroxidation in plasma of rats treated with Pb and Cd mixture and Al was previously reported by Andjelkovic *et al.,* (2019) and Hammoud and Shalaby (2019), respectively. Additionally, Pb binds to -SH groups in vital enzymes especially those responsible for antioxidant protection of body and impaired their function (Flora *et al.,* 2012). Also, AS conjugate with GSH

(glutathione, a key molecules of antioxidant) and decrease its level in body (Lee *et al.,* 2006). Once free radicals are generated they bind to macromolecules of cell and cell membrane leading to damage of cells and disturbance of metabolism and other vital biological mechanisms (Jan *et al.,* 2015 and Balali-Mood *et al.,* 2021).

In the current study FA mitigate oxidative stress through suppression of lipid peroxidation and preservation of TAC. Such effect was previously reported by Hammoud *et al.,* (2018) and Tawfik *et al.,* (2018). FA (4-hydroxy-3-methoxycinnamic acid) posses a unique structure of three active sites: 1-electron donating groups on the benzene ring, 2-the carboxylic acid group and 3-an adjacent unsaturated C–C double bond which play important role in free radicals scavenging activity of FA (Kanaski *et al.,* 2002).

4. Conclusion

Heavy metals are environmental pollutants that contaminate our food, water and air and cause serious health problem. Our observations clearly revealed that exposure to heavy metals mixture (Al, Pb, As, Hg, Ni, Cr and Cd) with different concentrations resulted in sever changes in hematological and biochemical parameters and structure of liver, kidney, intestine and brain associated with oxidative stress. On the other hand, ferulic acid showed notably antioxidant activity and reduces toxic effect of heavy metals on all tested parameters and organs.

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